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New Assay Procedure for Separation of Mycoplasmas from Virus Pools and Tissue Culture Systems

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Presence of mycoplasma organisms in tissue culture systems and virus pools was detected by titration of the contaminated material on agarose-suspended BHK21/13S cells. The use of this method permitted isolation of mycoplasmas which could not be detected by standard assay methods. Mycoplasma colonies at concentrations ranging from 10^4 to 10^6 colony-forming units/ml in agarose-BHK21/13S media could be distinguished from virus plaques, and the two populations of microorganisms could be easily disassociated either by electron microscopy or by biological methods. All isolated mycoplasmas were identified in growth inhibition tests as belonging to the GDL group. The growth inhibition test on agarose-BHK21/13S cell suspension plates could also be applied directly to those strains which could not be isolated by standard assay procedures.

Mycoplasma was first implicated in 1956 (37) as a contaminant of mammalian cells grown in tissue culture. Since then, the presence of mycoplasma has been detected in many tissue culture systems (15) which do not show, in most cases, changes either in growth patterns or morphology as the result of contamination (3, 38).

More recently, electron microscopy revealed the presence, in tissue culture systems, of agents structurally resembling *Mycoplasma*, in higher concentrations than could be estimated by standard assay procedures (D. Armstrong and K. Hummeler, *to be published*). These results led to the conclusion that many tissue cultures used for biological investigations may have been contaminated with mycoplasmas which escaped detection by the usual assay methods.

The purpose of this communication is to describe a method for demonstrating the presence of mycoplasmas in virus pools and in noninfected tissue culture systems, through formation of colonies in cell cultures maintained in agarose (6). By use of this method, it is possible to dissociate the virus pools from their mycoplasma contaminants and obtain mycoplasma-free preparations. The results are particularly interesting since the presence of mycoplasma contaminants cannot be easily detected by the use of standard isolation procedures.

MATERIALS AND METHODS

Materials tested for mycoplasma contamination. Materials tested are listed in Table 1. Two tissue culture systems, BHK21/13 and BHK21/13M [derived from a clone of BHK21/13 (43) established at this Institute], were maintained in continuous passage in Basal Medium, Eagle (BME) with 10% calf serum; the WI-38 culture (16) and one lot of BHK21/13M culture were recovered from the frozen state. Virus-infected culture stocks were kept in a frozen state prior to experimental use.

Laboratory-adapted strain of mycoplasma. T-11 strain, originally described by Armstrong et al. (1), was made available through the kindness of L. Hayflick of this Institute. This strain was maintained after 58 passages on PPLO Agar in frozen agar block. After thawing, the organism was passaged on PPLO Agar plates.

Virus plaque and mycoplasma colony assays. In general, the technique descibed by Cooper (6) for plaque assay on poliovirus to ERK cells suspended in an agar layer was followed, with the exception that in the present work BHK21/13S cells suspended in agarose medium rather than agar were used (43). This cell line represents progeny of a clone of BHK21/13 line adapted to growth in suspension.

The BHK21/13S cells, referred to in this work as

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TABLE 1. *Origin of mycoplasma-contaminated material*

Material	Cell line or strain	Origin
Tissue culture	WI-38	Man
	BHK21/13	Hamster
	BHK21/13M	
LCM virus pool, 1045, 825, 850-3	WI-38	Man
Rabies virus pool, 1152	Nil-2	Hamster

13S cells, were grown in Blake bottles and, after ethylenediaminetetraacetate-trypsin treatment, were placed in suspension (10^7 cells per ml) in BHK21 medium (28) with 2% inactivated fetal calf serum.

An 0.8% agarose (Seakem; Bausch and Lomb, Rochester, N.Y.) suspension was made in distilled water and diluted in an equal volume of MS medium (22) without bovine albumin and containing 4% fetal calf serum. A 4-ml amount of this suspension was plated on each 60-mm plastic petri dish (Falcon Plastics, Los Angeles, Calif.).

Samples of serial 10-fold dilutions of the test material in BHK21 medium were mixed with 13S cell suspension in a ratio of 1:9. To this suspension, an equal volume of 0.4 to 0.6% agarose medium was immediately added; 1 ml of the mixture was then pipetted onto the previously prepared agarose layer.

In some cases, the inoculum was not mixed with the cell suspension; instead, the agarose-13S suspension plates were overlaid with 1 ml of 0.2% agarose medium and the test material was plated on top of the agarose layer. The petri dishes were incubated from 4 to 8 days at 35 C in the presence of 2% CO_2.

Standard assay procedures for isolation of mycoplasmas. Solid medium consisted of PPLO Agar (Difco), 20% horse serum (Hyland Laboratories, Los Angeles, Calif.) and 10% yeast extract (Fleischmann Active Dry Yeast).

PPLO broth was made from Beef Heart for Infusion (Difco) (15) and contained 20% horse serum, 10% yeast extract, 1% glucose, and 0.002% phenol red.

In addition to the standard media, PPLO broth was enriched with 10% homogenate of L5178Y cells (13). The cell homogenate was prepared by ultrasonic treatment of the cell pellet, with the use of a Branson Sonicator.

Broth media were seeded directly with either a one-tenth volume of test material or with a mycoplasma-like colony from agarose-13S plates infected with a dilution of the test material. The agarose area with the mycoplasma-like colony was "punched out" with a coarse capillary pipette and then placed directly in the tube containing broth media.

Broth cultures were incubated at 37 C for a period of 6 weeks. A 0.1-ml amount from each broth culture was seeded on PPLO Agar plates at 2, 4, 6, 8, and 10 days after infection and at the end of the 6-week incubation period.

The PPLO Agar plates were also inoculated directly with 0.1 ml of undiluted test material and were incubated at 37 C, either aerobically or anaerobically (in an atmosphere of 5% carbon dioxide and 95% nitrogen). Plates were examined every 2 days during a 3-week period under a low-power microscope for the presence of mycoplasma colonies.

Colonies appearing on PPLO Agar plates were transferred at 5-day intervals to fresh agar plates.

Production of immune sera against mycoplasma strains. Three cloned mycoplasma strains: *M. hyorhinis* (Somerson strain), W-3 (13), and T-11 (1) were adapted to growth in broth cultures in the presence of 20% rabbit serum. The BT-3 strain (17) was grown in broth in the presence of 20% horse serum. Antigens from these four strains, prepared according to the method of Lemcke (26), were used for immunization of rabbits (30). Serum was obtained 1 week after the last injection of antigen. In addition, four sera against *M. hominis* type 1, *M. arthritidis* (hominis type 2 PG-27), *M. orale* type 1, and *M. pulmonis* (Negroni agent) were obtained from L. Hayflick.

Growth inhibition tests. Mycoplasma strains were identified by growth inhibition tests using the immune sera described above. The tests were performed either on agar medium as described by Clyde (4) or on agarose-13S medium with colony growth inhibition as the criterion for a positive reaction. The test was read on the 5th day postinfection, after the plates were stained with neutral red.

Electron microscopy. Virus plaques and mycoplasma colonies could be easily differentiated on agarose plates infected with higher dilutions (10^{-3} to 10^{-4}) of test material. These plates were fixed by addition of 2% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min. Subsequently, they were washed twice with PBS and postfixed for 10 min with 1% phosphate-buffered osmic acid. The osmic acid was removed, and the plate was washed with several changes of 70% ethyl alcohol. The plaques and colonies were further dehydrated in 80% and 95% ethyl alcohol and, after this partial dehydration, were harvested from the plates by cutting with a scalpel under a dissecting microscope. The top of the agarose

TABLE 2. *Presence of mycoplasma in tissue cultures demonstrated by growth of colonies on agarose-13S plates*

Cell cultures	No. of colonies per plate[a]					No. of colony-forming units/ml
	10^{-1}[b]	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
BHK21/13[c]	C	28	3	0	0	2.8×10^4
BHK21/13M[d]	C	C	80	7	0	8.0×10^5
BHK21/M13[c]	C	C	32	3	0	3.2×10^5
WI-38[d]	C	C	82	8	2	8.2×10^5

[a] C = confluent.

[b] Dilution of inoculum.

[c] Cell cultures maintained in continuous passage.

[d] Material kept in frozen state prior to use.

layer was peeled off, and the material was cut into smaller pieces, dehydrated further in absolute ethyl alcohol, and embedded in epoxy resin.

Thin sections were double-stained with lead citrate and uranyl acetate.

RESULTS

Presence of mycoplasma in tissue cultures. Four to six days after inoculation with tissue culture material listed in Table 2, the agarose-13S plates showed presence of nontransparent micro-areas easily distinguishable from the remaining area of the agarose plate by macroscopic examination (Fig. 1). These areas were confluent on plates exposed to a high concentration of the inoculum but could be easily counted on plates seeded with the 10^{-3}, 10^{-4}, and 10^{-5} dilutions of the test material. Under microscopic examination, these colonies could be distinguished from the surrounding area by their high concentration of granular material (Fig. 2A). Colonies displaying the same characteristics were observed on agarose-13S plates after exposure to a known laboratory strain of *Mycoplasma*, T-11, grown in PPLO broth (Fig. 2B).

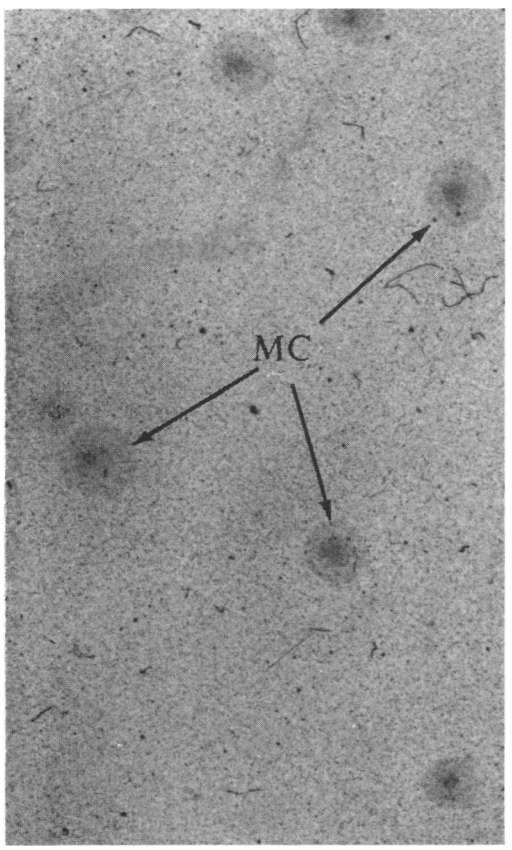

FIG. 1. *Mycoplasma colonies (MC) in unstained agarose-13S plate infected with a 10^{-3} dilution of tissue culture fluid from mycoplasma-contaminated WI-38 cells.* × 7.6.

Mycoplasma colonies with the typical "fried egg" appearance were obtained on agarose-13S plates when the tissue culture inoculum was seeded on top of the agarose layer, as outlined under Materials and Methods (Fig. 3). Routine examination, in the course of this study, of non-inoculated agarose-13S plates never revealed the presence of mycoplasma colonies.

Isolation of mycoplasmas from contaminated rabies and lymphocytic choriomeningitis (LCM) virus pools. Table 3 shows the results obtained by titration of four virus pools on agarose-13S plates. Virus plaques became visible, usually on the 5th day after inoculation, by the addition of 3 ml of 0.4% agarose overlay containing 10^{-4} g of neutral red per ml. At the same time, the mycoplasma colonies could be observed without staining and were easily differentiated macroscopically from the virus plaques (Fig. 4). After staining with neutral red, the mycoplasma colony appeared as a small, usually round, translucent area with a discrete opaque center. The translucent areas contained nonstained cells which probably undergo necrosis as the result of the cytopathic effect produced by the mycoplasma. Virus plaques were usually larger than mycoplasma colonies and could be easily distinguished from them by the absence of the opaque center (Fig. 4). As mentioned elsewhere (41), no differences in size and shape were noted between LCM and rabies virus plaques.

The results of titration of LCM pool 1045 indicate that, whereas virus plaques were observed on agarose-13S medium exposed to a 10^{-3} dilution of the inoculum, mycoplasma colonies appeared on plates exposed to a 10^{-5} dilution. However, since it was possible to distinguish the virus plaques from mycoplasma colonies, progeny of a virus plaque picked up from the plate exposed to the 10^{-3} dilution was passaged twice in WI-38 tissue culture in the presence of 50 μg of tylosin tartrate. Progeny of this virus (no. 1213) was again titrated on agarose-13S medium. The results show that, although the concentration of LCM was the same as in the original inoculum (no. 1045), no mycoplasma colony was found in the passaged material. Conversely, a mycoplasma colony picked up from the plate seeded with a 10^{-4} dilution of the original pool (no. 1045) was resuspended in 1 ml of medium and the material was titrated on agarose-13S plates; in this in-

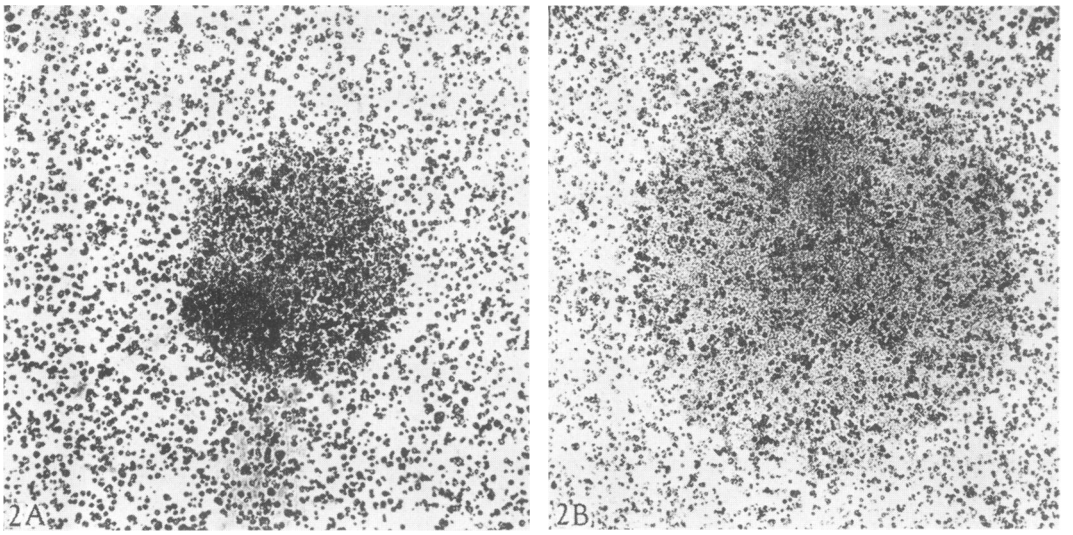

FIG. 2A. *Microscopic appearance of a mycoplasma colony isolated in agarose-13S medium from rabies poo 1152 5 days after inoculation.* × *100 approximately.*

FIG. 2B. *Microscopic appearance of T-11 mycoplasma colony on agarose-13S plate 5 days after inoculation.* × *50 approximately.*

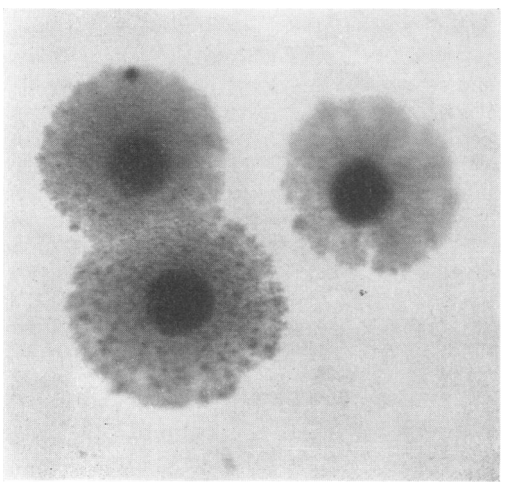

FIG. 3. *Agarose-13S plate showing mycoplasma colony with typical "fried egg" appearance. Test material placed on top of the agarose-13S layer (see Materials and Methods).* × *280.*

stance only mycoplasma colonies grew out (no. 1214).

Titration results of another LCM pool (no. 825) resulted in formation of colonies of mycoplasma on agarose-13S plates exposed to a 10^{-4} dilution of the inoculum; virus plaques were observed only on plates seeded with a 10^{-3} dilution of inoculum.

Titration results of rabies pool no. 1152 showed a higher concentration of rabies virus than of mycoplasmas in the inoculum. Progeny of virus plaques picked up from the plate exposed to a 10^{-5} dilution of the pool was found to be mycoplasma-free.

Attempts to grow mycoplasma in cell-free media. No mycoplasma colonies were observed on PPLO Agar plates incubated either aerobically or anaerobically after direct seeding with the inocula (undiluted and diluted at 10^{-1}, 10^{-2}, and 10^{-3}) listed in Table 4. However, a small number (five to seven) of mycoplasma colonies appeared when seeded with broth cultures of BHK21/13M material on PPLO Agar plates. The same result was obtained with broth culture enriched with cell homogenate and inoculated with WI-38 material; since the number of colonies in this case was also very small, it is doubtful whether the addition of cell homogenates to the broth was the factor responsible for the positive result. No mycoplasma colonies were isolated from other test material by direct seeding of broth cultures. The above tests were repeated five times with identical results.

When colonies from agarose-13S plates infected with four of the test materials were transferred into broth, and the broth culture was plated 4 to 6 days later on PPLO Agar (Table 4), colonies of mycoplasma appeared on the surface of the agar 4 to 12 days later. These colonies varied in number, size, and morphological ap-

pearance. With the exception of mycoplasma isolated from pool no. 1045, all isolated strains could be grown under aerobic conditions and maintained by transfer from one agar plate to another, by use of the agar-block technique. After several passages, the mycoplasma colonies showed the classical "fried egg" appearance.

Mycoplasma isolated from a colony on an agarose-13S plate exposed to pool no. 1045 grew out, after passage through broth culture, on PPLO Agar maintained anaerobically. This strain could not be maintained in continuous passages in cell-free media.

No mycoplasmas were isolated in either broth culture by direct assay, or after passage of virus pool no. 1152 in agarose-13S medium, in spite of a high concentration of mycoplasmas [1.2×10^5 colony-forming units (CFU)/ml] in the original inoculum.

Identification of mycoplasma strains by growth

TABLE 3. *Titration of mycoplasma-contaminated virus pools on agarose BHK-13S plates*

Material tested		Lesions	No. of plaques and colonies observed after exposure to dilution of inoculum[b]						
Pool no.	Virus[a]		Undiluted	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1045	LCM	Plaques	NT	NT	C	35	0	0	0
		Colonies	NT	NT	C	>100	62	6	0
1213[c]	LCM	Plaques	NT	NT	C	48	3	0	0
		Colonies	0	0	0	0	0	0	0
1214[d]	—	Plaques	0	0	0	0	0	0	0
		Colonies	NT	C	C	91	9	0	0
825	LCM	Plaques	NT	C	17	2	0	0	0
		Colonies	NT	C	>100	16	2	0	0
850-3	LCM	Plaques	NT	50	5	1	0	0	0
		Colonies	NT	5	3	0	0	0	0
1152	Rabies	Plaques	NT	NT	C	C	C	36	0
		Colonies	NT	C	C	61	8	0	0

[a] The LCM pools were produced on WI-38 cells; the rabies pool on Nil-2 cells.
[b] NT = not tested; C = confluent.
[c] Derived from virus plaque isolated from agarose plate exposed to 10^{-3} dilution of pool 1045; passaged twice in the presence of antibiotics.
[d] Derived from a mycoplasma colony isolated from agarose plate exposed to a 10^{-4} dilution of pool 1045.

TABLE 4. *Attempts to isolate mycoplasmas on cell-free media*[a]

Material tested	Colony-forming units per ml on agarose 13S plates	Mycoplasmas demonstrated by				
		Growth on PPLO Agar	Subculture on PPLO Agar from			
			PPLO broth		CH broth	
			D	A	D	A
BHK21/13	2.8×10^4	—	—	+ (4)[b]	—	+ (4)
BHK21/13M	8×10^5	—	+ (2)	+ (4)	+ (2)	+ (4)
WI-38	8.2×10^5	—	—	+ (6)	+ (2)	+ (6)
1045	6×10^6	—	—	+[c] (4)	—	+[b] (4)
1152	1.2×10^5	—	—	—	—	—

[a] D = broth directly seeded with test material; A = broth seeded with a colony from agarose BHK-13S plate; CH-PPLO broth + 10% cell homogenate; + = presence of mycoplasma colonies on PPLO Agar; — = absence of mycoplasma colonies on PPLO Agar.
[b] Days of incubation in broth.
[c] Growth observed only in agar plates incubated in 5% CO_2 and 95% N_2.

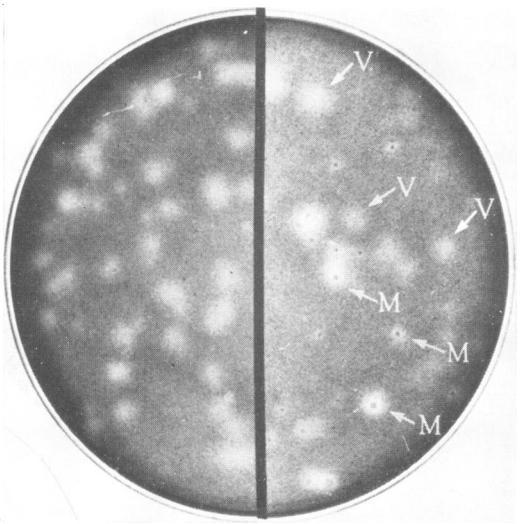

FIG. 4. *Macroscopic appearance of agarose-13S plate showing the presence of rabies virus plaques and mycoplasma colonies. On the left, typical rabies plaques. On the right, rabies plaques (v) interspersed with translucent areas of mycoplasma colonies with characteristic discrete opaque centers (M).* × *1.4*

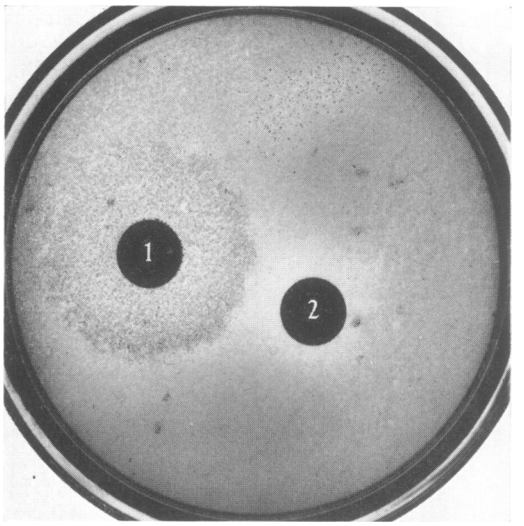

FIG. 5. *Growth inhibition of mycoplasma isolated from WI-38 cells. Inhibition area around disc 1 permeated with immune serum against T-11 strain; no growth inhibition around disc 2 permeated with normal serum. Neutral red stain.* × *1.9.*

inhibition tests. Results of the growth inhibition tests on agarose-13S plates (Fig. 5) indicated that mycoplasmas present in no. 1152 and WI-38 material were serologically identical with the GDL group (Table 5). For control purposes, the test was repeated, with a laboratory-adapted strain, T-11, belonging to the GDL group; the results were identical. The growth inhibition test was also performed on PPLO Agar plates with the three isolated strains from BHK21/13, BHK21/13M, and WI-38 cell cultures, and the results confirmed their serological identity with the GDL group.

Immune sera against four other strains of *Mycoplasma* listed in Table 5 did not inhibit growth of the isolated *Mycoplasma* strains.

Morphological appearance of Mycoplasma under electron microscopy. Agarose-13S plates were infected with serial dilutions of rabies pool no. 1152, and the plate showing the presence of distinct virus plaques and mycoplasma colonies was chosen for electron microscopy. Examination of the virus plaques showed cells in varying stages of necrosis, many of which contained virus particles with the morphological characteristics of rabies virus. Structures previously described

TABLE 5. *Identification of Mycoplasma organisms by growth inhibition test*[a]

Hyperimmune rabbit serum against	Growth inhibition of mycoplasma organisms seeded on:						
	Agarose-13S plates			PPLO Agar plates			
	1152	WI-38	T-11	BHK 21/13	BHK 21/13M	WI-38	T-11
T-11 (GDL group)	+	+	+	+	+	+	+
W-3 (GDL group)	+	+	+	+	+	+	+
BT-3 (GDL group)	+	+	+	+	+	+	+
M. hyorhinis (GDL group)	+	+	+	+	+	+	+
M. hominis type 1 (PG-21)	−	−	−	−	−	−	−
M. orale type 1	−	−	−	−	−	−	−
M. pulmonis (Negroni agent)	−	−	−	−	−	−	−
M. arthritidis (hominis type 2 PG-27)	−	−	−	−	−	−	−

[a] Growth inhibition, +; no inhibition of growth, −.

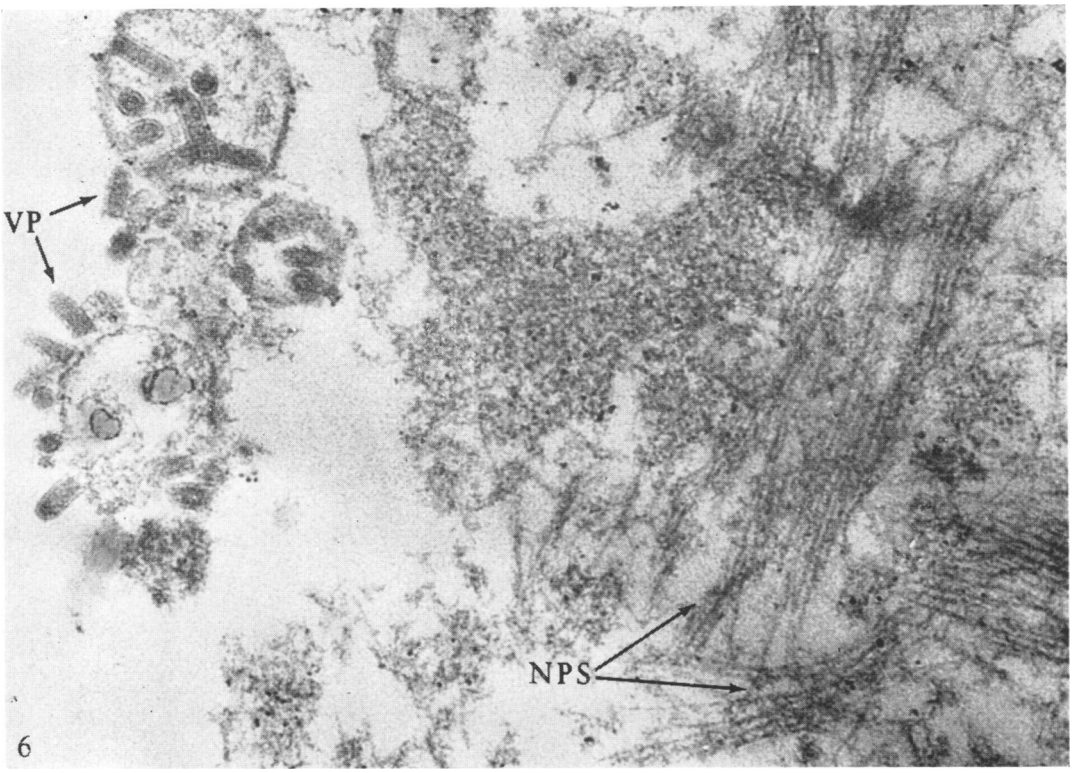

FIG. 6. *Electron micrograph of rabies virus particles (VP), and nucleoprotein strands (NPS) in cell debris. From a virus plaque on agarose-13S suspension plate.* × *52,500.*

as possible viral nucleoprotein strands (20) were also frequently encountered in infected cell material (Fig. 6). No mycoplasma-like structures were seen in virus plaques which had appeared clear in the light microscope.

The visible mycoplasma colonies gave the appearance of mature mycoplasma organisms, although lacking some of the characteristic features, such as the deoxyribonucleic acid (DNA) strands described previously (19; Fig. 7). The absence of these features was probably the result of improper fixation in the semisolid agarose.

Electron microscopy showed a clear separation of rabies virus and mycoplasma morphologically, thus supporting the findings of the biological assays.

DISCUSSION

The results reported in the preceding section indicate that contamination of tissue culture systems and virus pools by mycoplasma organisms of the GDL group could not be proven by the use of standard assay procedures for the isolation of mycoplasma.

Although most cell-free media used for growth of mycoplasmas, which are fastidious in their nutritional requirements, are much richer in their ingredients than ordinary bacteriological media, they failed (with the exception of two cases) to support the growth of the *Mycoplasma* strains present in the virus pools and tissue culture systems. Only after the contaminated material was titrated on agarose-13S medium did it become possible to demonstrate the presence of mycoplasmas in concentrations which, by direct count of macroscopically visible colonies, often exceeded 10^4 CFU/ml. Despite this high concentration of mycoplasmas in the inoculum, direct seeding of the contaminated material on PPLO Agar did not result in a single isolation of the organism. Direct seeding of broth cultures with the test material resulted in isolation of mycoplasmas on PPLO Agar plates in only two instances; however, in both cases, the number of colonies visible on agar was very small in comparison with the number of colonies isolated on agarose-13S plates. Subculture of agarose colonies on standard cell-free PPLO media resulted, with the exception of the contaminants of virus pool

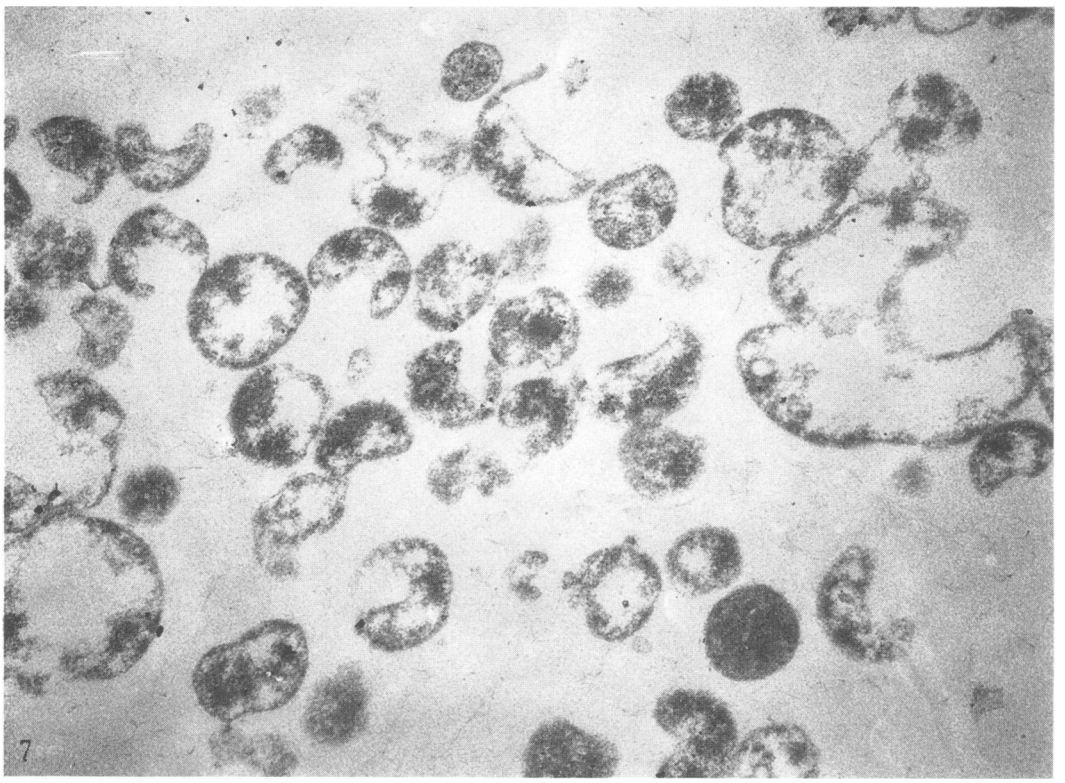

FIG. 7. *Electron micrograph of mycoplasma organisms. From a colony on agarose-13S suspension plate.* × *31,500.*

no. 1152, in the growth of mycoplasma organisms on PPLO broth. After serial passages in PPLO broth, the number of CFU/ml of mycoplasma obtained by titration either on PPLO agar or on agarose-13S plates was approximately the same.

The oxygen environment in which PPLO agar plates were incubated seemed to play an important role in the isolation of mycoplasma in some studies (23), since 83% of isolations were positive under anaerobic conditions, compared with 43% under aerobic conditions. It is difficult to say whether, in the studies reported here, this factor was significant; only one strain of *Mycoplasma* (no. 1045), isolated originally from agarose-13S plates, grew on PPLO plates which were anaerobically incubated. This strain could not be maintained, however, in continuous passage under the same conditions.

The relative insusceptibility of the standard media to infection with mycoplasma organisms isolated in the course of this work may be attributed either to the presence of growth inhibitors or to the absence of growth promoters in the medium. Smith (42) and Lynn and Morton (27) described the presence of growth inhibitors of *Mycoplasma* in several fractions of agar. Such inhibitors were also found by Morton et al. (32) among more than 50% of peptones tested as ingredients of the medium. The relatively low sensitivity of the standard broth medium may also be related to the fact that horse serum was used almost exclusively as a protein source in the medium. Originally, the choice of horse serum was justified because of its high cholesterol level and absence of mycoplasma antibodies; however, different lots of horse serum originating from the same source have been found to vary in their mycoplasma growth-supporting capacity (31). Thus, in spite of the apparent advantages of horse serum, it is possible that sera obtained from other species, such as rabbit or man, may display greater growth-promoting properties for certain *Mycoplasma* strains.

The possibility must also be considered that inhibitors of mycoplasma growth may have been produced in the contaminated tissue culture material, and that the presence of additional inhibitors in the PPLO medium infected with material diluted beyond the range of the inhibitors made it possible to isolate the organism.

It is feasible that the presence of growing mammalian cells in agarose-13S medium promoted growth of mycoplasmas. However, extracts of mammalian cells obtained from another source and added to the PPLO broth did not seem to have the same growth-promoting effect, since, as shown in Table 4, use of enriched broth resulted in only one additional positive isolation on direct seeding of the contaminated material.

One of the *mycoplasma* strains isolated from contaminated rabies virus pool no. 1152 failed to grow on standard media even after passage on agarose-13S medium. It was possible to identify it serologically as belonging to the GDL group, since the growth inhibition test could be performed on agarose-13S plates instead of PPLO agar. The fact that all mycoplasma contaminants belonged serologically to the GDL group does not make it easier to trace the source of the contamination. Strains classified serologically as the GDL group are of relatively recent origin (2). Past references dealt more frequently with the isolation of strains of *M. hominis* type 1 (5, 7) and *M. orale* of human origin (25), *M. hominis* type 2 (10), *M. gallisepticum* (9), and *M. laidlawi* (24); however, the majority of strains isolated recently in this Institute from a variety of diagnostic material belongs to the GDL group (18). These strains display a marked affinity for viable mammalian tissue, causing cytopathic effect (2, 13), but their origin remains obscure since they were found in tissues of diverse origin: T-11 from hemangioma (1), W-3 from human fibroblast culture WI-26 (13), and BT-3 from human bladder papilloma (17). Although this makes it difficult to trace the possible common origin of mycoplasmas found as contaminants of the tissue cultures and virus pools used in these studies, one should not overlook the possibility that the infection resulted from cross-contamination by a single *Mycoplasma* strain from one tissue culture to another by aerosols. Survival of mycoplasmas in the aerosol state has been observed by G. D. Baily, D. N. Wright, and M. T. Hatch (Bacteriol. Proc., p. 71, 1967), and mycoplasma aerosols as a source of laboratory contamination were described by O'Connell et al. (34).

Presence of mycoplasmas in tissue cultures may cause cytogenetic changes (12) and lead to metabolic disturbances (14, 29, 33, 35, 39, 40, 45), also affecting the metabolism of the host cell DNA which is particularly labile (36) in mycoplasma-infected cultures. Furthermore, production of virus antigens in mycoplasma-contaminated tissue culture systems may lead to unexpected cross-reactivity in serological and immunological reactions between serologically unrelated antigens (8).

Through the use of the agarose-13S system, it was possible not only to detect mycoplasmas in test material which was negative by standard assay methods, but also to separate virus populations from the mycoplasma organisms.

In the case of pool no. 1045, in which the concentration of mycoplasmas was higher than that of LCM virus, it was possible to isolate a mycoplasma strain from a colony visible on a plate infected with a high dilution of the inoculum (no. 1214, Table 3). Conversely, passage of the material in tissue culture in presence of tylosin tartrate depressed the concentration of mycoplasmas to such an extent that it became possible to isolate a pure virus plaque and propagate its mycoplasma-free virus progeny (pool 1213, Table 3).

The morphological evidence of virus-mycoplasma separation into distinct virus plaques and mycoplasma colonies in agarose, confirmed by electron microscopy, should aid in the description of as yet unknown virus structures. By identifying forms of mycoplasma which bear a close resemblance to some viruses, especially those of the myxovirus group (21), it will be possible to eliminate them from consideration in the study of these viruses.

More recently it was possible to isolate mycoplasma strains from sheep brains (kindly supplied by Clarence J. Gibbs of the National Institute of Neurological Diseases and Blindness) on agarose-cell suspension medium. Colonies formed by these strains on agarose, in contrast to those observed with the GDL strain, were almost invisible without staining. After staining with neutral red, it was possible to distinguish minute mycoplasma colonies less than 0.1 mm in diameter surrounded by a halo of nonstained cells. In this case, the mycoplasma "plaque" closely resembled plaques produced by virus, and the presence of mycoplasma could only be confirmed by successful seeding of the plaque material on PPLO broth and passage on PPLO Agar. These strains are as yet unidentified but do not belong to any of the 14 most commonly encountered *Mycoplasma* strains of human and animal origin.

We feel, therefore, that the use of agarose-mammalian cell medium for growth of mycoplasma should become a routine procedure in all cases in which standard assay methods give negative results.

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