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RAPID DETECTION BY TRANSMISSION ELECTRON MICROSCOPY OF MYCOPLASMA CONTAMINATION IN SERA AND CELL CULTURES

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Transmission electron microscopy has been employed for the rapid detection of mycoplasma in sera and cell cultures. High speed centrifugation of sera or low speed centrifugation of cell debris, followed by negative staining of the resuspended pellet, detected mycoplasma contamination more frequently than a culture method followed by direct fluorescence (DAPI), which was used as a control procedure. The appearance of the mycoplasma cell border and content gives some information about particle viability.

Key words: mycoplasmas – negative contrast – rapid method – sera and cell contamination

Mycoplasmas, bacteria and fungi are the most common contaminants of cell and tissue cultures. The last two are easy to identify by light microscopy because of their typical forms and configurations; the first however constitutes a more difficult problem as it is not easy to identifity it simply and quickly.

According to Maniloff & Morowitz (1972), the smallest mycoplasma genomes known are of the order of 5 x 10^8 daltons and such a molecule cannot be packed in a membrane bound sphere smaller than 150 nm external diameter. Negative staining with heavy metals and high speed centrifugation (greater than 3.090 g x for 15 min) without fixation modify the original pleomorphic form of mycoplasma. Fixed cells however are not distorted when high speed centrifugation (17.750 g x for 15 min) is applied (Robertson et al., 1975b). Filtration has also a considerable influence on mycoplasma forms; without fixation they pass throughout filter pores smaller than the cell diameter, but when fixed, they are retained (Maniloff & Morowitz, 1972).

Electron microscopic examinations of mycoplasma containing suspensions are frequently based on preparations made by negative staining (Chu & Horne, 1967; Boatman, 1973; Kirchhoff et al., 1984). Smooth bordered spheres, cup, flask, pear shaped or other pleomorphic forms of mycoplasmas are considered to be replicative, while particles with rough surfaces indicate a loss in viability (Robertson et al., 1975a). Single filaments with transverse diameter less than 100 nm as detached segments from living forms (Bieberfeld & Bieberfeld, 1970) or as a result of pressure filtration are not viable (Maniloff & Morowitz, 1972).

Mycoplasma contamination of sera and cell cultures has been studied by several authors: at ultrastructural level (Boatman et al., 1976), by its growth in cell cultures, broth or solid media (Hayflick, 1965; Hopps et al., 1973; Barile et al., 1973) and by enzymatic detection (Bonissol et al., 1984).

All these methods take several days to obtain satisfactory results. We have attempted to find a quicker method for the detection of mycoplasma contamination in fetal bovine sera and cell cultures used for research purposes in Virology.

MATERIAL AND METHODS

Sera – Commercially obtained fetal bovine sera, standard media which contained fetal bovine sera prepared in our Department and several other animal sera available in our Institute (mouse, rabbit, horse and human) were examined.

Cell cultures – Supernatants were examined as well as disrupted cells from a variety of cell cultures in use over the past six years in the Department of Virology and in other Departments of our Institute.

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PROCEDURES

Detection of mycoplasmas in sera – 1 ml of serum was centrifuged in a Spin I centrifuge (Eppendorf centrifuge) at 15.000 g for 15 minutes. The pellet was drained for 10 minutes in a humid chamber and resuspended with two drops of distilled water. A small amount of this suspension was applied to a formvar/carbon coated 400 mesh copper grid, left for 30 seconds and drained with a strip of filter paper. Immediately after, one drop of a 1% solution of phospho-tungstic-acid, pH 7.2 was applied for 30 seconds and the excess of fluid removed with filter paper. No additional washing was needed. The grid was then ready to be examined in a transmission electron microscope.

Detection of mycoplasmas in cell cultures – Cells were carefully scraped from the walls of the tissue culture bottles and transfered with medium to a 12 or 15 ml conical centrifuge tube. They were then spun at 1.500 rpm for 10 minutes. The supernatant was discarded and the pellet suspended in 0,5 ml of distilled water, frozen and thawed twice, mixed well and centrifuged at 1.500 rpm for 10 minutes. The supernatant was discarded and the sediment resuspended in two drops of distilled water. Grids were prepared as described for sera.

Electron microscopy – The preparations were examined with a Zeiss EM-10B transmission electron microscope at 20.000 or 25.000 x magnification and 60 or 80 KV.

Control method for mycoplasma detection – Some sera were tested on chicken embryo fibroblast primary cell cultures (CEF) and stained by direct fluorescent staining 4-6-Diamidino-2-phenylindole (DAPI) as described by DelGiudice & Hopps (1977).

RESULTS

Sera – Sediments from 136 sera were examined after negative staining by electron microscopy. Mycoplasmas were detected in 43 samples (32%). The size of the particles depended upon the method employed to clarify the sera. Larger particles (Fig. 1) were rarely found in sera from laboratory animals. Commercially available sera contained only small mycoplasma particles, whose sizes varied depending on the different mesh diameters of the filters employed. If a 200 nm filter was used, little round mycoplasmas were commonly detected. These sometimes had one or two small white lateral filaments (Figs. 2 and 3) denoting a living form. When filter membranes with larger pores were used, larger pleomorphic particles were detected; with smaller pores (100 nm) only thin filaments without plasma content were seen (Fig. 4).

Cell cultures – Cell suspensions from 211 different tissue cultures were examined and mycoplasmas detected at 99 (47%) of them. The particles were always larger than those seen in sera, and were pleomorphic, sometimes presenting exotic configurations (Figs. 5 and 6) or compact aglomerations of particles. Empirically, as a general rule, if the mycoplasmas presented a white and smooth cell periphery, as seen in fresh cell cultures, they are living forms that could multiply (Fig. 7); if the cell periphery was only a gray or dark line of irregular pattern (Figs. 8 and 9), as in older cell cultures, this particle was no longer viable. There was no correlation between the type of cell and the mycoplasma form.

Controls – 43 sera were also tested by the DAPI fluorescent method on CEF cell cultures. Twelve sera treated by exposure to a UV-light source became, according to the DAPI technique, toxic to CEF cells and could not be included in the comparision. But of the remaining 31 sera, 27 (87%) were either positive or negative by both methods. Four were positive only by electron microscopy, indicating a higher sensitivity by this method.

DISCUSSION

All sera were tested prior to be used in cell cultures by electron microscopy. Different sizes and forms of mycoplasmas were found in some of them. This may be related to the method employed for serum purification, such as different filter pore diameters (Maniloff & Morowitz, 1972) or different speeds for centrifugation (Robertson et al., 1975b). By negative staining of electron microscope preparations it was always possible to distinguish mycoplasma particles from other debris, such as lipid droplets or cell membrane fragments. It was also possible to recognise two types of mycoplasmas: a viable form with smooth and white bordered particles including the so called "elementary bodies" and a form which lacks this border, which suggest is probably non

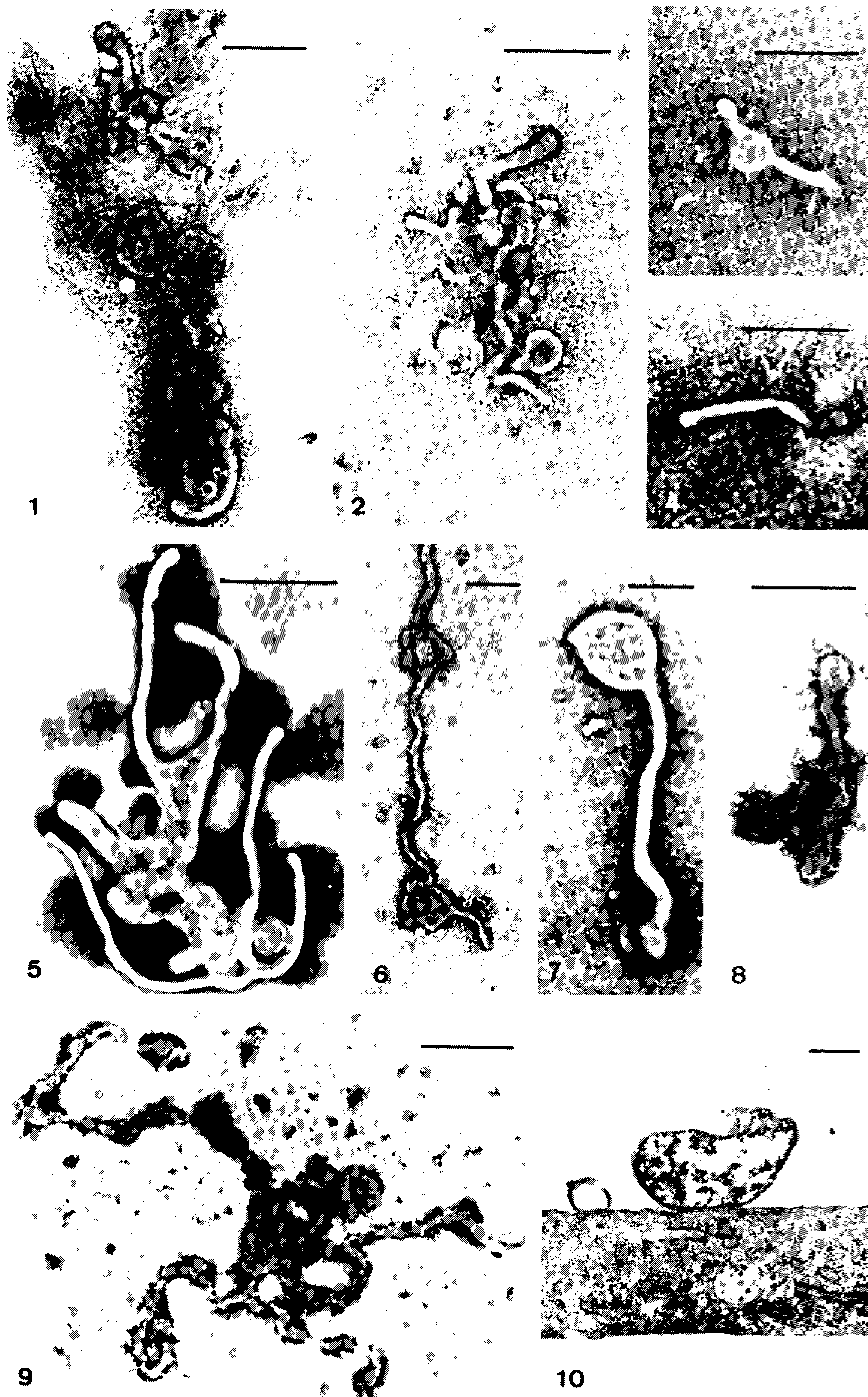

Fig. 1: Larger mycoplasma particle from animal serum (bar = 500 nm). Fig. 2: Group of small mycoplasma particles from fetal bovine serum (bar = 500 nm). Fig. 3: Viable mycoplasma particle from fetal bovine serum (bar = 500 nm). Fig. 4: Thin, probably non viable mycoplasma particle from fetal bovine serum (bar = 500 nm). Fig. 5: Mycoplasma particle from contaminated MDCK cell culture. Denote numerous thin filaments emerging from the cell body (bar = 500 nm). Fig. 6: Viable mycoplasma particle from contaminated cell culture with two cell bodies and long filaments (bar = 500 nm). Fig. 7: Viable white bordered mycoplasma particle with a white filament (bar = 200 nm). Fig. 8: Probably non viable mycoplasma particle from serum (bar = 500 nm). Fig. 9: Group of probably non viable mycoplasma particles from contamined Vero cell culture (bar = 500 nm). Fig. 10: Thin section of a mycoplasma particle attached to a Vero cell membrane (bar = 500 nm).

viable (Robertson et al., 1975a). Sera containing only single white filaments of mycoplasmas, without protoplasma, are considered useable for cell media preparation according to Bieberfeld & Bieberfeld (1970) and Maniloff & Morowitz (1972).

Mycoplasma particles in cell cultures (Fig. 10) attach intensely to the cell membrane (Boatman et al., 1976; Araake et al., 1984) and even after disrupting the cells by consecutive freezing and thawing cycles, mycoplasmas remain firmly attached to cell membrane fragments. Only a few small particles are found in the cell culture supernatant, and in general they cannot be detected by negative staining, even after high speed centrifugation. Observations by negative staining of cell debris obtained simply by low speed centrifugation constitutes a sensitive and rapid method for the detection of mycoplasma particles in cell cultures.

In comparison with other methods described for mycoplasma detection in sera and cell cultures, negative staining and electron microscopy is both faster and highly sensitive.

RESUMO

Detecção rápida da contaminação por micoplasmas de soros e culturas celulares por meio da microscopia eletrônica de transmissão – Para a detecção de micoplasmas em soros e culturas de células foi aplicada a microscopia eletrônica de transmissão. Os soros foram centrifugados em alta rotação, enquanto que aos detritos celulares aplicou-se uma centrifugação mais lenta. Os sedimentos ressuspendidos foram preparados por meio de contrastação negativa. Por esta técnica detectou-se contaminações por micoplasmas com maior freqüência do que pela aplicação do corante fluorescente direto (DAPI) em culturas celulares com micoplasmas, servindo de controle. A aparência das bordas celulares dos micoplasmas e de seu conteúdo fornece alguns dados sobre a viabilidade das partículas.

Palavras-chave: micoplasmas – contraste negativo – método rápido – contaminação de soros e células

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