Antimicrobial susceptibilities of *Chlamydia trachomatis* isolated from the urethra and pharynx of Japanese males

Running title: Drug susceptibilities of *Chlamydia trachomatis*

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Abstract

Objectives: Sexually transmitted infections due to *Chlamydia trachomatis* (*C. trachomatis*) are a worldwide public health problem. The aim of this study was to investigate the drug susceptibilities of *C. trachomatis* strains isolated from the urethra and pharynx of Japanese males.

Methods: Urethral and pharyngeal swabs were collected between 2013 and 2014 from Japanese males with urethritis. Using a McCoy cell line, 18 chlamydial strains were isolated from urethra in 18 patients and 7 from the pharynx in 7 of the 18 patients. The minimum inhibitory concentrations (MICs) of levofloxacin (LVFX) and azithromycin (AZM) were measured using the standard method of the Japanese Society of Chemotherapy.

Results: The MICs of LVFX and AZM against urethral chlamydial strains were 0.125-0.5 μg/mL and 0.125-0.25 μg/mL, respectively. In pharyngeal strains, the MICs of LVFX and AZM were 0.125-0.25 μg/mL and 0.125-0.25 μg/mL, respectively. In 7 patients with chlamydial strains isolated from both the urethra and pharynx, the MICs of LVFX between these strains were identical in 3 of 6 patients (no growth was observed

for one pharyngeal strain), while the MICs of AZM between these strains were identical in all 6 patients (not performed for one patient).

Conclusions: Our data suggest that *C. trachomatis* strains isolated from the urethra and pharynx of Japanese males are susceptible to LVFX and AZM. Although measuring the MICs of chlamydial strains is labor intensive, it is a significant surveillance tool for treating chlamydial infections and preventing the spread of STIs.

Key words: Levofloxacin, Azithromycin, *Chlamydia trachomatis*, Pharyngitis, Sexually transmitted infection, Drug susceptibility

Introduction

The spread of sexually transmitted infections, particularly male urethritis and uterine cervicitis due to *Chlamydia trachomatis* (*C. trachomatis*), is a major worldwide health concern [1-3]. In Japanese guidelines published between 2014 and 2016, macrolides, tetracyclines and fluoroquinolones are the recommended treatment for genitourinary tract infections due to *C. trachomatis* [4, 5]. However, male urethritis and cervicitis are also transmitted from the pharynx during oral sex [6, 7]. Several studies have reported that pharyngeal chlamydial infection is refractory to some antimicrobial regimens [8-11]. Since one of the reasons might be a low penetration rate of antimicrobial agents into the pharynx [11], there is the possibility that the lower drug susceptibility of *C. trachomatis* cannot be overcome. However, while drug-resistant strains of *C. trachomatis* have been observed in other countries [12-15], there have also been reports of high drug susceptibilities in strains isolated from the urethras of Japanese males [16]. The aim of this study is to investigate the drug susceptibilities of *C. trachomatis* strains isolated from both the urethra and the pharynx of Japanese males. The target drugs, levofloxacin and azithromycin, are recommended in the treatment

guidelines and are the most frequently administered antimicrobials by clinicians for *C. trachomatis* infection in Japan.

Materials and Methods

Specimens were obtained from male patients diagnosed with urethritis or patients who wanted to check for the presence of urethritis at Okayama University Hospital, Araki Urology Clinic and Hirashima Clinic located in Okayama, Japan between 2013 and 2014.

Patient characteristics

Patient characteristics were ascertained from the medical records of patients for whom *C. trachomatis* was isolated from the urethra or pharynx. The following characteristics were noted: age, presence of symptoms caused by urethritis, and results of nucleic acid amplification testing of urine samples. Antimicrobial administration and treatment outcome were not included because the objective of this study was to survey for the presence of drug-resistant *C. trachomatis* strains.

Clinical specimens and chlamydial cultures for isolation of C. trachomatis

A cotton swab (Copan Diagnostics Inc., Italy) was inserted into throat and swab the pharynx. For urethra, a cotton swab was gently inserted about 3 cm into the urethra and gently rotated. Each swab was placed in a tube containing 0.5 mL of 10mM

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) • NaOH buffer containing sucrose (0.075g/ml) and L-glutamic acid (0.72mg/ml) buffer with micro beads followed by preservation at -70°C until cultured for *Chlamydia spp*.

We performed chlamydial culture of clinical swab specimens using a previously described method [17]. Frozen tubes containing swabs were quickly thawed in a water tank set at 37°C, after which the tubes were stirred using a vortex mixer to release the epithelial cells and chlamydial organisms from the cotton swab. The epithelial cells were sonicated using the Bioruptor® UCD-200T Ultrasonic Wave Disruption System (Cosmo Bio Co. Ltd., Tokyo, Japan). Following centrifugation at 300 × *g* for 3 min at room temperature, 0.25 mL of supernatant was placed on McCoy cells that had been cultured as confluent monolayers in a 24-well cell culture plate (Corning Costar Corp., Corning, NY, USA). The plate was centrifuged (860 × *g*, 25°C, 60 min) using a Hitachi himac CR21E centrifuge (Hitachi Koki Co. Ltd., Tokyo, Japan) to adhere chlamydial organisms to epithelial cells. One mL of Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) including 1 μg/ml of cycloheximide, 10 μg/ml of kanamycin, 10 μg/ml of vancomycin, 10 μg/ml of amphotericin B and

supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) was added to each well, and the inoculated cells were incubated at 37°C in 5% CO_2. Cell conditions were monitored, at appropriate intervals using a phase-contrast microscope, for evidence of the cytopathic effect. Immediately following observation of cell bursts, the cells were removed from the plates with sterile rubber fragments, suspended in 1 mL/well of sucrose-phosphate-glutamate (SPG) buffer and preserved at -70°C.

Fluorescent staining

Fluorescent staining to observe chlamydial inclusion was performed as previously described [17]. McCoy cell monolayers prepared on a cover glass (14 mm in diameter) were stained 48-50 h post-inoculation, during the preservation on incubated McCoy cells detailed in the previous section. The cells were fixed with 99.5% ethanol and stained with fluorescein-conjugated monoclonal antibody directed against a genus-specific antigen (*Chlamydia* FA Seiken [DFA stain]; Denka Seiken, Tokyo, Japan) Matsumoto *et al*. [18].

Drug susceptibility testing

Drug susceptibility testing was performed according to the standard method of the Japanese Society of Chemotherapy [19]. Two antimicrobial agents, levofloxacin (LVFX; Daiichi Sankyo, Co., Ltd., Tokyo, Japan) and azithromycin (AZM; Pfizer Inc., New York, NY, USA), which are recommended in treatment guidelines [4, 5] were chosen for susceptibility testing. LVFX and AZM were obtained for a fee from Sigma-Aldrich Co. Ltd. and LKT Laboratories, Inc., respectively.

Preliminary experiments

Preliminary experiments using standard strains of *C. trachomatis*, including a reference strain, were performed to evaluate the quality of the HeLa229 cell line and the drug-susceptibility measuring system [19]. HeLa229 cells purchased from the National Institute of Infectious Diseases were cultured as described above in DMEM containing 10% heat-inactivated FBS. Cell conditions were monitored at appropriate intervals using a phase-contrast microscope. Chlamydial strains used in these preliminary experiments included serovar A, C, D/UW-3/Cx (reference strain), F, G and H. The MICs of LVFX and AZM against these strains were measured before testing of the clinical isolates.

Ethics

This clinical study was approved by the Okayama University Institutional Review Board prior to study initiation (Registration no.; 1519). The study was registered with the University Hospital Medical Information Network (UMIN), Japan (Registration no.; R000027274). Participants reviewed the informed consent document and received individual counseling with a thorough discussion as to alternative treatment, including nonparticipation.

Results

Patient characteristics

A total of 18 patients diagnosed as urethritis due to *C. trachomatis* were picked up from our database and enrolled in this study. The mean age of the 18 patients was 25.7±7.8 years. Symptoms included micturition pain in 8 patients, pus discharge in 4 patients, both in 4 patients and none in 1 patient. Pharyngitis symptomology was not observed. All patients were diagnosed with chlamydial urethritis by polymerase chain reaction (PCR) or standard displacement amplification (SDA). During the 18 patients, single-dose AZM 2000 mg were administered for 10 patients, single-dose AZM 1000 mg for 6 patients and once-daily 500 mg LVFX for 7 or 14 days for 2 patients. Cure in 14 patients out of 18 have been confirmed using their urine samples, however, examination using urine samples after antimicrobial administration were not performed in 4 patients. Because of residual symptoms, once-daily 500 mg LVFX for 7 days were additionally administered for 2 patients; 1 with single-dose AZM 2000 mg (Patient No. 10), 1 with single-dose AZM 1000 mg (Patient No. 14), and the 2 patients were diagnosed as cure after additional administration.

Preliminary experiments

HeLa229 cells were appropriately prepared and infected with the standard strains. LVFX MICs of serovar A, C, D/UW-3/Cx (reference strain), F, G and H were 0.25, 0.25, 0.125, 0.25, 0.25, and 0.125 μg/mL, respectively (Table 1). The AZM MICs of serovar A, C, D/UW-3/Cx (reference strain), F, and G were all 0.25 μg/mL (Table 1). Neither the HeLa229 cell line nor the standard *C. trachomatis* strains, including the D/UW-3/Cx reference strain, exhibited unsuitability for use in the study.

In vitro drug susceptibility

During the 18 patients, *C. trachomatis* strains were isolated from only their urethra in 11 patients, and *C. trachomatis* strains were isolated from both their urethra and pharynx in 7 patients. Namely, 18 strains isolated from urethra and 7 strains isolated from pharynx were evaluated.

Drug susceptibilities of the clinical isolates are shown in Table 2. The MICs of LVFX and AZM against urethral isolates ranged from 0.125 μg/mL to 0.5 μg/mL. MIC_{50}/MIC_{90} values of LVFX and AZM were 0.25/0.5 μg/mL and 0.25/0.25 μg/mL, respectively. In contrast, the LVFX and AZM MICs of chlamydial strains isolated from

the pharynx ranged from 0.125 μg/mL to 0.5 μg/mL and from 0.125 μg/mL to 0.25 μg/mL, respectively. MIC_{50}/MIC_{90} values of LVFX and AZM were 0.5/0.5 μg/mL and 0.25/0.25 μg/mL, respectively. In patients from whom chlamydial strains were isolated from both the urethra and the pharynx, the MICs of LVFX and AZM between strains isolated from urethra and pharynx were identical in all six patients (1 pharyngeal strain could not be evaluated and was not harvestable due to bacterial contamination that prevented measurement of the AZM MIC).

Discussion

In the present study, the MICs of LVFX and AZM of 18 chlamydial strains isolated from urethra and 7 strains isolated from pharynx were measured using the standard method published by the Japanese Society of Chemotherapy.

Reports on the drug susceptibilities of *C. trachomatis* are available for both Japan and other countries. According to reports from foreign countries such as the USA and Russia, drug resistance of *C. trachomatis* has been observed with isolates from treatment refractory STIs [12-15]; however, similar results have not been reported from Japan. Takahashi et al. did not isolate drug-resistant *C. trachomatis* from the urethras of Japanese males [16]. In contrast, studies of drug susceptibilities in *C. trachomatis* pharyngeal isolates have not been published from any part of the world. Despite the small number of isolates in the present study, our results support the report from Takahashi et al. No drug-resistant strains of *C. trachomatis* were isolated from the urethra and pharynx in Japanese patients.

In the present study, the MICs of LVFX and AZM in chlamydial strains isolated from the urethra were 0.125-0.5 μg/mL and 0.125-0.25 μg/mL, respectively.

MIC_{50}/MIC_{90} values of LVFX and AZM were 0.25/0.5 μg/mL and 0.25/0.25 μg/mL, respectively. According to Takahashi et al. [16], the MIC_{50}/MIC_{90} values of LVFX were 0.25/0.5 μg/mL in 2009 and 0.25/0.5 μg/mL in 2012. The MIC_{50}/MIC_{90}s of AZM were 0.063/0.063 μg/mL in 2009 and 0.031/0.031 μg/mL in 2012. AZM MICs reported in the present study were higher than those in their study. Regarding chlamydial culture using McCoy cells before MIC measurement, and complex protocol for measuring the MICs of chlamydial strains might have affected the results. However, the results of our preliminary experiments showed that the LVFX MIC of the reference strain, *C. trachomatis* D/UW-3/Cx, was reasonable at 0.125 μg/mL [19]. Thus, our procedures consist of the master dilution of antimicrobials, preparation of HeLa229 cells, inoculation and incubation steps, and fluorescent staining, with the ultimate decision that the MICs are suitable for the MIC measurement of chlamydial strains. Therefore, there might be other reasons; narrow recruitment area; recruiting time difference (from 2009 to 2012 in the study reported by Takahashi et al. and from 2103 to 2014 in the present study); method of collecting swab specimens.

Pharyngeal infection of *C. trachomatis* is a relatively recent focus of

investigation, and it has been reported that pharyngeal infection of *C. trachomatis* is refractory against some antimicrobials [8-11]. Chlamydial culturing from a pharyngeal specimen is very difficult due to the lower number of chlamydial organisms in a mouth wash; the invasive procedure for obtaining pharyngeal swab specimens; and frequent contamination by other organisms. The present study is the first report of *C. trachomatis* strains isolated from the pharynx. No drug-resistant strains were observed in pharyngeal *C. trachomatis* isolates. Our results might be significant for clinicians who must treat patients at the first visit. Based on current surveillance trends, Japanese clinicians should administer antimicrobials against chlamydial infections as recommended by the published guidelines. However, drug-resistant *C. trachomatis* strains or treatment refractory STIs might appear in Japan in the near future, as they have in other countries [12-15]. If clinicians face treatment-refractory cases, they should consider the presence of a drug-resistant *C. trachomatis* strain, penetration of antimicrobials into the tissue and other factors. Thus, the surveillance of drug-resistant *C. trachomatis* is a significant tool, and MICs should be measured despite the complicated (multi-step, time- and labor-intensive) procedure that must be used.

There are some limitations in this study; first, as mentioned above, the present study included a small number of strains collected from a limited recruitment area; second, specimens were cultured before MIC measurement without cloning (e.g., plaque cloning [20]); third, pairs of chlamydial strains, isolated from urethra and pharynx of the same patient, should be evaluated their identity by ompA sequencing or multilocus sequence typing [21, 22]. While the collection of swab specimens is an invasive procedure, and the cloning of chlamydial strains and procedures for MIC measurements can be quite troublesome, larger and long-term surveillance for drug-resistant *C. trachomatis* is necessary in any country.

In conclusion, our data suggest that both LVFX and AZM are effective antimicrobials for sexually transmitted infections due to *C. trachomatis* in Japan. Further studies that include a larger number of isolates, not only from the urethra and uterine cervix but also from the pharynx, are necessary for the surveillance of drug-resistant *C. trachomatis*.

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This work was not supported by any company.

Conflict of interest

None to declare

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Figure legends

Table 1. MICs of *C. trachomatis* standard strains against levofloxacin and azithromycin.

Table 2. Levofloxacin and azithromycin MICs of clinical isolates from urethra and pharynx.

Table 1

	Serovar					
	A	C	D/UW-3/CX	F	G	H
Levofloxacin MIC (μg/mL)	0.25	0.25	0.125	0.25	0.25	0.125
Azithromycin MIC (μg/mL)	0.25	0.25	0.25	0.25	0.25	0.25

Table 2

	Clinical isolates No.																							Standard strains	
Patient No.	1		2		3		4		5	6	7		8		9	10	11	12	13	14	15	16		17	18		
Clinical specimen No.	394	395[a]	534	535[a]	546	547[a]	648	649[a]	844	848	908	909[a]	916	917[a]	942	946	950	958	974	998	1002	1008	1009[a]	1052	1068	D/UW-3/CX	H
Levofloxacin MIC (μg/mL)	0.25	NE	0.25	0.25	0.25	0.25	0.125	0.125	ND	0.25	0.5	0.5	0.5	0.5	0.125	0.125	0.125	0.125	0.25	0.125	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Azithromycin MIC (μg/mL)	ND	ND	0.125	0.125	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.25	0.25	0.125	0.125	0.125	0.125	0.25	0.25	0.125	0.25	0.25	0.125	0.25	0.25	ND

[a] Clinical isolates from pharynx
ND: Not done
NE: Not evaluable and harvestable due to bacterial contamination