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Mycoplasma infection followed by time-lapse microscopy

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ABSTRACT

Early detection of mycoplasma infection is crucial for saving precious often irreplaceable data from the tissues of patients. Mycoplasma infections cause diseases in the upper and lower respiratory tracts, urethritis in men resulting in painful dysuria, urgency and urethral discharge. Cough, fever, headache, urethritis may persist for several weeks and convalescence is slow.

The symptoms of these diseases are aggravated by the detection of mycoplasma infections, that takes either a long time, besides being expensive or is specific and restricted to only a limited number of contaminant strains. Mycoplasmas are hard to detect visually but could be seen and followed by time-lapse microscopy. Our hypothesis is that one can detect mycoplasma infection irrespective of its origin and type of mycoplasma.

Main lines of supporting evidence are provided by the time-lapse microscopy showing dynamic morphological alterations caused by mycoplasmas before changes in human cell cultures become visible. Morphometric measurements of mycoplasma infections revealed four subphases: *i*) detachment of infected cells, *ii*) aggregation, iii) biofilm formation and iv) shrinkage of infected cells. The applicability of time-lapse microscopy for the detection of mycoplasma infection was validated by a mycoplasma test Kit.

Most important implications related to morphometric parameters include the observation of mycoplasma infected cultures for an extended period of time instead of applying static snap-shot microscopy. A reliable method is offered to estimate the time of mycoplasma exposure that elapsed during the cell growth. This microphotometric approach served a more economical detection of mycoplasma contamination at its early stage of cell growth and spread, irrespective of the origin of contaminated serum, without defining the type of mycoplasma.

Introduction and background

Mycoplasmas are the smallest (0.3–0.8 μm) and simplest self-replicating microorganisms that can grow on artificial substrates. The mycoplasma genus of the fungus-like bacteria is lacking cell wall around the cell membrane, consequently, these bacteria are resistant to common antibiotics including penicillin. Beta-lactam antibiotics that affect bacteria target cell wall synthesis. Although mycoplasmas are bacteria, they are not to be confused with mycobacteria, that may cause serious diseases such as tuberculosis (*Mycobacterium tuberculosis*) or leprosy (*Mycobacterium leprae*) [1]. Infections caused by mycoplasmas in cell cultures can induce alterations in growth rate, morphological changes, cell metabolism, chromosomal aberrations, and ultimately generate a different cell line. To prevent mycoplasma infection it is recommended to grow cells in antibiotic-free media, except for primary cell culture, to follow a strict policy of not using incoming cell cultures until the absence of mycoplasma infection has been confirmed and to observe the Good Cell Culture Practice. These precautions indicate that these measures are not sufficient to filter out infections, as contaminations are invisible without changes in turbidity up to 10^8 cells/ml. The several mycoplasma tests with their advantages and disadvantages also indicate that there is no generally accepted method that could be used to show the presence at the early stage of infection, not speaking about the identification of mycoplasma species.

One of the most often occurring *Mycoplasma pneumonia* and other contagious respiratory infections necessitate not only the fast, reliable and easy to execute recognition, but also to understand the mechanism of spread of mycoplasma. Unfortunately, methods to follow mycoplasma infection are neither simple nor fast and may require special facilities and capabilities. Electron microscopic, light microscopic identification of mycoplasmas with orcin, fluorescent microscopy after fluorescence labeling is time-consuming methods and not suitable for monitoring the dynamics of mycoplasma infection.

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Hypothesis

The problems with the rarely occurring, but the more annoying mycoplasma infections in cell cultures led to our hypothesis, whether one could use time-lapse photomicrography to test cell cultures and screen serums for mycoplasma contaminations without carrying out expensive experiments. The novel approach is expected have the advantage of following any type of infection without the identification of the type of mycotoxin species. Our video microscopy technique combined with digital image analysis has been adapted to investigate the dynamics of mycoplasmic spread in human cell cultures and to trace morphological changes in infected cells. To support the validity of our hypothesis and that prediction can be made regarding its applicability, pilot experiments were carried for the selection of calf serums containing mycoplasmas. These experiments served also the purpose of finding proper conditions for the detection and verification of mycoplasma infections in three human cell cultures (HaCaT, OCM-1, and SCC). Infections were validated by mycoplasma PCR test. The PCR products were analyzed by agarose gel electrophoresis providing a reliable means for real time analysis.

experimental approach

Cell cultures

HaCaT cells were derived from human skin keratinocytes [2,3], the OCM-1 malignant (human choroidea melanoma-1) cell line was obtained from the local clinical Department of Ophthalmology [4], originally established from the biopsied specimen of choroidal melanoma [5]. Squamous cell carcinoma (SCC) cells [6] were obtained from Prof. Reinhard Zeidler, Helmholtz Zentrum München.

Cell growth

Healthy cells were cultured in RPMI-1640 medium (Sigma Aldrich, Budapest, Hungary) containing 5% bovine calf serum (BCS) (Hyclone, Logan, UT). Infection with mycoplasma was induced with 5% bovine calf serum obtained from the local slaughterhouse. Bovine calf serums were filter sterilized but not heat inactivated and selected for mycoplasma infection. The number of living host cells was determined by trypan blue staining in a Burker chamber. Due to their small size, mycoplasma cells remained invisible by light microscopy.

PCR mycoplasma test

The first line of defense against mycoplasma contamination is detection. Bovine calf serum without heat inactivation could serve as a potential source of mycoplasma infection. The direct assay for the detection of mycoplasmas is a slow process, that requires an annoying long incubation time for at least 14 days under anaerobic atmosphere and two more weeks sampled on agar plates. Such a long wait is unacceptable in a pandemic scenario when none of the vaccines could afford a 4 weeks release period. Beside the test being complicated, it does not detect some of the mycoplasma species. Therefore, the direct but slow mycoplasma assay was replaced by the fast PCR mycoplasma test.

The PCR mycoplasma test was performed with the Promokine Test Kit, suitable to detect major contaminating species such as *M. orale*, *M. hyorhinis*, *M. arginini*, *M. fermentans*, *M. salivarium*, *M. hominis* and *Acholeplasma laidlawii* usually present in cell cultures. This test Kit does not detect the clinically relevant *M. pneumoniae* and *Ureaplasma urealyticum*.

Infection of cell cultures with mycoplasma

Several batches of bovine calf serum were filter sterilized without heat inactivation and tested for mycoplasma infection in HaCaT cell culture. The bovine calf serum that was found to be mycoplasma infected by the Promokine Test was added as a source of mycoplasma infection to different cell cultures (HaCaT, OCM1, SCC) and grown in RPM 1640 medium, then mycoplasma test was performed as described by the Promokine Kit. The growth of mycoplasma infected cell cultures was followed by time-lapse microscopy and image analysis.

Time-lapse microphotography

Time-lapse image microscopy was established to study structural chromosomal changes [7] and the adherence and growth patterns of the infectious yeast *Candida albicans* [8]. The equipment of time-lapse microphotography consisted of a carbon dioxide incubator (Sanyomco18-AC, Wood Dale, IL, USA) hosting custom-built inverted microscopes (Olympus, Tokyo) with couple charged (CCD) cameras attached to them. Led diodes emitting 940 nm near-infrared light (5 mm in diameter, 1.2 V, 20 mA, 5 V-directed series connection of 220 Ohm resistance) illuminated the cells to minimize heat- and phototoxicity. Carl Zeiss (Jena, Germany) dry objectives (x10:0.25 NA) were used to secure a wide visual field and long working distance to penetrate the cell culture. Two megapixel (1600 × 1200 pixel) UVC USB 2.0 cameras (Asus Computer International, Fremont, CA, USA) were used with their infrared filters being removed.

Digital image analysis

To improve image analysis the Fiji analysis software was used. Thresholding (image processing) as the simplest method of image segmentation was applied, where the pixels of objects seen in the photos were separated on the basis of their gray scale intensity values. This way the surface (e.g. the monolayer of cells) to be determined could be distinguished and separated from the rest of the picture. The quantitative parameters of the selected area could then be characterized and plotted as diagrams.

To determine the confluency values, the monolayer was separated from the background of the monitored surface, then the segregated area, i.e. the cells attached to the flask, was expressed in percentages of the visible surface of the cell culture. A similar method was applied when the spread of the biofilm of mycoplasma infection was determined. Only the biofilm of mycoplasma infection was visible, individual mycoplasma cells could not be seen during the time-lapse image analysis.

To judge the motility of the mammalian cells the background was removed from the rest of the images, and the differences in motion of consecutive pictures were expressed in square pixels. These numerical values provided information with respect to the shift of cells relative to the previous state of motion. The motility values were also used to determine the set time when the cells peeled off the surface of the tissue flasks. At set time the adhesion connections ceased and the movement of the cells suspended in the medium intensified similarly to other minute particles due to the Brownian motion. This way the time of detachment of individual cells could be determined. Digital image analysis was performed at five randomly selected, representative regions in each cell culture.

Validation of mycoplasma infection

Proper conditions for the detection of mycoplasma infection were tested in cell cultures (HaCaT, OCM-1, and SCC) using the mycoplasma PCR test and products were analyzed by agarose gel electrophoresis. Cells that were not infected gave negative results The presence of mycoplasma could be reliably detected in infected cultures. Internal positive control served the verification of the results.