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Note

In vitro evaluation of minimum inhibitory concentration of several antibacterial agents against *Rickettsia japonica* using a plaque reduction assay system☆

Masaaki Satoh, Hirofumi Kato, Mutsuyo Takayama-Ito, Motohiko Ogawa, Shuji Ando, Masayuki Saijo*

Department of Virology I, National Institute of Infectious Diseases, Japan

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ABSTRACT

The study was conducted to determine the minimum inhibitory concentrations (MICs) of several antibacterial agents against *Rickettsia japonica*, which causes Japanese spotted fever. A plaque reduction assay as an *in vitro* culture method was conducted to determine the MICs of antibacterial agents (4 types of tetracyclines: tetracycline, doxycycline, minocycline, and tigecycline; 3 types of quinolones: ciprofloxacin, ofloxacin, and levofloxacin; and 2 types of macrolides: azithromycin and clarythromycin) against *R. japonica*. *R. japonica* was sensitive to the antibacterial agents tested with MICs similar to those against other spotted fever rickettsia determined in previously described plaque reduction assays.

JSF is a type of spotted fever rickettsiosis caused by *Rickettsia japonica*. JSF was first discovered in 1984 in Japan [1] and is endemic to Japan, Korea, and China [2–4]. Patients with JSF generally show fever, eschar, and rash [2].

Rickettsia japonica is a gram-negative, obligate intracellular bacterium that exerts its pathogenicity in the endothelial cells of blood vessels [5]. This pathogen was isolated or detected by polymerase chain reaction in *Haemaphysalis longicornis*, *H. flava*, *H. formonensis*, *H. hystricis*, *H. cornigera*, *H. monosspinosa*, *Ixodes ovatus*, and *Dermacentor taiwanensis* [6].

Tetracyclines, mainly doxycycline and minocycline, are first-line antibacterial agents used to treat JSF [2,7]. The *in vitro* susceptibility using plaque reduction assay of *R. conorii* and *R. rickettsii*, which are the causative pathogens of Mediterranean spotted fever and Rocky mountain spotted fever, respectively, to these agents was reported previously [8–11]. Although the *in vitro* susceptibility of *Rickettsia* species isolated from patients of JSF in Japan (named as *R. japonica* later [5]) to some antibacterial agents was also reported previously with Giemsa or Macchiavello staining methods [12,13], we examined that of *R. japonica* to several antibacterial agents by conducting plaque reduction assays in this study.

The YH strain of *Rickettsia japonica* [5] was grown in Vero cell monolayers cultured in MEM-2FBS (MEM, Sigma-Aldrich, St. Louis, MO, USA).

The minimum inhibitory concentrations (MICs) of the following 9 antibacterial agents were measured: 4 types of tetracyclines (tetracycline, doxycycline, minocycline, and tigecycline), 3 types of quinolones (ciprofloxacin, ofloxacin, and levofloxacin), and 2 types of macrolides (clarythromycin and azithromycin). No information on the MIC of tigecycline on *Rickettsia* spp. is available. The range of concentrations were as follows: 0.008, 0.016, 0.031, 0.062, 0.125, 0.25, and 0.5 μg/mL for tetracyclines and 0.125, 0.25, 0.5, 1, 2, 4, and 8 μg/mL for quinolones and macrolides. Each dilution was prepared before use.

The *in vitro* susceptibility assay was performed using a plaque reduction assay as described below. Vero cells were grown in MEM-5FBS in a 24-well plate (Techno Plastic Product AG, Trasadingen,

Abbreviations: JSF, Japanese spotted fever; MEM-2FBS, Eagle's minimal essential medium supplemented with 2% fetal bovine serum and 2 mM L-glutamine, but without antibacterial agents; MEM-5FBS, Eagle's minimal essential medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine, but without antibacterial agents.

☆ All authors meet the ICMJE authorship criteria.

* Corresponding author. Department of Virology 1, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
E-mail address: m-satoh@nih.go.jp (M. Saijo).

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Switzerland) at the day before the inoculation. The cells monolayer was adsorbed with MEM-2FBS, which contained 50 plaque-forming units of *R. japonica* with tilting the plate at 15-min intervals for 1 h at 34 °C. After absorption, the cells were overlaid with 0.5 mL of MEM-2FBS containing 0.5% methylcellulose (Fujifilm Wako Pure Chemicals, Osaka, Japan) and diluted antibacterial agent on the wells, and were incubated for 7 days at 34 °C in a 5% CO_2 incubator. The cells were fixed with formalin (Fujifilm Wako Pure Chemicals) for 1 h and stained for 1 h with 0.04% methylene blue. The MIC was determined as the lowest concentration of antibacterial agent that completely inhibited plaque formation.

The inhibitory effect of each antibacterial agent in the plaque reduction assay is shown in Fig. 1. Tetracyclines showed the strongest inhibitory effect on *R. japonica* among the 3 types of antibacterial agents. The MIC of tigecycline was the highest among the tetracycline group agents. The MIC of levofloxacin was lower than other quinolones and the MICs of macrolides showed the same value (Fig. 1). The MICs shown in Table 1 were calculated using the MICs obtained from three independent experiments and are shown as the average with standard error. Thus, *R. japonica* was sensitive to all the antibacterial agents tested. The MIC of each antibacterial agent was similar to the MICs against other rickettsia species, *R. japonica*, *R. rickettsii* and *R. conorii*, as determined in previous studies (Table 1, [9–13]).

Comparing previous data about MICs against *R. japonica* with our results, values of the MICs for tetracycline, minocycline, ciprofloxacin, and ofloxacin indicated higher than those of this study (Table 1). It is supposed that this difference might be due to disparity of method for evaluating the MICs against *R. japonica*; Giemsa or Macchiavello staining [12,13] and plaque reduction assay using methylcellulose (this study). Since the limitation of Giemsa or macchiavello staining is lack of detection of bacterial viability [14], results might be reflected as higher values of MICs.

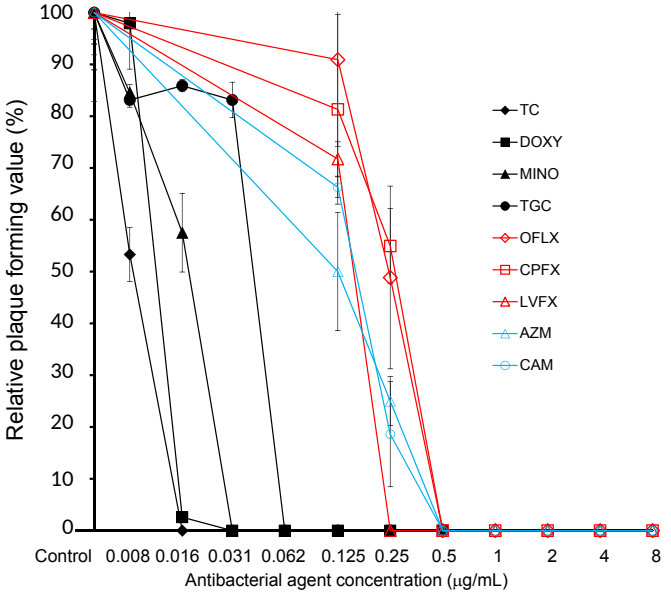

Fig. 1. Inhibitory effects of tetracyclines [black line; tetracycline (TC: ◆), doxycycline (DOXY: ■), minocycline (MINO: ▲), tigecycline (TGC: ●)], quinolones [red lines; ofloxacin (OFLX: ◊), ciprofloxacin (CPFX: □), levofloxacin (LVFX: Δ)], and macrolides [blue lines; azithromycin (AZM: Δ), clarithromycin (CAM: ○)] are represented. The number of plaques with no antimicrobial agent (control) is defined as 100%. The ratio of the number of plaques at each concentration divided by the number in the control was defined as the relative plaque forming value (%). The number of plaques at each antibacterial agent concentration was counted in triplicate wells and the average was calculated. The vertical bars indicate the standard error. This figure shows the data for one experiment from among 3 independently performed experiments.

Table 1
Summary of MICs of antibacterial agents against *R. japonica*, *R. rickettsii*, and *R. conorii*.

Antibacterial agents	*Rickettsia* spp.	MIC (μg/mL)	Reference
Tetracycline	*R. japonica*	0.026 ± 0.005	This study
		0.31	[12][a]
		0.04	[13][b]
	R. rickettsii	0.25	[9]
	R. conorii	0.25	[9]
Doxycycline	*R. japonica*	0.041 ± 0.010	This study
	R. rickettsii	0.06	[9]
	R. conorii	0.06	[9]
Minocycline	*R. japonica*	0.052 ± 0.010	This study
		0.15	[12][a]
Tigecycline	*R. japonica*	0.104 ± 0.021	This study
Ciprofloxacin	*R. japonica*	0.5	This study
		1.56	[13][b]
	R. rickettsii	1	[10]
	R. conorii	0.25	[10]
Ofloxacin	*R. japonica*	0.5	This study
		0.78	[13][b]
	R. rickettsii	1	[10]
	R. conorii	1	[10]
Levofloxacin	*R. japonica*	0.25	This study
Azithromycin	*R. japonica*	0.5	This study
Clarithromycin	*R. japonica*	0.5	This study
	R. rickettsii	2	[11]

All of other MICs were evaluated using plaque assay using Vero cells.
[a] MICs were evaluated by Macchiavello staining using GM cells.
[b] MICs were evaluated by Macchiavello and Giemsa staining using L cells.

The plaque reduction assay is useful for evaluating various biological features of spotted fever rickettsia and other intracellular bacteria species [14]. In this study, methylcellulose was used rather than agarose to inhibit the mechanical release of bacteria into the culture medium, allowing only cell-to-cell bacterial propagation. This reagent is used for several viruses in plaque assays, as it makes it easier to conduct the plaque reduction assay in terms of incubation temperature control of the overlay medium to form a stable viscous solution [14]. Thus, the plaque reduction assay, in which methylcellulose was used to prepare viscous culture solution, can be used to determine the MICs of antibacterial agents for intracellular bacteria species.

In summary, we confirmed that the plaque reduction assay developed in this study is useful for evaluating the inhibitory effect of antimicrobial agents on *R. japonica*. Additionally, all tested antibacterial agents, including tetracyclines, quinolones, and macrolides, showed inhibitory effects.

Conflicts of interest

None.

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