METHOD FOR *IN VITRO* DETERMINATION OF CHLAMYDIAL SUSCEPTIBILITY (MINIMAL LETHAL CONCENTRATION; MLC) TO ANTIMICROBIAL AGENTS

—STANDARD METHOD OF THE JAPAN SOCIETY OF CHEMOTHERAPY (in 1991)—

[Manual for the Determination of MLC for *Chlamydiae*]

Principle: The method is based on the determination of the minimum drug concentration which completely inhibits the re-formation of chlamydial inclusions in infected HeLa 229 cells even after elimination of the drug from the culture medium.

Caution in Handling *Chlamydia*:

All the cautions stated in the Manual for the Determination of the MIC for Chlamydia (Chemotherapy 40 (3): 308~314, 1992) remain valid in this Manual.

Note 1: Although MLC (minimal lethal concentration)[2,3] MCC (minimal chlamydicidal concentration)[4,5] and MBC (minimal bacteriocidal concentration)[6] have the same meaning, the Committee decided to use MLC as the standard term.

Note 2: The Committee conducted a comparative study on the method based on the above principle (method A) and the following method (method B). That is, in method B, the drug is eliminated, *Chlamydiae* are recovered from the infected HeLa cells by ultrasonic treatment, etc., and the recovered organisms are reinoculated into a freshly prepared HeLa cell culture. After repeating this procedure twice for, i.e. done a total of 3 times, the presence or absence of chlamydial inclusions is examined. However, the procedures of method B are onerous, and it was found that measured values differ greatly between institutions since the recovery rate of *Chlamydiae* is not always constant; it depends on the sonicator, time of treatment, etc. Accordingly, the Committee decided to select method A, which is simple and convenient and reflects relatively well the clinical efficacy of drugs.

Note 3: When performing MLC determination, it is necessary to determine the MIC at the same time, employing the same infection system. That is, an MLC value is considered to be valid only when the result of MIC determination is confirmed to be within the MIC range established for the reference strain by the Committee.

Note 4: Although the Committee does not require the MLC as essential data for evaluating the efficacy of a drug against *Chlamydiae*, it is desirable to determine the MLC together with the MIC.

Note 5: The details regarding the host cell line, culture medium, inoculum size including determination of the infectious titer (IFU), reference strains, method of dilution of drugs, temperature conditions and method of judgment should be in accord with the Manual for MIC Determination.

[Preparation of Cell Cultures]

1. Use plastic, 24-well cell-culture plates for MLC determination.
2. Dispense 1 ml of culture medium [Eagle's MEM+heat-inactivated fetal calf serum (FCS)] containing $1.5-2.0\times10^5$ HeLa 229 cells per ml into each well (in which a cover glass with a diameter of 14 mm has already been placed) on the plate.
3. Culture for 24 hrs in an incubator maintained at 37°C with an atmosphere containing 5% CO_2. After confirming the growth of a confluent HeLa cell monolayer, remove the culture fluid from the wells by aspiration. As a rule, DEAE-dextran treatment should not be carried out prior to *Chlamydiae* inoculation.

[Inoculation of *Chlamydiae*]

4. Inoculate 10^4 IFU (0.25 ml of a 4×10^4 IFU/ml suspension) of the *Chlamydiae* strain to be tested into each well. At the same time, it is necessary to also inoculate the reference strains designated by the Committee so that it can be verified that the determination method is being properly executed.
5. Perform centrifugal adsorption at $500-900 \times g$ for 1 hr at ambient air temperature.
6. Remove the supernatant by aspiration.

[Culture in the Presence of Drug]

7. Dispense 1 ml of each preparation of culture medium (Eagle's MEM+heat-inactivated FCS+cycloheximide at a final concentration of 1 μg/ml) containing one concentration of the test antibiotic (diluted in accordance with the master dilution method) into each well.
8. Perform the culture under the following conditions.

   Culture conditions for *Chlamydia trachomatis*:

   37°C (permissible range: ±1°C) for 72 hrs, in a 5% CO_2 incubator.

   Culture conditions for *Chlamydia psittaci*:

   37°C (permissible range: ±1°C) for 48 hrs, in a 5% CO_2 incubator.

   Culture conditions for *Chlamydia pneumoniae*:

   35°C (permissible range: ±1°C) for 72 hrs, in a 5% CO_2 incubator.

[Removal of Drug]

9. Remove the culture fluid by aspiration.
10. Rinse the infected cells 3 times each with 1 ml of Eagle's MEM prewarmed to 37°C.
11. Dispense 1 ml of the drug-free medium (Eagle's MEM+heat-inactivated FCS+cycloheximide at a final concentration of 1 μg/ml) to each well.
12. Culture for 24 hrs in a 5% CO_2 incubator at 37°C for *C. trachomatis* and *C. psittaci*, and at 35°C for *C. pneumoniae*.
13. Replase the culture fluid with drug-free, fresh medium (Eagle's MEM+heat-inactivated FCS+cycloheximide at a final concentration of 1 μg/ml) and continue culturing for another 48 hrs for *C. trachomatis* and *C. psittaci*, and 24 hrs for *C. psittaci*.

[Determination of MLC]

14. After removing the culture fluid, stain by the DFA method in accordance with the Manual for the Determination of MIC for *Chlamydiae* and determine the MLC.
15. Observe the entire visual field with a fluorescence microscope at a magnification of ×100 and classify chlamydial inclusions into the following 3 types (refer to the attached photographs).

   Normal inclusions (photo A): Inclusions as seen in the control samples when determining MIC or MLC or inclusions with a similar size. Inclusions of this type are clearly observed at ×100.

   Small inclusions (photo B): The size of inclusions of this type is clearly smaller than that of the normal type, but at ×100 they can be easily identified as inclusions.

   Microinclusions (photos C & D): At ×100, these inclusions are observed as smaller or larger spots, but at ×200 the fluorescent microbodies can be identified as inclusions each containing several chlamydial bodies.
16. Re-formation of inclusions is judged to have been inhibited if no normal inclusions or small inclusions are observed.

   Microinclusions are interpreted as an inhibition of re-forming inclusion, since they are not reformed by repeating culture. The minimal concentration which inhibits the re-formation of normal or small inclusions is designated as the MLC of the drug tested.

[Reference Data]

The results of the Committee's determination of the MLCs of the reference strains are shown in the table

below. The variation in the MLC values is larger than that in the MIC values, and thus the ranges of the MLC are rather large. The Committee considers this to be unavoidable.

	C. trachomatis D/UW-3/Cx	*C. psittaci* Budgerigar No.1	*C. pneumoniae* TW-183
Minocycline	0.125-0.5	0.5-4.0	0.063-0.125
Doxycycline	0.125-1.0	4.0-8.0	2.0-4.0
Erythromycin	2.0-4.0	16-32	2.0-8.0
Clarithromycin	0.03-0.125	0.5-2.0	1.0-2.0
Ofloxacin	2.0-16	64-128	1.0-2.0

(μg/ml)

References

1) Committee for Investigating Method on MIC of *Chlamydiae* (Chairman: Yoshiaki Kumamoto): Method for *In Vitro* Determination of Chlamydial Susceptibility (Minimum Inhibitory Concentration; MIC) to Antimicrobial Agents-Standard Method of Japan Society of Chemotherapy-(revision in 1991), Chemotherapy 40: 308~314, 1992
2) Nagayama A, Nakano T, Taen H: *In vitro* activities of ofloxacin and four other new quinoline-Carboxylic acids against *Chlamydia trachomatis*. Antimicrob. Agents Chemotherapy 32: 1735~1737, 1988
3) Hessen F W, Muytjens H: *In vitro* activities of ciprofloxacin, norfloxacin, pipemidic acid, cinoxacin and nalidixic acid against *Chlamydia trachomatis*. Antimicrob. Agents Chemotherapy 25: 123~124, 1984
4) Mourad A, Sweet R L, Sugg N, Schachter J: Relative resistance to erythromycin in *Chlamydia trachomatis*. Antimicrob. Agents Chemotherapy 18: 696~698, 1980
5) Hammgret M R, Gleyzer A: *In vitro* activity of a group of broad-spectrum cephalosporins and other β-lactam antibiotics against *Chlamydia trachomatis*. Antimicrob. Agents Chemotherapy 23: 493~494, 1983
6) Bowie W R, Lee C K: Prediction of efficacy of antimicrobial agents in treatment of infections due to *Chlamydia trachomatis*. J. Infect. Dis. 138: 655~659, 1978

[Appendix]

This report was prepared by the Committee for Investigating the Method for Determining the MIC for *Chlamydiae*. Effort was made to standardize the determination method so that it would be internationally acceptable. This Manual was presented at the 39th meeting of the Japan Society of Chemotherapy, on June 5, 1991.

The members of the Committee:

Chairman Yoshiaki Kumamoto (Dept. of Urology, Sapporo Medical College)

Deputy Chairman Akira Matsumoto (Dept. of Microbiology, Kawasaki Medical School)

Ariaki Nagayama (Dept. of Microbiology, School of Medicine, Fukuoka University)

Rinzo Soejima (Division of Respiratory Disease, Dept. of Medicine, Kawasaki Medical School)

Katsuya Hirai (Dept. of Veterinary Microbiology, Faculty of Agriculture, Gifu University)

Sou Hashizume (Japan Poliomyelitis Research Institute)

Toshikatsu Hagiwara (Central Virus Diagnostic Laboratory, National Institute of Health)

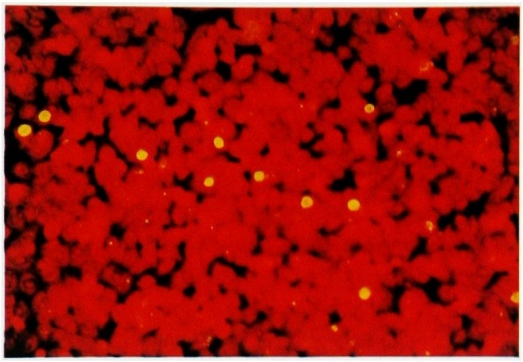

Photo. A Normal inclusions [Magnification: × 100 (10×10)]
Inclusions formed in the absence of drug.

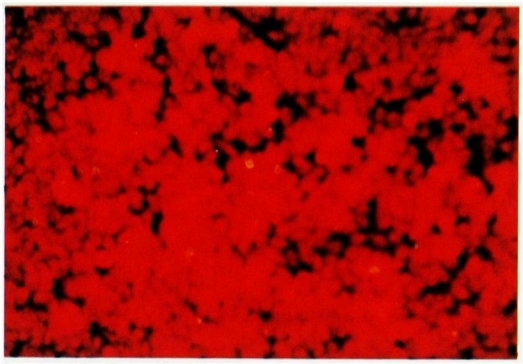

Photo. B Small inclusions [Magnification: ×100 (10×10)]
Incomplete inclusions formed in the presence of 0.5 μg/ml of Ofloxacin.

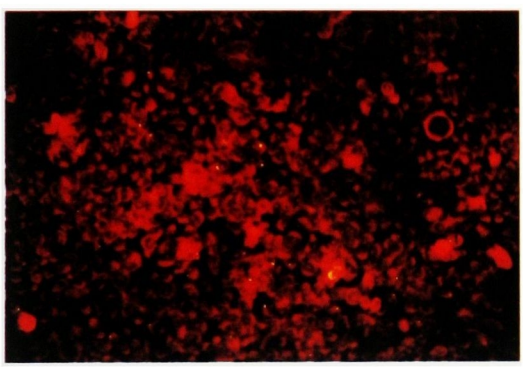

Photo. C Microinclusions [Magnification: ×100 (10×10)]
Fluorescent microbodies formed in the presence of 1.0 μg/ml of Ofloxacin. These are not judged as formation of inclusions.

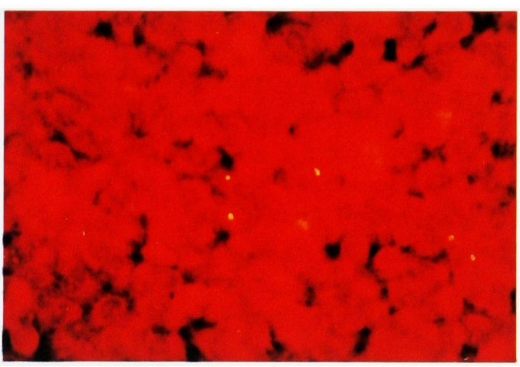

Photo. D Microinclusions [Magnification: ×200 (20×10)]
At ×200, fluorescent microbodies can be identified as microinclusions each containing several chlamydial bodies. These are not judged as formation of inclusions.