クラミジア MIC 測定法

―日本化学療法学会標準法―

≪クラミジア MIC 測定法マニュアル≫

〈原理〉: 被験クラミジアを接種した HeLa 229 細胞を薬剤存在下で培養し，封入体形成抑制を示す最小薬剤濃度を検定する。

〈クラミジア取扱い上の注意〉: クラミジアは実験室内感染を起こす可能性がある。特に *C. psittaci* は危険度3に指定され，その取扱いは2重ドア，陰圧室内に設置した安全キャビネット内で行なうことが義務付けられている。*C. trachomatis* については安全キャビネットのみで取扱うことができるが，いずれにおいても汚染器具類，廃液等は原則としてオートクレーブ滅菌を要する。クラミジア浮遊液を取扱う際に，ピペットを口で吸引することは以上のバイオハザードの観点から絶対に行なってはならない。

1) MIC 測定には24穴細胞培養用 plastic plate を用いる。

2) 1.5～2.0×10^5/ml の HeLa 229 細胞を含む培養液［Eagle's MEM＋熱非働化牛胎児血清（FCS)］を plate の各 well（直径 14 mm のカバーガラスを予め入れておく）に 1 ml ずつ分注する。
   ① HeLa 229 細胞の使用に関しては＜附記 A.1.a.＞参照
   ② HeLa 229 細胞の維持使用法に関しては＜附記 A.1.b.＞参照
   ③ Eagle MEM に関しては＜附記 A.2.a.＞参照
   ④ 熱非働化牛胎児血清（FCS）に関しては＜附記 A.2.b.＞参照

3) 37℃, 5% CO_2 incubator で24時間培養して confluent monolayer の形成を確認後，培養液を吸引除去する。原則的にクラミジア接種前の DEAE-dextran 処理は行なわない。
   ① DEAE-dextran の使用に関しては＜附記 A.3.＞参照

4) 各 well に 10^4 IFU/well（4×10^4 IFU/ml, 0.25 ml）の検討すべきクラミジア株を接種する。なお当委員会にて決定した reference strain も同時に接種し，測定法に誤りがないことを確認することが望ましい。
   ① 接種菌量に関しては＜附記 A.4. および A.5.＞参照
   ② Reference strain に関しては＜附記 A.6.＞参照

5) 室温 500～900×*g* にて1時間遠心吸着を行なう[1]。

6) 上清を吸引除去する。

7) 各薬剤濃度（Master dilution 法にて希釈）を含む培養液（Eagle's MEM＋熱非働化 FCS＋終末 1 μg/ml Cycloheximide）を各 well に 1 ml ずつ分注する。
   ① Master dilution 法に関しては＜附記 A.7.a.＞参照
   ② 薬剤濃度の上限に関しては＜附記 A.7.b.＞参照
   ③ 薬剤濃度の表示法に関しては＜附記 A.7.c.＞参照
   ④ Cycloheximide に関しては＜附記 A.7.d.＞参照

8) 下記の条件にて培養する。
   ［*C. trachomatis* 培養条件］: 37℃（±1℃ は許容範囲), 5% CO_2 incubator にて 72 時間培養し判定する。
   ［*C. psittaci* 培養条件］: 37℃（±1℃ は許容範囲), 5% CO_2 incubator にて 36 時間培養し判定する。
   C. psittaci は *C. trachomatis* に比べ，増殖が速い。
   ［*C. pneumoniae*（TWAR）関連株培養条件］
   : 35℃（±1℃ は許容範囲), 5% CO_2 incubator にて 72 時間培養し判定する。
   ① 温度条件に関しては＜附記 A.8.＞を参照

9) 培養液を吸引後，IF 法にて染色判定する。
   ① IF 法の使用に関しては＜附記 A.9.a.＞参照

10) 100 倍を判定倍率とし蛍光顕微鏡にて全視野を観察する。封入体がまったく認められないものを陰性と判定し，

封入体形成を抑制した最小薬剤濃度を MIC とする。

① 観察倍率に関しては<附記 A. 9. b. >参照

≪附記(A)≫

1. 使用細胞

a. 使用細胞の種類

HeLa 229 細胞を使用する。HeLa 229 細胞, McCoy 細胞いずれも良く使われているが[2~5], HeLa 229 細胞は人由来細胞株であるところから, これを用いた data は国際的により通用するものと考えられる。また, *C. psittaci* および *C. pneumoniae* (TWAR) 関連株では McCoy 細胞より HeLa 229 細胞のほうが感受性が高いと考えられる。以上の様な理由により HeLa 229 細胞を使用することにする。

なお, HeLa 229 細胞は国立予防衛生研究所ウイルス中央検査部(山崎修道部長)の保存株を使用することにする(連絡先:〒190-12 東京都武蔵村山市学園 4-7-1 Tel. 0425-61-0771)。

b. HeLa 229 細胞の継代法

3～4日間隔で継代する(1/3～1/4 に希釈し継代する時には3日～4日で confluent になる)。もし発育速度の悪い時は *Mycoplasma* の contamination の可能性があるため, マイコプラズマ除去剤 MC 210 (大日本製薬)を説明書に従って培養液に加え, 2～3代継代し, *Mycoplasma* を除去する。

細胞を長期保存する場合は凍結保存する。その凍結保存細胞を使用する場合は, 少なくとも2回以上継代の後使用する。

2. 培養液

a. MEM

HeLa 229 細胞には, Eagle's MEM が適している。

b. 熱非働化牛胎児血清 (Fetal calf serum)

使用する牛胎児血清(FCS)は熱非働化して用いる。メーカーによるよりも lot によるバラツキがあるので, この点を留意して使用する。濃度は細胞継代には 10% FCS, MIC 測定時の FCS 濃度は *C. psittaci* 5%, *C. trachomatis* 8%, *C. pneumoniae* (TWAR) 関連株は10%を使用する[6] (*Chlamydia* の growth rate の遅いもの程 FCS 濃度を上げる)。

3. DEAE-dextran

細胞が傷み, 操作が繁雑になるため, 原則的に使用しない。しかし, 標準株と比較して MIC が2管以上異なった場合, ならびに *C. pneumoniae* (TWAR) 関連株などの特殊な場合には使用する。

4. 接種菌量(感染力価)

C. trachomatis 分離株は, HeLa 229 細胞にて感染増殖させ, 充分に封入体が観察できた時点で, 培地を除去後, 等量の SPG (次項参照)を加えラバーポリスマンにて感染細胞を剝離する。超音波などにてその感染細胞からクラミジアを遊離させた上, 500～900×*g* にて5分遠心する。その上清を *C. trachomatis* 浮遊液とする。*C. psittaci* 浮遊液も同様に調整する。作成した浮遊液は −70℃ 以下にて凍結保存し, 使用時に解凍する。

C. trachomatis, *C. psittaci* いずれも接種時の濃度が高すぎる(10^6 IFU/well 以上)とクラミジアの immediate toxicity[7,8] により細胞に損傷を与え, 感染率もそれほど上がらない。また低すぎる(10^2 IFU/well 以下)と封入体数が少なく判定が困難である。10^4 IFU/well が適当な接種量である[5]。接種菌液の容量は吸着時に乾燥しないように 0.25 ml (4×10^4 IFU/ml) とする。

5. IFU (inclusion forming unit) の測定法

a. 原 理

confluent monolayer の HeLa 229 細胞に段階希釈した一定量のクラミジア浮遊液を接種, 培養の後, 生じた封入体数を測定して, 封入体数, 接種量, 希釈倍数から単位浮遊液量当りの封入体形成単位, すなわち IFU を算定する。

b. 方 法

① 未知感染力価のクラミジア浮遊液の 10 倍段階希釈系列を以下の方法に従って準備する。

i）増殖培養した感染2～3日後の感染細胞から培地を除去する。

ii）適量の SPG を加え，ラバーポリスマンにて細胞をかき取り，感染細胞浮遊液として集める（24穴プレート使用の場合には SPG 約 1 ml/well が適量である）。

SPG（sucrose-phosphate-glutamic acid medium）は以下の処方により調整する。

sucrose 37.5g，KH_2PO_4 0.26g，$Na_2HPO_4 \cdot 2H_2O$ 0.7645g，glutamic acid 0.36g を蒸留水 500 ml に溶解後，0.2 μmφ メンブランフィルターにて濾過滅菌する（ストレプトマイシンまたはカナマイシン，およびバンコマイシンを各終末 100 μg/ml 加えるとよい）。

iii）超音波（15～20秒でよい）などにて感染細胞からクラミジアを放出させ，500～900×g にて5分遠心し，上清をクラミジア浮遊液の原液とする。

iv）原液をもとに 1～2 ml 単位で SPG を用いて10倍希釈系列を作る。希釈は10^{-8}～10^{-9}までで充分である。原液や希釈系列は氷中に保存する。

② マニュアル 1)～3) に従って準備した confluent の HeLa 229 細胞に各希釈液 0.25 ml を接種する。同一希釈液を4穴に接種し，封入体数を4穴の平均値として算出することが望ましい。したがって，実際に用いる希釈液は 10^{-3}～10^{-8} の6段階である。残りの原液は－70℃以下に凍結保存し，使用に際して軽く超音波処理後，実測して求めた IFU に合せて SPG にて希釈後，感染に用いる。

③ 吸着，培養，封入体の染色，観察はすべてマニュアル 4)～9) に従って実施する。封入体数はカバーガラス全域について測定し，封入体数測定可能な希釈液の間，例えば 10^{-5} と 10^{-6} の間で4穴の平均値がほぼ10倍の違いがあれば力価測定値は良好と断定できる。下表のごとく，多少のズレがあっても異なった希釈度間で平均値を求めて，原液の IFU 値を算定し，これをもとに 10^4 IFU/well になる希釈倍数を求めても，MIC 値に影響を及ぼすことはほとんどない。

IFU（*C. trachomatis* D 株）の算定例

希釈倍数	封入体実測数	平均値/well	原液の IFU/ml	
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	

この値の原液なら SPG にて 1,100 倍に希釈し，その 0.25 ml/well を接種して MIC 測定に用いる。

なお，臨床分離株の MIC を測定する場合は可能な限り継代数の少ない株について測定することが望ましい。このため増菌培養に際しては接種材料を希釈し過ぎないよう配慮する必要がある。

6. Reference strain

臨床分離株の測定に際しては reference strain を同時に使用して MIC 測定法の正確性の確認をすることが望ましい。

なお報告に際しては reference strain に対する薬剤の MIC も附記する。

Reference strain としては，*C. trachomatis* では D/UW-3 Cx 株，*C. psittaci* では Budgerigar No. 1 株を用いる。いずれも国立予防衛生研究所ウイルス中央検査部（山崎修道部長）より分与を受ける（連絡先は〈附記A. 1. a.〉に準ずる）。

7. 薬剤希釈

a. 薬剤希釈法

薬剤の希釈法の違いにて薬剤濃度の違いが生じ，MIC に影響を与える可能性がある。特に少量の溶液にて希釈系列を作製した場合，または2倍希釈を何度も繰返し施行した場合，その誤差は大きくなる。したがって，下記の方法にて実施する。

各薬剤に関し，目的とする最大薬剤濃度の 20 倍を蒸留水など（各薬剤により指定された溶液）にて作製し（Master dilution），使用時には medium 9 ml に 20 倍濃度薬剤液 1 ml を加え，10 ml の2倍濃度溶液とする。その後 5 ml 単位でピペットを使用し，2倍希釈系列を作製するのが望ましい。2倍希釈は6回までに留める。

b. 薬剤濃度の上限

β-lactam 系薬剤などの高 MIC 薬剤では高濃度にても封入体が観察されるので 128 μg/ml まで測定して打切る。

c. 薬剤濃度の表示

1 μg/ml を基準とし，濃度表示は下記のごとくにする。

例：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)]

Chlamydia 増殖は宿主細胞の蛋白合成を阻害すると増強され，封入体が観察され易くなる。したがって，MIC 測定に際しては終末 1 μg/ml の cycloheximide を加える。

◎ Cycloheximide 原液の処方

グルコース 5.4 mg, cycloheximide 10 mg を蒸留水 100 ml に溶解後，0.2 μmφ フィルターにて濾過滅菌，4℃ に保存する。使用に際して培地の 1/100 量を加える。

8. 温度条件

Incubation 温度が高くなると成績に著しい影響を及ぼすので温度条件は厳守すること。

なお *C. pneumoniae* (TWAR) 関連株は一般に他のクラミジアより発育速度は遅いが，37℃ と 35℃ の培養後の感染力価を比較すると 35℃ 培養の感染力価が 37℃ 培養のそれに比べ有意に高い[6)]。これは *C. pneumoniae* (TWAR) の生物学的特性と考えられる。したがって *C. pneumoniae* (TWAR) 関連株の MIC 測定時には 35℃±1℃ を培養温度とする。

9. 判定法

a. 染色法

MIC の判定には IF 法 [MicroTrak® は *C. trachomatis* のみ，オーソクラミジア (FA), Culture set® は *C. trachomatis*, *C. psittaci*, および *C. pneumoniae* (TWAR) 関連株の染色に使用可能] で行なうこととする。カバーガラスを取り出し，エタノールにて室温 15 分間固定，乾燥後遮光して染色する。ただちに染色できない場合は，固定乾燥後，−20℃ に保存できる。

HeLa 229 細胞は細胞質内にヨード陽性小体が存在し，封入体と混同される可能性があるのでヨード法は HeLa 229 細胞を用いる封入体観察には適さない。

b. 観察倍率

変形した microinclusion は，400〜1,000 倍の高倍率で見ると少数が数段階にわたり残存することがあるので消失判定が困難である。そこで変形した microinclusion が比較的見えにくい 100 倍にて観察する。

≪参考資料≫

Reference strain に対する当委員会の MIC 測定成績は次のごとくである。

	C. trachomatis D/UW-3/Cx	*C. psittaci* Budgerigar No. 1
MINO	0.016〜0.063(μg/ml)	0.016〜0.063(μg/ml)
DOXY	0.031〜0.063	0.016〜0.063
EM	0.125〜0.5	0.25 〜0.5
TE-031	0.008〜0.031	0.008〜0.031
OFLX	0.25 〜1.0	0.25 〜1.0
ABPC	128<	128<

≪参考文献≫

1) DAROUGAR S, KINNISON J R, JONES B R: Simplified irradiated McCoy cell culture for isolation *Chlamydiae*; in Nichols Trachoma and related disorders caused by chlamydial agents. Excerpta med. Int. Congr. Series No. 223, pp. 63〜70, 1971

2) SCHERER W F, SYVERTON J T, GEY G O : Studies on propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exp. Med., 97 : 695～709, 1953
3) JENKIN H M : The continuous passage of agents of trachoma in cell culture. J. Infect. Dis., 116 : 390～399, 1966
4) KUO C C, WANG S P, WENTWORTH B B, GRAYSTON J T : Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. J. Infect. Dis., 125 : 665～668, 1972
5) NAGAYAMA A, NAKAO T, TAEN H: *In vitro* activities of ofloxacin and four other new quinoline-carboxylic acid against *Chlamydia trachomatis*. Antimicrob. Agents Chemother., 32 : 1735～1737, 1988
6) KUO C C, GRAYSTON J T : Factors affecting viabilitiy and growth in HeLa 229 cells of *Chlamydia sp.* strain TWAR. J. Clin. Microbiol., 26 : 812～815, 1988
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
8) KUO C C : Immediate cytotoxicity of *Chlamydia trachomatis* for mouse peritoneal macrophages. Infect. Immun., 20 : 613～618, 1978

≪附　記（B）≫

1) 本報告書はクラミジア MIC 測定法検討委員会により，国際的に通用する測定法の統一化を目的にして検討されたものである。1989年5月18日第37回日本化学療法学会にて報告された。

委員（委 員 長）熊本悦明（札幌医大　泌尿器科）
〃 （副委員長）松本　明（川崎医大　微生物）
〃 永山在明（福岡大　医学部微生物）
〃 副島林造（川崎医大　内科）
〃 平井克也（岐阜大　農学部家畜微生物）
〃 橋爪　壮（千葉大　看護学部病態学）
〃 荻原敏且（国立予防衛生研究所　ウイルス中央検査部）

2) MLC (minimum lethal concentration) 測定法については，別途報告する。

METHOD FOR *IN VITRO* DETERMINATION OF CHLAMYDIAL SUSCEPTIBILITY (MINIMUM INHIBITORY CONCENTRATION ; MIC) TO ANTIMICROBIAL AGENTS

—STANDARD METHOD OF JAPAN SOCIETY OF CHEMOTHERAPY—

[Manual for *Chlamydia* 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 *Chlamydia* :

There is the danger of laboratory personnel being infected by *Chlamydia*. 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 *Chlamydia*, suspensions of *Chlamydia* must absolutely never be pipetted using the mouth.

1. Use plastic, 24-well cell-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 14mm 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 maintained at 37°C with an atmosphere containing 5% CO_2. After confirming growth of a confluent monolayer, remove the culture fluid from the wells buy aspiration. As a rule, DEAE-dextran treatment should not be carried out prior to *Chlamydia* 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 *Chlamydia* 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 $500-900\times g$ 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 36 hrs since this species grows more rapidly than *C. trachomatis.*
   Culture conditions for *C. pneumoniae* (TWAR)-related strains :
   35°C (permissible 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 the 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 the 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* suspension. 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 *Chlamydia* 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 *Chlamydia* 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 37.5g, KH_2PO_4 0.26 g $Na_2HPO_4\cdot2H_2O$ 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 100μg/ml each).
         c) To release the *Chlamydia*, 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 *Chlamydia* suspension.
         d) Prepare a 10-fold dilution series using SPG with 1—2 ml of the stock *Chlamydia* 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℃. 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	$\fallingdotseq 4.4\times10^7$
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 *Chlamydia*, 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 (Chief : SHUDO YAMAZAKI, 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 *Chlamydia* 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 *Chlamydia* 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 *Chlamydia* 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 two reference strains.

Antibiotic	*C. trachomatis* D/UW-3/Cx	*C. psittaci* Budgerigar No. 1
MINO	0.016～0.063(μg/ml)	0.016～0.063(μg/ml)
DOXY	0.031～0.063	0.016～0.063
EM	0.125～0.5	0.25 ～0.5
TE-031	0.008～0.031	0.008～0.031
OFLX	0.25 ～1.0	0.25 ～1.0
ABPC	128<	128<

REFERENCES

1) DAROUGAR S, KINNISON J R, JONES B R : Simplified irradiated McCoy cell culture for isolation *Chlamydiae* ; in Nichols Trachoma and related disorders caused by chlamydial agents. Excerpta med. Int. Congr. Series No. 223, pp. 63～70, 1971
2) SCHERER W F, SYVERTON J T, GEY G O : Studies on propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J. Exp. Med., 97 : 695～709, 1953.
3) JENKIN H M : The continuous passage of agents of trachoma in cell culture. J. Infect. Dis., 116 : 390～399, 1966
4) KUO C C, WANG S P, WENTWORTH B B, GRAYSTON J T : Primary isolation of TRIC organisms in HeLa 229 cells treated with DEAE-dextran. J. Infect. Dis., 125 : 665～668, 1972
5) NAGAYAMA A, NAKAO T, TAEN H : *In vitro* activities of ofloxacin and four other new quinoline-carboxylic acid against *Chlamydia trachomatis*. Antimicrob. Agents Chemother., 32 : 1735～1737, 1988
6) KUO C C, GRAYSTON J T : Factors affecting viability and growth in HeLa 229 cells of *Chlamydia sp.* strain TWAR: J. Clin. Microbiol., 26 : 812～815, 1988
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
8) KUO C C : Immediate cytotoxicity of *Chlamydia trachomatis* for mouse peritoneal macrophages. Infect. Immun., 20 : 613～618, 1978

[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 Manual will be presented at the 37 th meeting of the Japan Society of Chemotherapy, on May 18, 1989.

   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 (Dept. of Microbiology and Pathology, Chiba University, School of Nursing)

   TOSHIKATSU HAGIWARA (Central Virus Diagnostic Laboratory, National Institute of Health)

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