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TEHNIČKI ASPEKTI TESTIRANJA ANTIMIKROBNE OSJETLJIVOSTI BAKTERIJE *CHLAMYDIA TRACHOMATIS* U STANIČNOJ KULTURI

TECHNICAL ASPECTS OF *CHLAMYDIA TRACHOMATIS* ANTIMICROBIAL SUSCEPTIBILITY TESTING IN CELL CULTURE SYSTEM

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Stručni članak

Sažetak: *Chlamydia trachomatis (C. trachomatis) najčešći je bakterijski uzročnik spolno-prenosivih infekcija i obligatna unutarstanična bakterija s jedinstvenim životnim ciklusom. Za testiranje antimikrobne osjetljivosti ovog učestalog patogena danas se najčešće koristi stanična kultura McCoy stanica uz imunofluorescentno bojanje za detekciju karakterističnih klamidijskih inkluzija. Sam postupak testiranja može se podijeliti u četiri osnovna koraka – predizolacijska faza, izolacija C. trachomatis u staničnoj kulturi, priprema za testiranje antimikrobne osjetljivosti te, najzad, faza testiranja uz određivanje minimalne inhibitorne koncentracije (MIC) i minimalne klamicidne koncentracije (MCC). Kako ova metoda zahtjeva preciznost i posvećivanje posebne pozornosti čimbenicima koji mogu utjecati na sposobnost klamidija da inficiraju stanice u kulturi, ali i na učinkovitost testiranog antibiotika uslijed različitih mehanizama kao što je pravodobni ulazak u stanicu, pravilno izvođenje svakog od ovih koraka je ključno kako bi se osigurala reproducibilnost i usporedivost dobivenih rezultata.*

Ključne riječi: *Chlamydia trachomatis, testiranje antimikrobne osjetljivosti, stanična kultura, imunofluorescentno bojenje, minimalna inhibitorna koncentracija, minimalna klamicidna koncentracija*

Professional paper

Abstract: *Chlamydia trachomatis (C. trachomatis) is the most common bacterial agent of sexually transmitted infections and an obligate intracellular bacterium with a unique life cycle. Cell culture systems using McCoy cells and immunofluorescent staining to identify characteristic chlamydial inclusions represent the most common methodology employed in antimicrobial susceptibility testing of this prevalent pathogen. The testing process can be broken down into four technical steps – preisolation stage, isolation of C. trachomatis in cell culture, preparatory steps for antimicrobial susceptibility testing and, finally, testing phase with a determination of minimal inhibitory concentration (MIC) and minimal chlamydicidal concentration (MCC). As this technique requires precision and careful attention to conditions which may influence both the ability of chlamydial organisms to infect cells in culture and the efficacy of a tested antibiotic through mechanisms such as intracellular uptake, following these steps is pivotal in order to ensure reproducibility and comparability of obtained results.*

Keywords: *Chlamydia trachomatis, antimicrobial susceptibility testing, cell culture system, immunofluorescent staining, minimal inhibitory concentration, minimal chlamydicidal concentration*

1. INTRODUCTION

Chlamydia trachomatis (*C. trachomatis*) is the most common bacterial agent of sexually transmitted infections in Croatia and around the world. The World Health Organization (WHO) estimates that there are over 100 million new cases of C. trachomatis infections every year [1]. In the developed countries, chlamydial infections are most often found in young heterosexual adults under 25 years of age, ranging from 3 to 6% among those who are sexually active [2,3]. Recent study that investigated *C. trachomatis* infections among sexually active Croatian young women and men aged 18–25 revealed 5.3% and 7.3% prevalence, respectively [4].

This obligate intracellular bacterium has a unique life cycle characterized by the transformation of an extracellular, infectious elementary body (EB) in the intracellular, noninfectious, metabolically active reticulate body (RB) and vice versa (Figure 1) [5]. Due to its small size and reliance on the biosynthetic machinery, *C. trachomatis* was once considered to be a virus; however, presence of a cell wall, DNA, RNA and ribosomes, as well as its susceptibility to antimicrobial agents, are undoubtedly placing this organism in the bacterial domain of life [6].

C. trachomatis is a causative factor of a plethora of symptoms and clinical syndromes, ranging from urethritis, epididymitis and prostatitis in men, to cervicitis, endometritis and pelvic inflammatory disease in women [7]. Although molecular techniques are currently considered the gold standard for the detection of *C. trachomatis*, cultivation in cell culture system remains a method of choice – not only in terms of medico-legal investigations and follow-up after completed therapy, but also as a valuable (and thus far only) tool for determining the antimicrobial sensitivity of this microorganism [8].

In an era of increasing resistance to antimicrobials in many bacterial species, resistance has been exceedingly rare in *C. trachomatis*; the primary reason is the intracellular nature of the pathogen which eliminates the opportunity for rapid evolution of cell surface components [9]. In addition, not a lot of patients have chlamydia cultures obtained, tests of cure evaluations are not routinely done, and even if cultured, hardly a few isolates are subjected to antimicrobial susceptibility testing due to the laborious methodology. However, clinical treatment failures that have been described and attributed to multidrug-resistant *C. trachomatis* strains, are the reason for antimicrobial susceptibility testing and surveillance for resistance remain essential in detecting shifts in chlamydial sensitivity to antibacterial agents [10].

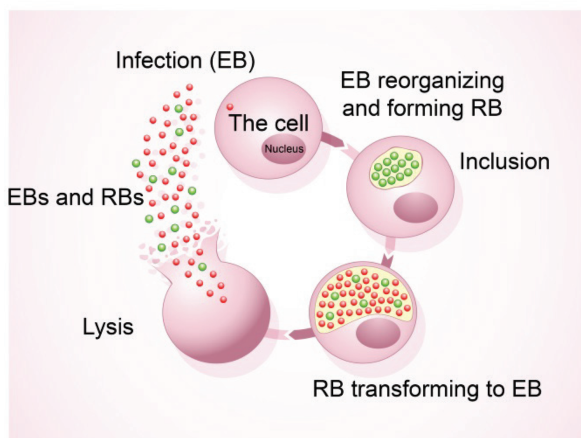

Figure 1 Diagram depicting life cycle *of Chlamydia trachomatis* [5]

2. ANTIMICROBIAL SUSCEPTIBILITY TESTING IN CELL CULTURE SYSTEM

Antimicrobial susceptibility testing of *C. trachomatis* differs greatly from standard procedures in bacteriology, since it is necessary to demonstrate the ability (or inability) of this pathogen to multiply in cells in the presence of different concentrations of antibiotics [11]. Its growth is assessed by the detection of the inclusions, which are intracellular membrane-bound structures that represent a sign of active chlamydial replication [11].

Today, cell culture systems using immunofluorescence staining to identify aforementioned inclusions are the most common methodology employed in antimicrobial susceptibility testing of *C. trachomatis* [12]. Several different cell lines of human or animal origin can be used for that purpose, albeit McCoy cells derived from mouse fibroblasts offer the most reliable and consistent results. HeLa (human cervical adenocarcinoma), HEp-2 (human epidermoid laryngeal carcinoma) and HL (human epithelial cells) cell cultures can also be employed, whilst primate kidney cell line BGMK and Vero cells are utilized less frequently [12].

Laboratory conditions such as temperature, pH, nutritional content of the medium, the polarity of infected cells and the secretion of cytokines by infected cells may play a role in penetration and activity of certain antimicrobial agents [13-15]. It has been shown that in a medium with a high concentration of glucose, neutral pH value and a higher temperature during centrifugation (33-35 °C) one can expect a higher yield of *C. trachomatis* inclusions [14]. Furthermore, the use of polarized host cells results in more efficient transport and internal concentration of antimicrobial drug azithromycin within cells [15].

Additional variables that may influence the results of sensitivity testing in *Chlamydiae* (but which have been incompletely studied) include the inoculum size, the interval between the establishment of infection and the administration of an antibiotic, as well as the timing of antimicrobial removal [16]. In addition, it is pivotal to implement adequate quality control of the cell line that is being used for the testing purposes [5].

It must be noted that cell culture system for antimicrobial susceptibility testing of *C. trachomatis* is not completely physiologic in nature, since during natural infection this microorganism is usually exposed to antimicrobials long after the establishment of intracellular infection and the induction of a host inflammatory response [12]. Conversely, with the *in vitro* systems, tested antimicrobial drugs are usually added simultaneously with or shortly after the infection; hence the inability to detect infectious chlamydiae in cell cultures does not exclude a viable state that could recur after antibiotics are removed [17].

The testing process can be broken down into four fundamental steps – preisolation stage, isolation of *C. trachomatis* in cell culture, preparatory steps for antimicrobial susceptibility testing and, finally, testing phase with the determination of concentration values. Each of these steps will be explained in detail in order to provide comprehensive review and further standardization of the method.

2.1 Preisolation stage

Before antimicrobial susceptibility testing can be pursued, clinical materials for isolation of *C. trachomatis* must be collected. The most commonly used specimens in chlamydial susceptibility testing are cervical swabs in women and urethral swabs (and sometimes expressed prostatic secretions) in men [5]. Conjunctival swabs can be also used as a sample, namely in communities of the world where trachoma is still endemic [18]. All samples that can be processed in the laboratory within 24 hours can be held and shipped at 4 °C (on wet ice); if this is not

achievable, they are stored at -70 °C before isolation procedure can ensue [19].

Only swabs with a Dacron tip and an aluminum or plastic shaft should be used to collect specimens. Swabs with calcium alginate or cotton tips and wooden shafts are unacceptable, as growth inhibition of the organism can occur. Specimens should be placed in sucrose phosphate (2SP) transport medium (sucrose, 68.46 g; potassium phosphate dibasic, 2.01 g; potassium phosphate monobasic, 1.13 g; gentamicin, 25 µg/mL; nistatin, 25 µg/mL; vancomycin, 100 µg/mL; and 10%–20% fetal calf serum, made up to a final volume of 1000 mL, pH 7.2-7.4) and not removed before transport [5,19].

Growth medium must be prepared as well. Eagle's minimum essential medium (MEM) is used as a base, with a formulation that usually incorporates either Hanks' or Earle's balanced salts in order to provide adequate buffering of the medium [20]. As MEM is a simple medium, the recommendation is to add additional supplements or higher levels of serum to improve cell growth. Most commercially available liquid media already contain the appropriate bicarbonate levels, therefore do not require additional bicarbonate. Fetal calf serum (inactivated at 56 °C to inhibit components of the complement), glucose and cycloheximide are then added in order to prepare a growth medium [5].

The preferred cell line for isolation of *C. trachomatis* are McCoy cells, a continuous cell culture of adherent fibroblasts derived from the unknown tissue of the house mouse (*Mus musculus*) [21]. Those fibroblasts possess structurally abnormal marker chromosomes characteristic of strain L mouse fibroblasts. The subcultivation of McCoy cells should be done following these steps: removing the growth medium, adding 0.25% of trypsin-versene (EDTA) solution for 1-2 minutes, removing the trypsin-versene solution, waiting for the cells to detach from the culture vessel surface into suspension, adding new growth medium, and then reinoculating the suspension into new culture vessels [5].

2.2 Isolation of *C. trahcomatis* in cell culture

As *C. trachomatis* represents an organism that belongs to biosafety level 2 (BSL 2) agents, biosafety practices that include personal protective equipment, decontamination and appropriate biohazardous material waste must be followed. The specimen is inoculated by centrifugation onto a confluent monolayer of McCoy cells that support the growth of this obligate intracellular bacterium [22]. Inoculated cells are harvested after 48–72 hours of growth; characteristic intracytoplasmic inclusions that contain substantial numbers of *C. trachomatis* elementary and reticulate bodies are developed upon infection.

The cell monolayers are then reacted with either genus specific or species-specific fluorescein-conjugated monoclonal antibodies to permit specific visualization of the chlamydial inclusions with an epifluorescent microscope (Figure 2) [23]. High specificity of cell culture detection is achieved if a *C. trachomatis* major outer membrane protein (MOMP)–specific stain is used. Although monoclonal antibodies directed against the family-specific lipopolysaccharide (LPS) of *Chlamydiaceae* cost less, they might stain bacteria that share LPS antigens. LPS stains might be suitable for routine use, but a species-specific (MOMP) stain is recommended in situations requiring increased specificity [23]. Less specific inclusion-detection methods using iodine, Giemsa stain or Wright stain are not recommended.

Cell culture methods can differ among laboratories, resulting in considerable interlaboratory variation in performance [24]. For isolation purposes, the shell vial method of culturing is a better choice than the 96-well microtiter plate method, as it uses a larger inoculum with a reduced risk for cross-contamination and thus provides better accuracy [22]. Some laboratories can achieve higher sensitivities if they perform a blind pass where an inoculated cell monolayer is allowed to incubate for 48–72 hours, after which it is disrupted and used to inoculate a fresh monolayer that is stained after 48–72 hours of incubation to allow for another cycle of growth [23].

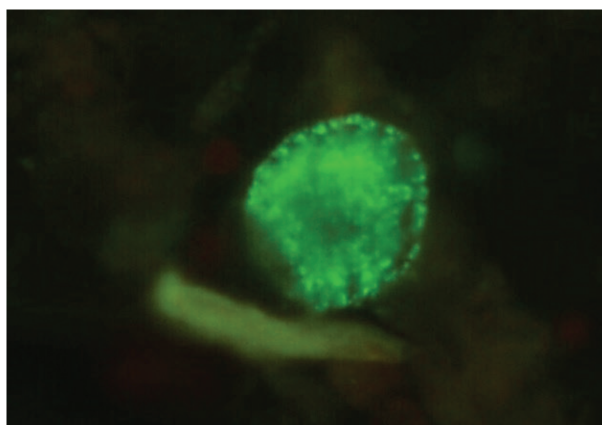

Figure 2 Enlarged inclusion body of *C. trachomatis* in infected McCoy cells stained with monoclonal antibodies [5,27]

2.3 Preparatory steps for antimicrobial susceptibility testing

Isolated chlamydial strains that are selected for antimicrobial susceptibility testing must by amplified by multiple passages so that they infect approximately 10 to 50% of the cells [12]. This prerequisite must be met in order to achieve sufficient amount and inoculum size of 5 000 – 10 000 inclusion forming units (IFU) per well of the microtiter plate used for susceptibility testing [8,12]. These plates usually have 96 wells that contain McCoy cell monolayers, 24-h old and grown in the medium without cycloheximide and antibiotics (Figure 3). Prior to the inoculation of chlamydial strains, the cells are checked under the microscope to assess the adequacy of growth and to rule out eventual presence of infection.

Antimicrobial agents should be obtained in powder form, weighed and adjusted for purity, and reconstituted according to the manufacturer's instructions. Each drug should be prepared as a stock solution at a concentration of 1024 µg/ml, according to its potency calculated from assay purity by high-performance liquid chromatography (HPLC), water content and active fraction of each

substance, as specified by the manufacturer [25]. Such stock solutions can be kept at −70 °C for a maximum period of two weeks.

Figure 3 Microtiter plate with suspension of McCoy cells for antimicrobial susceptibility testing [5]

2.4 Susceptibility testing phase and determination of concentration values

After the growth medium from the previously prepared microtiter plates is aspirated and discarded, each well can be inoculated with 100 µg of the previously amplified chlamydial test strain. Two batches of plates are incubated in parallel, and subsequently centrifuged for one hour at 1200 × g for at 37°C. After centrifugation plates should immediately be transferred into a thermostat at 37 °C with 5% CO_2 for an additional two hours [5].

Previously prepared antimicrobial stock solutions are then serially diluted from the initial concentration of 1024 µg/ml to 0.008 µg/ml in MEM containing 10% heat-inactivated fetal bovine serum and cycloheximide (1.0 µg/ml). At the end of a two-hour incubation period, the plates can be removed from the thermostat and the supernatants aspirated with multichannel pipette. In each well 100 µl of the appropriately diluted antimicrobial drug is added to the appropriate wells for a minimum final concentration range of 0.008 µg/ml to 8 µg/ml (and preferably up to 128 µg/ml) [12]. Antibiotic-free controls should also be used.

After the 72-hour incubation period at 37 °C in 5% CO_2, the cultures in one microtiter plate can be fixed with methanol and then stained for inclusions with either genus specific or species-specific fluorescein-conjugated monoclonal antibodies [12]. The plate is then transferred to a moist chamber and incubated protected from light for 30 minutes at a room temperature (23 °C ± 3 °C). Upon washing the wells twice, they can then be fixated with glycerol and analyzed under the invert fluorescent microscope (Figure 4) to determine minimal inhibitory concentration (MIC) values – the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism [8,26].

Following the incubation of the infected cells overlaid with appropriate two-fold drug dilution for 72 hours, the cells on the second plate are frozen at −70 °C in order to make a passage of tissue culture plates employing a freeze-thaw method [12]. After thawing the methodological procedure is similar as described for the first plate, *i.e.* cultures are passed onto new cells, centrifuged, overlaid with antibiotic-free medium (MEM with cycloheximide only), incubated for 72 hours, fixed and stained. The analysis of this plate under the invert fluorescent microscope is used to determine minimal chlamydicidal concentration (MCC) values, which are akin to minimal bactericidal concentration (MBC) in other domains of bacteriology, defined as a lowest dilution where the culture has been completely sterilized [5].

The reproducibility of *C. trachomatis* antimicrobial susceptibility testing in cell culture system relies on standardized definitions of MIC and MCC. To achieve this, it is necessary to introduce the transition point MIC (MIC_{TP}), which can be defined as the concentration of drug in which 90% or more of the inclusions are altered in their morphology and size [12]. The MIC can then be defined as the concentration of drug that is one twofold dilution more concentrated than the MIC_{TP} (Figure 5). The MCC is defined as the lowest concentration of drug at which there were no visible inclusions after one passage from the cell culture with the addition of antimicrobial drugs to the cell culture without them [5,26].

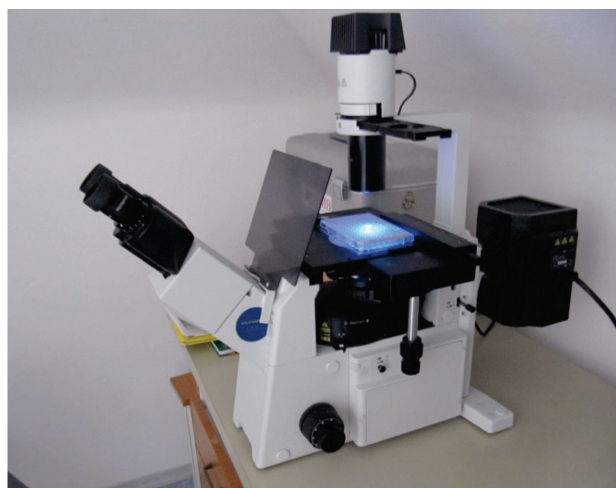

Figure 4 Invert fluorescent microscope [5]

3. CONCLUSION

Antimicrobial susceptibility testing for *C. trachomatis* (but also other chlamydial species) requires precision and careful attention to conditions which may influence both the ability of chlamydial organisms to infect cells in culture and the efficacy of a tested antibiotic through mechanisms such as intracellular uptake [12,26]. Therefore all four aforedescribed steps should be followed meticulously in order to ensure reproducibility and comparability of results. One cell line should be consistently used for *C. trachomatis* susceptibility testing; based on the literature and our experience, we recommend using McCoy cells.

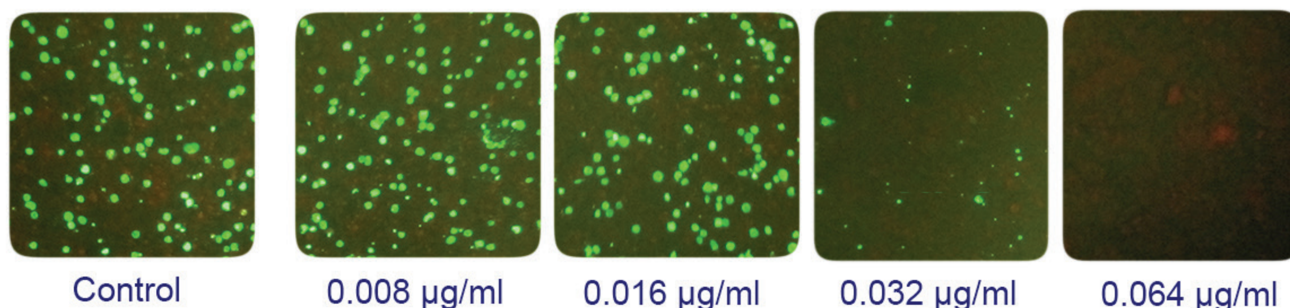

Figure 5 Photomicrographs of growth pattern of *C. trachomatis* at various concentrations of a tested antimicrobial drug showing determination of minimal inhibitory concentration (MIC) which is defined as the lowest concentration of antibiotic without visible inclusions (0.064 µg/mL). Transition point MIC (MIC_{TP}) is observed at the concentrational value of 0.032 µg/mL.

A paramount methodological aspect of susceptibility testing in cell culture system is the endpoint utilized for defining the MIC, since differentiating aberrant from normal inclusions can be tiresome, and subjective interpretation may lead to severalfold variation in the MICs [8]. Hence, the MIC_{TP} provides a consistent endpoint where practically all chlamydial inclusions are inhibited (Figure 5), and one twofold concentration above the MIC_{TP} provides a reasonable standardized endpoint to define the MIC [12].

Studies on antimicrobial susceptibility of *C. trachomatis* conducted in Croatia and other countries using first-line and second-line antibiotics demonstrate good sensitivity of this microorganism, although cases of heterotypic resistance have been repeatedly described in the literature [5,10,12,27]. As we still lack information on whether *C. trachomatis* is evolving *in vivo* in response to antibiotic selection pressure, we must continue to test them in order to detect eventual shifts in antimicrobial susceptibilities *in vitro*. Although the methodology is cumbersome and not completely standardized, cell culture systems with immunofluorescence staining for the identification of chlamydial inclusions represent the most common methodology employed in antimicrobial susceptibility testing of this prevalent pathogen.

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