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Short Communication

Hydroxychloroquine susceptibility determination of *Coxiella burnetii* in human embryonic lung (HEL) fibroblast cells

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ABSTRACT

Coxiella burnetii, the causative agent of Q fever, survives and replicates in the acidic environment of monocytes/macrophages; hydroxychloroquine, through alkalinisation of the acidic vacuoles, is critical for the management of Q fever. In this study, a collection of *C. burnetii* strains isolated from human samples was tested to evaluate the in vitro minimum inhibitory concentrations (MICs) of doxycycline and hydroxychloroquine. Serial two-fold dilutions of doxycycline (0.25–8 mg/L) and hydroxychloroquine (0.25–4 mg/L) were added to *C. burnetii*-infected human embryonic lung (HEL) fibroblast cells after 48 h of incubation, in duplicate. DNA was detected by *C. burnetii*-specific semi-quantitative PCR with primers and probes designed for amplification of the IS*1111* and IS*30A* spacers. A total of 29 *C. burnetii* isolates obtained from 29 patients were tested. Doxycycline MICs ranged from 0.25 mg/L to 0.5 mg/L and hydroxychloroquine MICs from 0.25 mg/L to >4 mg/L. Four *C. burnetii* stains had hydroxychloroquine MICs ≤ 1 mg/L. The concentration of hydroxychloroquine was associated with a significant decrease in *C. burnetii* DNA copies in HEL cells based on linear regression analysis ($P = 0.01$). Recommended serum concentrations of hydroxychloroquine significantly reduced the growth of *C. burnetii*. Moreover, some *C. burnetii* strains presented hydroxychloroquine MICs below the recommended serum concentrations, indicating that, for these cases, hydroxychloroquine treatment alone may even be effective.

1. Introduction

Q fever is a potentially life-threatening worldwide zoonosis caused by an obligate intracellular bacterium, *Coxiella burnetii* [1]. Doxycycline in combination with hydroxychloroquine is recommended for the treatment of *C. burnetii* localised infections, and its doses are adjusted so that their concentration in serum is >5 mg/L and 0.8–1.2 mg/L respectively [2]. To date, studies have determined the in vitro minimum inhibitory concentrations (MICs) of doxycycline against *C. burnetii* [3–5] and most strains were found to be sensitive to doxycycline, however the existence of resistant isolates has been also described [5]. Establishment of *C. burnetii* infection is based on a specific strategy that the bacteria adopt in order to enter monocytes/macrophages [6]; hydroxychloroquine, through alkalinisation of the acidic vacuoles, is critical for the management of Q fever [7]. Indeed, it was found that increasing the phagolysosomal pH of infected chicken embryo fibroblasts inhibited the multiplication of *C. burnetii* [8]. However, to the best of our knowledge, the in vitro MICs of hydroxychloroquine against *C. burnetii* have not been determined. In this study, a collection of *C. burnetii* strains isolated from human samples was tested to evaluate the in vitro MICs of doxycycline and hydroxychloroquine.

2. Materials and methods

2.1. C. burnetii *strains*

A total 29 *C. burnetii* isolates from our laboratory, obtained from 29 patients, were tested and cultured in human embryonic lung (HEL) fibroblast cells (Table 1). Microscopic examination of cell monolayers as well as Gimenez and immunofluorescence staining was performed every 3 days over a 15-day period.

2.2. *Antibiotic susceptibility testing*

For antibiotic susceptibility testing, cells that were heavily infected with *C. burnetii* were incubated for 3 weeks. Dilutions of doxycycline (0.25–8 mg/L) (Pfizer, Neuilly, France) and hydroxychloroquine (0.25–4 mg/L) (Sanofi Synthélabo, Paris, France) were then added at serial two-fold dilutions to the culture medium of

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Table 1
Patient characteristics for the 29 *Coxiella burnetii* strains analysed.

Sex	Age (years)	Sample	Clinical manifestation	IFA phase I			IFA phase II			Serum concentration (mg/L)		Log_{10} DNA copies in sample	Weeks of culture	MIC (mg/L)	
				IgG	IgM	IgA	IgG	IgM	IgA	DOX	HCQ			DOX	HCQ
M	55	Serum	Acute Q fever	Neg.			Neg.			NP	NP	3	10	0.25	>4
F	15	Blood	Acute Q fever	Neg.			Neg.			NP	NP	3	6	0.25	4
F	26	Knee	Osteoarticular infection	200	0	0	400	0	0	NP	NP	2.3	6	0.25	>4
M	69	Psoas abscess	Osteoarticular infection	6,400	0	400	12,800	0	800	NP	NP	2.6	12	0.25	4
M	62	Synovial fluid	Osteoarticular infection	1,600	0	0	400	0	0	NP	NP	2.3	5	0.25	4
M	88	Psoas abscess	Osteoarticular infection	200	0	0	200	0	0	NP	NP	2.3	8	0.25	>4
M	73	Knee	Osteoarticular infection	800	0	0	800	0	0	4.8 ± 2.8	1.3 ± 0.5	2.7	4	0.25	>4
F	75	Cardiac valve	Infective endocarditis	6,400	0	0	1,600	0	0	NP	NP	2.7	5	0.25	0.5
M	45	Cardiac valve	Infective endocarditis	6,400	0	200	6,400	0	100	NP	NP	2.7	12	0.25	>4
F	63	Cardiac valve	Infective endocarditis	1,600	0	0	400	0	0	5.3 ± 0.9	0.9 ± 0.3	2	2	0.25	1
F	78	Aortic thrombus	Infective endocarditis	3,200	0	1,600	400	0	0	16	1.4	3.1	12	0.25	>4
F	45	Cardiac valve	Infective endocarditis	6,400	0	800	1,600	0	200	NP	NP	1.8	2	0.25	4
M	81	Blood	Infective endocarditis	1,600	400	3,200	1,600	400	3,200	4.6 ± 0.9	0.9 ± 0.2	3.2	6	0.25	0.25
M	59	Cardiac valve	Infective endocarditis	NP						NP	NP	1.7	8	0.25	>4
M	56	Blood	Infective endocarditis	102,400	800	12,800	25,600	800	0	NP	NP	3.2	4	0.25	0.5
M	13	Cardiac valve	Infective endocarditis	25,200	200	800	51,200	400	1,600	NP	NP	2.9	5	0.25	>4
M	80	Blood	Infective endocarditis	51,200	100	0	102,400	200	0	4.9 ± 0.4	0.9 ± 0.3	2	8	0.25	2
M	79	Blood	Infective endocarditis	12,800	800	400	12,800	100	400	NP	NP	2.6	4	0.25	>4
M	71	Cardiac valve	Infective endocarditis	25,600	0	400	3,200	0	100	5.2 ± 1.9	0.6 ± 0.2	2	5	0.25	>4
M	48	Cardiac valve	Infective endocarditis	51,200	100	25	102,400	200	50	NP	NP	2.1	2	0.5	>4
M	60	Cardiac valve	Infective endocarditis	6,400	50	100	3,200	50	100	NP	NP	2.9	9	0.25	>4
F	63	Cardiac valve	Infective endocarditis	1,600	0	1,600	1,600	0	3,200	12 ± 2.3	1.2	3	7	0.25	2
M	70	Aortic aneurysm	Vascular infection	6,400	0	200	1,600	0	0	9.4 ± 1.2	0.7 ± 0.3	2.2	9	0.25	>4
M	64	Aortic aneurysm	Vascular infection	800	0	200	400	0	0	6.7	0.6	2.6	12	0.25	>4
M	62	Aortic aneurysm	Vascular infection	800	50	50	1,600	100	100	7.4 ± 2.3	0.5 ± 0.2	2.7	4	0.25	4
M	64	Aortic aneurysm	Vascular infection	1,600	0	100	200	0	100	6.2 ± 0.9	0.6 ± 0.2	2.6	16	0.25	>4
M	68	Aortic aneurysm	Vascular infection	3,200	0	25	6,400	0	50	5.2 ± 0.9	0.8 ± 0.2	2.1	4	0.25	4
M	47	Aortic aneurysm	Vascular infection	3,200	0	200	3,200	0	3,200	NP	NP	2.6	9	0.25	>4
M	63	Aortic aneurysm	Vascular infection	12,800	0	800	400	0	800	5.3 ± 1.9	0.8 ± 0.3	2.3	13	0.25	4

IFI, indirect fluorescent antibody; DOX, doxycycline; HCQ, hydroxychloroquine; MIC, minimum inhibitory concentration; NP, not provided.

individual tissue culture plate wells after 48 h of incubation, in duplicate. Untreated infected HEL cell wells served as negative controls. Cell cultures from wells were harvested every 3 days over a 15-day period. The MIC was defined as the lowest antibiotic concentration at which complete inhibition of bacterial growth was determined by measurement of DNA copies by *C. burnetii* quantitative PCR (qPCR) assay [9].

2.3. Molecular assays

Total genomic DNA was extracted from samples using a QIAamp® Tissue Kit (QIAGEN, Hilden, Germany). DNA was detected by *C. burnetii*-specific semi-quantitative PCR with primers and probes designed for amplification of the IS*1111* and IS*30A* spacers [9]. DNA extracted from the supernatant of a culture of *C. burnetii* L929 was used as a positive control, whilst samples from rats livers that were negative for *C. burnetii* (by culturing and qPCR analysis) and sterile water were used as negative controls in each run.

2.4. Quantification of C. burnetii using flow cytometry

C. burnetii particle quantification was performed by flow cytometry as previously described [9,10]. Serial 10-fold dilutions (from 10^{-1} to 10^{-11}) of phase 2 antigens from *C. burnetii* L929 were tested using qPCR targeting the IS*1111* spacer to express the cycle threshold (Ct) as the number of bacteria/μL and copies of IS*1111*/mL of sample (Supplementary Fig. S1). When calculating the log_{10} bacterial copies from the number of IS*1111* copies, it was assumed that the number of IS*1111* elements in the genome of the Nine Mile strain of *C. burnetii* was 20 [11].

2.5. Statistical analysis

Epi Info v.6.0 software (US Centers for Disease Control and Prevention, Atlanta, GA) was used for data comparison. Kruskal–Wallis and Mann–Whitney tests were used to compare *C. burnetii* concentrations, calculated as log_{10} number of DNA copies, between the doxycycline or hydroxychloroquine groups. Significant results were systematically confirmed using Dunn's multiple-comparison test. Linear regression analysis, adjusted systematically for *C. burnetii* and doxycycline or hydroxychloroquine concentrations, was used to estimate the effect of the presence of antibiotics on the *C. burnetii* concentration. All tests were bilateral and were considered significant at $P > 0.05$.

3. Results

Overall, 15 *C. burnetii* strains were isolated from patients with infective endocarditis, 7 strains from patients with Q fever vascular infection, 5 strains from patients with *C. burnetii* osteoarticular infection and 2 strains from patients with acute Q fever. Doxycycline and hydroxychloroquine serum levels were measured for 12 patients and ranged from 4.6 to 12 mg/L and from 0.5 to 1.3 mg/L, respectively (Table 1). qPCR was positive for *C. burnetii* in all samples and the mean time of incubation was 7.2 weeks (range 2–16 weeks). No connection was found between the log_{10} *C. burnetii* DNA copies in the patients' samples and the length of incubation required to obtain a positive *C. burnetii* culture ($y = 0.014x + 2.42$; $R^2 = 0.015$).

3.1. Doxycycline susceptibility testing

Quantification of growth with doxycycline revealed inhibition of growth for MICs between 0.25 mg/L and 0.5 mg/L (Table 1).