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)

[Manual for *Chlamydiae* MIC Determination Method]

Principle: The method is based on the determination of the minimum drug concentration which completely inhibits the formation of chlamydial inclusions in HeLa 229 cell cultures *in vitro*.

Causions in Handling of *Chlamydiae*:

There is the danger of laboratory personnel being infected by *Chlamydiae*. In particular, *C. psittaci* is given a pathogenic rating of 3, and laboratories intending to work with this microbe are required to perform all operations in a safety cabinet placed in a laboratory equipped with double-doors and maintained under negative air pressure. For *C. trachomatis*, it is sufficient to perform all operations in a safety cabinet, but in any case all contaminated equipment, waste liquids, etc., must, in principle, be sterilized in an autoclave. In view of the biohazards presented by *Chlamydiae*, suspensions of *Chlamydiae* must absolutely never be pipetted using the mouth.

1. Use plastic, 24-well culture plates for the MIC 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.
   a Refer to [Appendix A. 1. a.] for details regarding the use of HeLa 229 cells.
   b. Refer to [Appendix A. 1. b.] for details regarding the method for maintenance and use of HeLa 229 cells.
   c. Refer to [Appendix A. 2. a.] for details regarding Eagle's MEM.
   d. Refer to [Appendix A. 2. b.] for details regarding the heat-inactivated fetal calf serum (FCS).
3. Culture for 24 hrs in an incubator mainained at 37°C with an atmosphere containing 5% CO_2. After confirming growth of a confluent 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.
   a Refer to [Appendix A. 3.] for details regarding the use of DEAE-dextran.
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 will be desirable to also inoculate the reference strains designated by the Committee so that it can be verified that the determination method is being properly executed.
   a. Refer to [Appendixes A. 4. & A. 5.] for details regarging the size of the inoculum.
   b. Refer to [Appendix A. 6.] for details regarding the reference strains.
5. Perform centrifugal adsorption at 500—900×g for 1 hr at ambient temperature[1].
6. Remove the supernatant by aspiration.
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.
   a. Refer to [Appendix A. 7. a.] for details regarding the master dilution method.
   b. Refer to [Appendix A. 7. b.] for details regarding the upper limit for the antibiotic concentration.

c. Refer to [Appendix A. 7. c.] for details regarding the method for expressing the antibiotic concentration.

d. Refer to [Appendix A. 7. d.] for details regarding cycloheximide.

8. Perform the culture under the following conditions.

Culture conditions for *Chlamydia trachomatis*:

37°C (permissible range: ±1°C), in a 5% CO_2 incubator; check for the presence/absence of inclusion bodies after 72 hrs.

Culture conditions for *Chlamydia psittaci*:

37°C (permissible range: ±1°C), in a 5% CO_2 incubator; evaluate the results after 48 hrs since this species grows more rapidly than *C. trachomatis*.

Culture conditions for *C. pneumoniae* (TWAR)-related strains:

35°C (permissble range: ±1°C), in a 5% CO_2 incubator; evaluate the results after 72 hrs.

a. Refer to [Appendix A. 8.] for details regarding the incubation temperature.

9. Remove the supernatant by aspiration, then stain the cover glasses by the IF method and evaluate.

a. Refer to [Appendix A. 9.] for details regarding the IF method.

10. Observe the entire visual field with a fluorescence microscope at a magnification of ×100. Total absence of inclusion bodies should be rated as negative for chlamydial growth, and the lowest drug concentration which completely inhibits inclusion body formation should be designated as the minimal inhibitory concentration (MIC) of the test drug for the test strain.

a. Refer to [Appendix A. 9. b.] for details regarding the magnification to be used for observations.

[Appendix A]

1. Cells to be Employed

a. Cell Line

Use the HeLa 229 cell line. Both HeLa 229 cell and McCoy cells are widely employed[2-5], but since HeLa 229 cells are of human origin, it can be reasoned that data obtained with this cell line will be more accepted internationally. In addition, HeLa 229 cells are considered to be more susceptible than McCoy cells to infection by *C. psittaci* and *C. pneumoniae* (TWAR)-related strains. For these reasons, the HeLa 229 cell line is stipulated for use in this test method.

Furthermore, the HeLa 229 strain being maintained at the Central Virus Diagnostic Laboratory (Chief: Shudo Yamazaki) of the National Institute of Health (address: 4-7-1 Gakuen, Musashimurayama City, Tokyo, Japan 190-12; phone: 0425-61-0771) is stipulated as the strain to be employed for this test method.

b. Method for Maintenance of HeLa 229 Cell Line

Cells of HeLa 229 should be transferred at intervals of 3-4 days (when a 1/3 to 1/4 dilution is transferred, confluent growth should be attained in 3-4 days). In the event that the growth rate is poor, the culture may be contaminated with *Mycoplasma*. On the basis of that assumption, MC 210, a mycoplasma-eliminating agent (a product of Dainippon Pharmaceutical Co., Ltd.), should be added to the HeLa 229 cell culture in accordance with the instructions accompanying the drug, and any contaminating *Mycoplasma* should be purged from the culture by performing 2-3 transfers. For long-term preservation of the HeLa 229 cell line, cultures should be frozen. Prior to the use of such a frozen culture of cells, perform at least two subcultures.

2. Liquid Culture Media

a. MEM

Culture HeLa 229 cells in Eagle's MEM.

b. Heat-Inactivated Fetal Calf Serum

Employ FCS after it has been heat-inactivated. Be aware that the variation between lot is greater than that between products of different makers. The concentration of FCS to be added to the medium should be 10% for cell subcultures, and, for the MIC determination, 5% for *C. psittaci*, 8% for *C. trachomatis* and 10% for *C. pneumoniae* (TWAR)-related strains[6] (Increase the added FCS concentration when the growth rate is slow).

3. DEAE-dextran

In principle, do not employ DEAE dextran because it would damage the cells and make the operations more complicated. However, use DEAE dextran in the case that the MIC of test strain is 2 or more dilution tubes different from that of the standard strain, and in special cases such as *C. pneumoniae* (TWAR)-related strains[6].

4. Inoculum Size (Infectious Titer)

Inoculate each isolated strain of *C. trachomatis* to HeLa 229 cells and allow to propagate. After a sufficient number of inclusion bodies is observed to have been formed, replace the culture medium with an equal volume of SPG, scrape the infected cells from the vessel with a rubber policeman, release the *Chalmydia* from the cells by disruption with ultrasonic waves, etc, and centrifuge for 5 min. at 500—900×g. The supernatant is the *C. trachomatis* suspention. Prepare suspensions of *C. psittaci* in the same manner. Store the suspensions by freezing at blow −70°C, and use after thawing. If the concentrations of *C. trachomatis* and *C. psittaci* are too high (10^6 IFU/well of more) at the time of inoculation, the cells will be damaged due to the immediate toxicity[7,8] of the *Chlamydia*. Thus, care must be taken that the infectious titer is not excessive. Conversely, if the titer is too low (10^2 IFU/well or less), the number of inclusion bodies formed will be too small for ready evaluation of the results. Therefore, the appropriate inoculum size is 10^4 IFU/well[5]. The volume of the inoculum should be large enough so that it will not evaporate dry at the time of adsorption; thus, a volume of 0.25 ml (4×10^4 IFU/ml) is stipulated.

5. Method for Determining IFU (Inclusion-forming Unit)

a. Principle

Inoculate a constant volume of *Chlamydiae* suspensions prepared as serial dilutions onto HeLa 229 cells grown as a confluent monolayer. After culturing, count the number of inclusion bodies formed. Finally, calculated the IFU per unit volume of *Chlamydiae* suspension from the number of inclusion bodies, the volume of inoculum and the dilution factor.

b. Method

1) Follow the procedures described below to prepare 10-fold dilution series of a chlamydial suspension of unknown infectious titer.

a) Discard the medium from wells containing HeLa 229 cells that have been cultured for 2-3 days after being infected.

b) Fill each well with a suitable volume of SPG (sucrose-phosphate-glutamic acid medium), and scrape off the cells with a rubber policeman. Pool the fluid from each well as the infected cell suspension (in the case of using a 24-well plate, it will be appropriate to dispense approximately 1 ml of SPG to each well).

Prepare SPG as follows:

Dissolve sucrose 37.5 g, KH_2PO_4 0.26 g $Na_2HPO_4 \cdot 2H_2O$ 0.7645 g and glutamic acid 0.36 g in 500 ml of distilled water, and then pass this solution through a 0.2-μm membrane filter to sterilize it (it is recommended to add streptomycin, or kanamycin and vancomycin to a final concentration of 100 μg/ml each).

c) To release the *Chlamydiae*, disrupt the infected cells by ultrasonic treatment (15-20 sec. is sufficient) or by repeated aspiration and ejection with a syringe equipped with a

needle. Centrifuge for 5 min. at 500—900×g, and employ the supernatant as the stock *Chlamydiae* suspension.

d) Prepare a 10-fold dilution series using SPG with 1−2 ml of the stock *Chlamydiae* suspension. Dilution to $10^{-8}-10^{-9}$ will be sufficient. Hold the stock suspension and the dilution series in ice.

2) Dispense 0.25 ml of each dilution to the wells containing confluent growth of HeLa 229 cells prepared in accordance with steps 1 through 3 of the Manual. It will be desirable to place the same dilution in 4 wells, and then calculate the mean number of inclusion bodies for those 4 wells. Six different dilutions should be tested: from 10^{-3} to 10^{-8} Excess stock chlamydial suspension should be stored at blow −70°C. At the time of use, it should be subjected to mild ultrasonic treatment, diluted with SPG to 10^4 IFU/ml on the basis of the IFU that has been actually determined in the above, and then employed to infect cultured HeLa 229 cells.

3) Follow the instructions provided in items 4 through 9 of the Manual to carry out the steps of adsorption, culture, staining for inclusion bodies and observation for inclusion bodies. Count the number of inclusion bodies for the entire area of the cover glass. The determined titer can be considered accurate if there is approximately a 10-fold difference between the titers determined for two consecutive dilutions for which it was possible to calculate the mean number of inclusion bodies for 4 wells, for example, between dilutions of 10^{-5} and 10^{-6}. However, even if the mean number of inclusion bodies for two consecutive dilutions of the chlamydial suspension is determined, the IFU of the stock chlamydial suspension is calculated, and the dilution factor is calculated on the basis of that value so that the inoculum will be 10^4 IFU/well, there will be almost no influence on the determine MIC value, even if there is some difference between the mean values of two dilutions.

Example of Calculation of IFU (*C. trachomatis* D strain)

Dilution	No.of inclusion bodies	Mean No./well	IFU of stock suspension
10^{-5}	112, 118, 101, 125	114	4.6×10^7 ≒4.4×10^7
10^{-6}	12, 10, 8, 11	10.3	4.1×10^7

On the basis of the mean value in the above table, the stock chlamydial suspension should be diluted 1, 100-fold with SPG and dispensed at 0.25 ml/well to infect the HeLa 229 cells, and the MIC should then be determined.

For the determination of the MICs of clinical isolates of *Chlamydiae*, it will be desirable to employ the strain with as little subculturing as possible from the time of isolation. To achieve this, at the time of propagating the microbes, care must be taken to avoid excessive dilution of the inoculum.

6. Reference Strains

At the time of determining the MICs of a clinical isolate, it will be desirable to simultaneously determine the MICs of the appropriate reference strain so that the accuracy of the measurement system can be confirmed.

When reporting the MIC results for the test strain, the MICs of the tested antibiotics for the reference strains should also be recorded.

For clinical isolates of *C. trachomatis*, the reference strain is *C. trachomatis* D/UW-3/Cx, while in the case of clinical isolates of *C. psittaci*, the reference strain is *C. psittaci* Budgerigar No.1. Both of these reference strains are available upon request from the Central Virus Diagnostic Laboratory, National Institute of Health address: 4-7-1 Gakuen, Musashimurayama City, Tokyo, Japan 190-12; phone: 0425 -61-0771)

7. Antibiotic Dilutions

a. Method for Diluting Antibiotics

Differences in the method employed for diluting antibiotics can result in differences in the concentrations of the drugs, which influences the MICs determine. The error can be large, especially in the case of preparing a dilution series using small volumes, or in the case of carrying out 3-fold dilutions many times. Accordingly, apply the following method to prepare the antibiotic dilutions. For each antibiotic, prepare a master dilution by dissolving the drug in distilled water (or whichever other diluent is stipulated for use with the antibiotic in question) at a concentration to be prepared for the MIC determination. At the time of use, add 1 ml of this master dilution to 9 ml of medium, thereby obtaining 10 ml of a 2-fold concentration solution. Next, by transferring a volume of 5 ml, it will be desirable to employ a pipet to prepare a 2-fold dilution series. This series should consist of a maximum of 6 dilutions.

b Upper Limit of Antibiotic Concentration

In the case of antibiotics such as β-lactams, which have high MICs, 128/μg/ml should be the maximum concentration prepared and employed in the MIC determination since inclusion bodies are observed even at high concentrations.

c Method for Describing Antibiotic Concentrations

With 1 μg/ml as the standard, employ the following figures to express the concentration:
Example: 0.002, 0.004, 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 (μg/ml).

d. Cycloheximide[4)]

The growth of *Chlamydiae* is enhanced if the protein synthesis of the host cells is inhibited, and the observation of inclusion bodies becomes easier. Therefore, for the determination of the MICs of the *Chlamydiae* isolate, Cycloheximide should be added to the medium to a final concentration of 1 μg/ml.

Prepare cycloheximide 100×stock as follows;

Dissolve glucose 5.4 mg, cycloheximide 10 mg in 100 ml of distilled water and then pass this solution through 0.2 μm membrane filter.

8. Incubation Temperature

From this reason, high incubation temperature influence the MICs determined. Incubation temperature must be kept strictly. Commonly *C. pneumoniae* (TWAR)-related strains grow more slowly than other *Chlamydiae* strains. However, infections titers after the culture of *C. pneumoniae* (TWAR)-related strains in incubation at 35°C are higher districtly than in incubation at 37°C[6)] This fact is expected the biological characteristic. For this reason, *C pneumoniae* (TWAR)-related strains should be cultured in incubation temperature at 35°C (permissible range: ±1°C).

9. Method for Evaluation of Results

a. Staining Method

The judgment of the MICs should be carried out by the IF method [MicroTrak® can be used only for *C. trachomatis*, while Orthochlamydia (FA), culture set® is capable of staining *C. trachomatis*, *C. psittaci* and *C. pneumoniae* (TWAR)-related strains. Remove the cover glass from each well, perform fixation for 15 min. in ethanol at room temperature, and perform staining in a dark place after drying. In the event that staining can not be performed immediately, the cover glass should be stored at −20°C after the fixation and drying steps have been completed. HeLa 229 cells show iodine-positive microbodies in the cytoplasm, and there is potential for these to be mistaken for inclusion bodies. For this reason, the iodine method is not appropriate for use in the detection of inclusion bodies in HeLa 229 cells.

b. Magnification for Observations

When a high power of magnification of ×400−1,000 is employed, a small number of deformed microinclusions in the cytoplasm of HeLa 229 cells can still be observed over several dilutions, and this makes it difficult to judge a complete disappearance of inclusion bodies. Accordingly, observation of the cells should be performed at ×100, so that such deformed microinclusions will be comparatively difficult to see.

[Reference data]

The following table presents the MICs determined by this Committee for the reference strains.

Antibiotic	*C. trachomatis* D/UW-3/Cx	*C. psittaci* Budgerigar No.1	*C. pneumoniae* TW-183
MINO	0.016~0.063 (μg/ml)	0.016~0.063 (μg/ml)	0.016~0.031 (μg/ml)
DOXY	0.031~0.063	0.016~0.063	0.031~0.063
EM	0.125~0.5	0.25 ~0.5	0.25 ~0.5
CAM	0.008~0.031	0.008~0.031	0.008~0.031
OFLX	0.25 ~1.0	0.25 ~1.0	0.5 ~1.0
ABPC	128<	128<	128<

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7) Moulder J W, Hatch T P, Byrne G I, Kellog K R: Immediate toxicity of high multiplicities of *Chlamydia psittaci* for mouse fibroblasts (L cells). Infect. Immun., 14: 277~289, 1976
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[Appendix B]

1. This report was prepared by the Committee for Investigating Method on MIC of *Chlamydiae*. Effort was made to standardize the determination method so that it would be internationally acceptable. This revision Manual was presented at the 39 th 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)

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

Sou Hashizume (Japan poliomyelitis reserch institute)

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2. A separate report deals with the method for determination of the MLC (minimum lethal concentration).