JOURNAL OF BACTERIOLOGY, Mar. 1978, p. 1452-1456
0021-9193/78/0133-1452$02.00/0

Vol. 133, No. 3
Printed in U.S.A.

Positive Detection of Mycoplasma Contamination by the Whole-Mount Preparation of Cell Cultures for Transmission Electron Microscopy

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Received for publication December 1977

Low-level mycoplasma contamination of cell cultures is difficult to recognize with presently available techniques. This report describes the adaptation of the whole-mount technique, usually used for scanning microscopy, for transmission electron microscopy. The differentiation between microvilli and the equal-sized filamentous mycoplasma is based on the differential density obtained by the use of the method described. This method allows positive identification of mycoplasma and reduces the preparation time and the time necessary for scanning the preparation.

In the early days of electron microscopy, whole specimens had to be mounted on grids for examination, due to the inadequacies of the sectioning process. Critical-point drying achieved a good preservation of surface structure (1), but mycoplasma have not taken very well the harsh treatment necessary for placing them on an adequate support and have responded with a variety of changing forms. Mycoplasma contaminants of cell cultures, in our experience, have been mostly of the filamentous type and, if fixed in situ (i.e., without being removed from the substrate), have shown a remarkable structural integrity. With the advent of scanning microscopy (SEM), the direct visualization of mycoplasma contaminants has shifted to this new technology (see also 11). Because only the surfaces of the mycoplasma organisms can be seen, and because many types of cells have microvilli of comparable diameter and length, SEM is inadequate for making a positive diagnosis in many cases. Whole-mount electron microscopy has, therefore, been adopted as a method of detecting low-level mycoplasma contamination that is undetectable by the enzyme method of Becker and Levine (2).

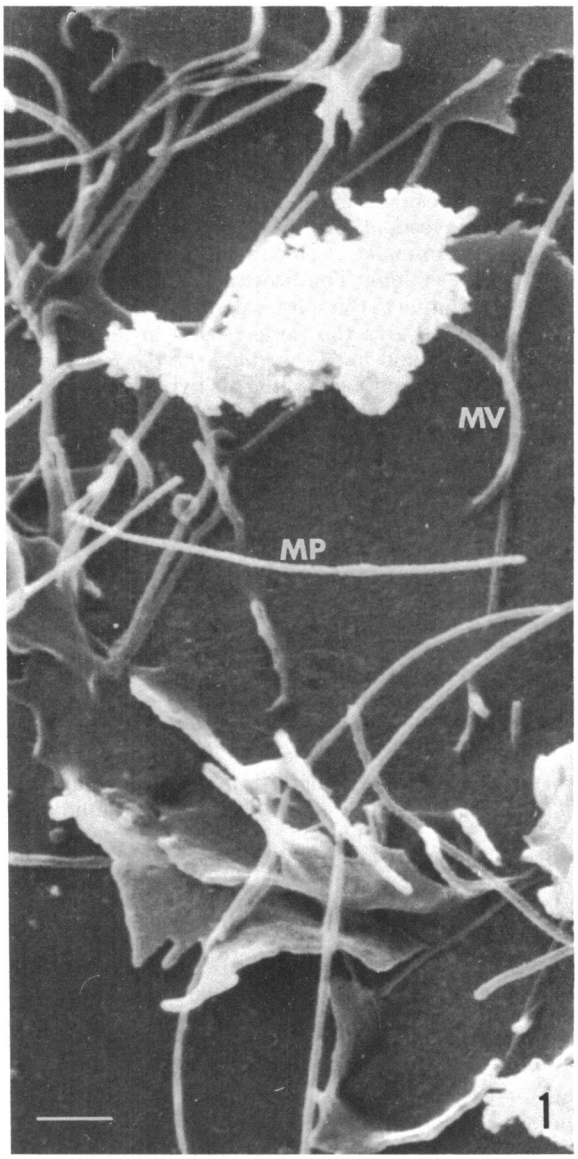
MATERIALS AND METHODS

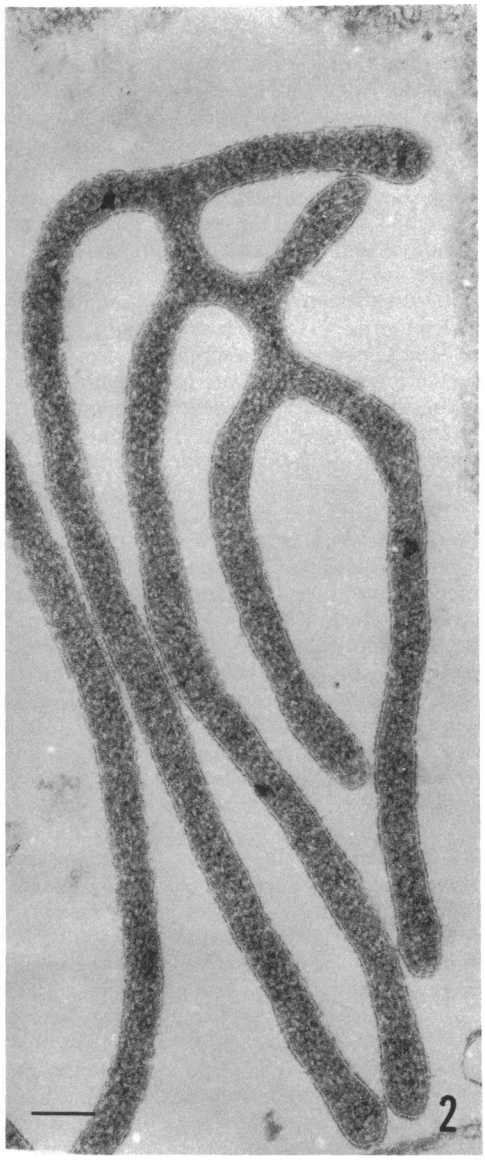
The method described is an adaptation of that used by Kilarski and Koprowski (8), who modified previous methods (6, 7, 10).

Clean glass or plastic cover slips were used to pick up 100-mesh grids floating on a Formvar or collodion film on water (1% collodion or Formvar in amyl acetate). After the coated cover slips with grids were dried, they were carbon coated and sterilized by exposure to UV light overnight. Mouse L cells were seeded onto the cover slips (8) in Falcon plastic petri dishes in Eagle minimum essential medium with 10% serum to a density that allowed them to grow in a CO_2 incubator for 48 h without becoming confluent. Low initial cell densities and seeding with the original medium also enhanced the chance that mycoplasma would settle and multiple in cell-free areas, which would greatly aid in their detection. For fixation, the medium in the petri dishes was gently removed, and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2, 1 mM $MgCl_2$) was added to the tilted dish. Tilting the dish back flooded the grids gently. This precaution was not necessary for *Mycoplasma pneumoniae*, which attached firmly to the substrate. After 15 min of fixation, the initial fixative was replaced with a 1% osmium solution in the same buffer for an additional 15 min. After being washed with distilled water, the cells were stained for different times with uranyl acetate from 10 min to 16 h, dehydrated in an alcohol series within 30 min, and placed in amyl acetate or acetone for critical-point drying (1). The grids were either carbon coated again or removed without coating and observed in the transmission microscope (Zeiss 10A) at 100 kV. For SEM (Jeol, JSM-U3), the specimens were shadowed with platinum. For sectioning, cells were grown directly in 3.5-cm Falcon plastic petri dishes, fixed and dehydrated as described, and then flat embedded according to Brinkley et al. (5). To preserve the original orientation, the cells were sectioned perpendicular or parallel to the substrate they grew on after being remounted.

RESULTS

Since the filamentous mycoplasma (undetermined species) and the microvilli of mouse L cells are of similar diameter, it is extremely difficult to distinguish between the two by SEM with any certainty. More specifically, false positive or false negative diagnoses may result if no general features of the filamentous mycoplasma are visible (Fig. 1). Also, it is often difficult to





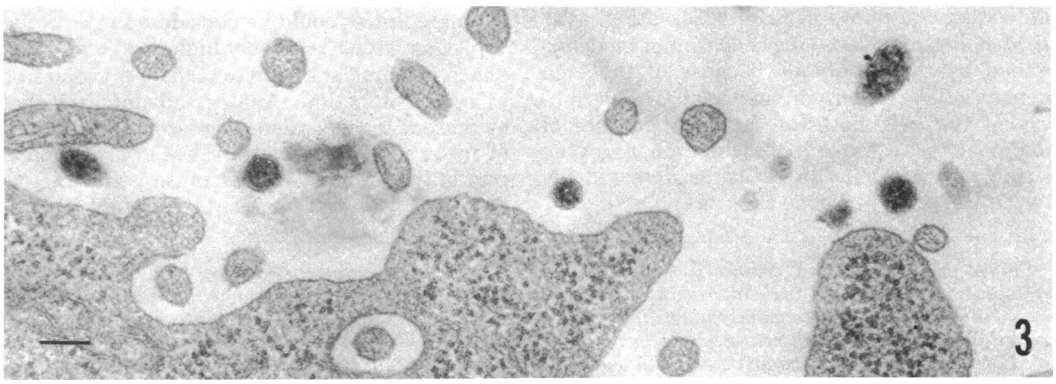


FIG. 1. *SEM micrograph of a mouse L cell contaminated with an unidentified mycoplasma. The microvilli (MV) are not readily distinguished from the mycoplasma (MP). Scale marker, 1 μm.*

FIG. 2. *Longitudinal section of an entire mycoplasma from a mouse L-cell culture. Scale marker, 100 nm.*

FIG. 3. *Cross section through microvilli and mycoplasma of a mouse L-cell culture. The sizes of microvilli and mycoplasma are about equal, but the mycoplasma have a high intracellular density that distinguishes them from the cytoplasm of the mammalian cells. Scale marker, 100 nm.*

determine whether direct contact or continuity with the cell membrane exists. A three-dimensional analysis with stereo pairs might overcome this handicap, but it would be too time-consuming for a general screening procedure. The mycoplasma contaminating the mouse L cells is a branched filamentous species, as seen in a longitudinal section of an entire specimen (Fig. 2). There are no immediately recognizable internal structures as in *M. gallisepticum* (9) and *M. pneumoniae* (3). In cross section, the mycoplasma appears with a circular outline that can be distinguished from the microfilaments by its higher density (Fig. 3). Sectioning and scanning of cross-sectioned cells are extremely time consuming, but allow positive identification not possible with SEM.

The density difference was exploited by combining the fast preparation method normally used for SEM and the possibility for positive recognition given by transmission electron microscopy. In Fig. 4a, a microvillus is crossed by mycoplasma filaments. The low density of the cytoplasm allows the resolution of the unit membrane, whereas the density of the mycoplasma precludes the resolution of the inner leaflet of the unit membrane. Even when the filamentous mycoplasma is intertwined with a microvillus, one is able to resolve the two structures (Fig. 4b). Most often it is the mycoplasma that is bent and twisted, but in Fig. 4c the microvillus is twisted and bent and could have caused the "correct" diagnosis for the wrong reason. Mycoplasma can also often be diagnosed if it has two ends (i.e., is not attached to the cell). In Fig. 4c the microvillus has probably been torn from the cell during the drying procedure, a rather common finding that may result in false positive determination of mycoplasma contamination. In Fig. 4d, a microvillus ends at a mycoplasma and seemingly is attached to it, as indicated by the bend in the mycoplasma at the point of contact, indicating continuity in SEM observation.

Mycoplasma overlaying cells cannot be determined by this technique because of the high density of the underlying material. The periphery of the cell, however, is often the site of microcolonies after 48 h of incubation, and even single mycoplasma can easily be detected between the cell extensions (Fig. 4e).

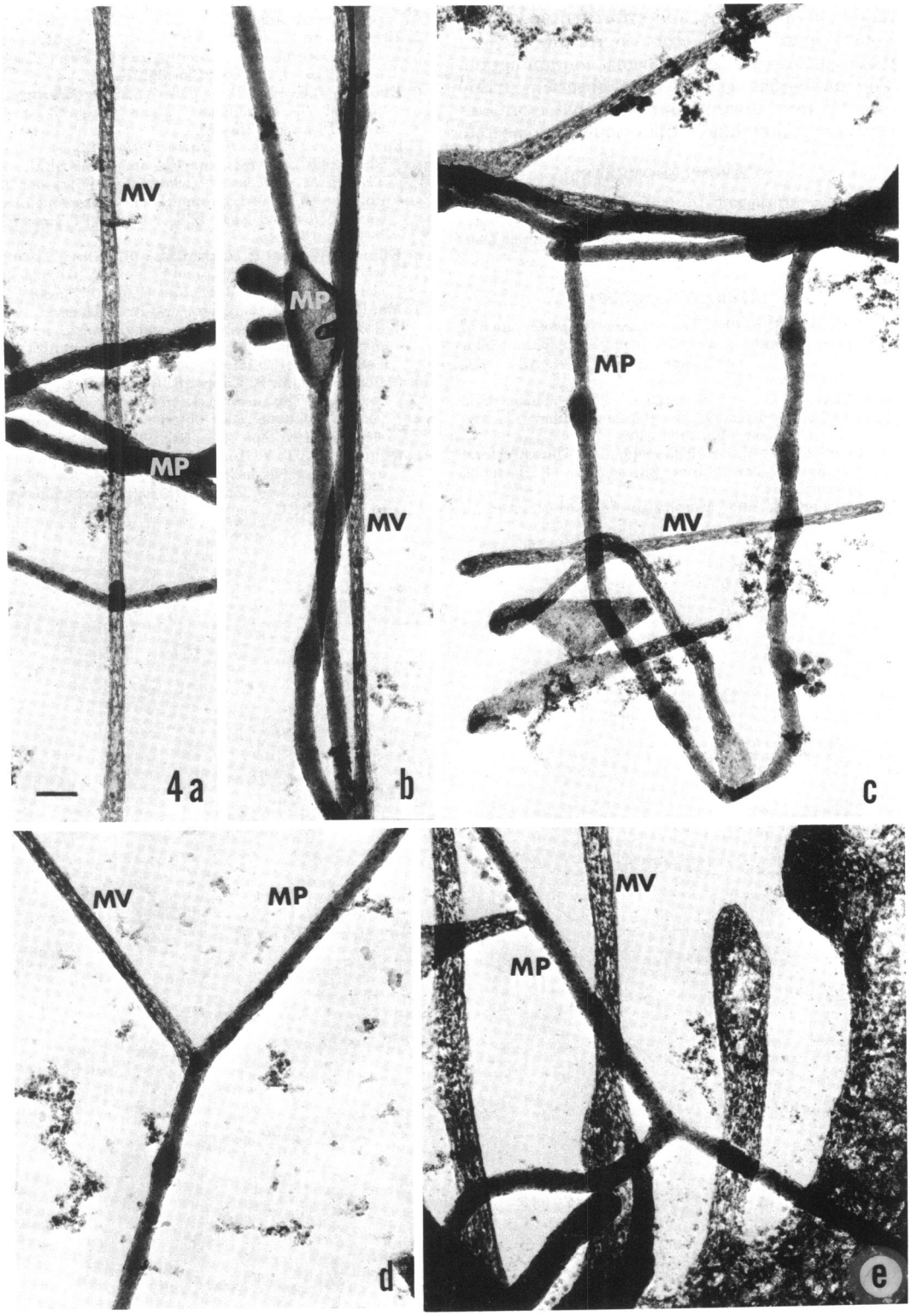
DISCUSSION

Whole-mount procedures for the visualization of mycoplasma species have been used previously. Boatman (4) reviewed some of the pitfalls of fixation particularly with respect to the different osmolarities used. The fixative used in this investigation shows the best ultrastructure for mammalian cells in culture as found by freeze-etching. The fixative has a high osmolarity relative to the solutes used for freeze-etching, but both give the same results in mammalian cultures and in *M. pneumoniae* (G. Maul, submitted for publication). This hypertonic fixative was, therefore, used for the procedures designed to identify mycoplasma.

The main advantages of the whole-mount technique are the speed of preparation, which is also characteristic of SEM, and the ability to positively identify and distinguish mycoplasma from microvilli. Therefore the time spent on the microscope is reduced significantly, since a single mycoplasma is definite proof of contamination. SEM does not allow positive identification or the resolution possible with transmission electron microscopy. The disadvantage of the whole-mount technique is that it cannot recognize mycoplasma on top of cells.

This new technique is particularly valuable for identification of low levels of contamination not recognizable by the enzyme method of Becker and Levine (2) or by autoradiographic methods, which all need substantial levels of mycoplasma. The recognition of the filamentous form depends on its higher density relative to microvilli of eucaryotic cells after staining with uranyl acetate. Scanning of large areas is facilitated by the easily observable density differences; 200 cells and the surrounding intercellular spaces can be searched for mycoplasma in 1 h. High levels of contamination can be observed immediately, low levels in no more than 10 min. If no mycoplasma were found within 1 h, mammalian cultures could be considered free of mycoplasma with a relatively high degree of confidence. Technicians learn to scan for mycoplasma in a very short time. Another advantage is that at present transmission microscopy is available in more institutes than is SEM. Furthermore, part of the sample used for transmission microscopy can be saved for SEM. This simple tech-

FIG. 4. *(a) Mycoplasma crosses a straight microvillus. The mycoplasma has the higher density. (b) Mycoplasma intertwined with microvilli; this relationship could not be identified with SEM. (c) Broken microvilli that may be mistakenly identified as mycoplasma in SEM. The mycoplasma contains bulges at irregular intervals. (d) Connecting mycoplasma and microvillus, which could be identified as only microvillus in SEM if the microvillus branch connected with a cell. (e) Mycoplasma between larger microvilli close to the mouse L cell. No identification is possible when mycoplasma are over the cell body itself. MP, Mycoplasma; MV, microvillus. All micrographs are the same scale. Scale marker, 100 nm.*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nique should be fast and effortless enough to induce more investigators to regularly check their cultures for mycoplasma contamination. The impression gained from observing many cultures from diverse sources is that more cultures are contaminated than would be expected.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants GM-21615 from the National Institute of General Medical Sciences and CA-10815 from the National Cancer Institute.

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