DETECTION AND SPECIATION OF COMMON CELL CULTURE MYCOPLASMAS BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY WITH BIOTIN-AVIDIN AMPLIFICATION AND MICROPOROUS MEMBRANE SOLID PHASE

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SUMMARY

An enzyme-linked immunosorbent assay (ELISA) was developed in order to serve in detecting and speciating mycoplasmas isolated from cell cultures. Its main features included a biotin-streptavidin amplification step and a solid phase consisting of a microporous membrane. Cell samples in the form of suspensions were applied to nitrocellulose or ion exchange membranes immobilized in commercially-available microtiter, multiwell manifolds. The blocking buffer contained 1% purified α-casein. The primary antibodies were monoclonal and the polyclonal secondary antibody was biotinylated. The enzyme utilized was streptavidin-horseradish peroxidase. The substrate-dye complex consisted of either 4-chloro-1-naphthol and hydrogen peroxide or ortho phenylene diamine (OPD) and hydrogen peroxide. The presence of homologous antiserum in the reaction sequence gave clearly visible, colored reactions on the membrane when 50 ul with approximately 10^5 or more cfu/ml were present. This new biotin-avidin microporous membrane (BAMM-ELISA) test can be used both to detect mycoplasmas and to speciate them. The BAMM-ELISA is simple, rapid, sensitive, specific and economical. As such, it has potential for aiding in the control of mycoplasma contamination in cell culture, and could prove useful in clinical diagnostic applications as well.

Key words: enzyme-linked immunosorbent assay; ELISA; mycoplasma; contamination; identification; streptavidin; peroxidase.

INTRODUCTION

Covert contamination of cell lines by mycoplasmas continues to be a major problem in cell biology laboratories. Estimates of contamination levels range from 15% (26) to more than 50% (2). The consequences of contamination are significant since mycoplasmas can perturb virtually every parameter used in cell culture analyses. Effects range from alterations in the yield of viruses and interferon (4) to altered nucleic acid metabolism (44) and interference in hybridoma selection (16,21).

There currently are available a number of mycoplasma detection methods. The most sensitive is assumed to be direct culture (2,26). Other assays include the DNA-fluorochrome stain (10) and RNA probes (37). Relative reliability and sensitivities are good, but each of these assays requires excessive time or complex, expensive equipment.

Our laboratory has developed a novel enzyme-linked immunosorbent assay (ELISA). It employs a biotin-avidin amplification step, and a microporous filter membrane as the solid phase. This approach provides several distinctive advantages over other assays, including speed, economy, and sensitivity. Data are presented on the utility of this ELISA assay, along with a study on detectability limits and specificity.

MATERIALS AND METHODS

Mycoplasmas. Stock mycoplasmas were obtained from the American Type Culture Collection. They consisted of: M. arginini (ATCC 23838), M. orale (ATCC 23714), M. hyorhinis (ATCC 17981), M. salivarium (ATCC 23064) and A. laidlawii (ATCC 23206). They were cultivated in Fortified Commercial Broth (24) which has a Mycoplasma Broth Base (Difco Laboratories, Detroit, MI) supplemented with glucose, arginine, thymic DNA, and several vitamins and cofactors. It was fortified with 20% horse serum and 10% fresh yeast extract prior to use. Stock cultures were stored frozen at $-70°$ C, and typically were used within 20 passages since receipt. Identity of the mycoplasma stocks were verified by growth inhibition assays of colonies on agar plates with antiserum on filter paper discs.

Cell lines. VERO cells (CCL 81) of monkey kidney origin were grown in medium 199 with 5% fetal bovine serum and no antibiotics. Routine culture was done in disposable plastic flasks, and cells were routinely screened for mycoplasmas. The basic screening procedure of Chen (10) was followed, but with the following modifications. Basically, cell suspensions (approximately 3 ml) were added to a 40 X 40 mm glass coverslip. After an attachment period of 60 minutes, cells were fixed twice in acetic acid:methanol (1:3) per the "CELLshipper"

directions (Bionique Laboratories, Inc., Saranac Lake, NY). Air dried samples were stained with the Hoechst 33258 fluorochrome dye and examined with a UV microscope at 200× magnification.

ELISA Protocol. Microporous membranes (0.45 micrometer pores; BA-85 nitrocellulose or NA-49 cation exchange) were obtained from Schleicher & Schuell (Keene, NH). They were secured in commercial manifolds (96-well, by V&P Scientific, San Diego, CA; or slot-blot apparatus by Schleicher and Schuell). Gaskets in the manifolds were lightly coated with silicone vacuum grease before use.

Once assembled, 50 ul/well of phosphate buffered saline (calcium-magnesium free, pH 7.4; CMF-PBS) were added to wet the membrane. Excess CMF-PBS were removed by vacuum filtration, and 50 ul cell samples were added. In the current study, cell input samples were either broth cultures of stock mycoplasmas or those isolated from naturally-infected cell lines. Cells were immobilized onto the microporous membranes by vacuum pressure (approximately 25 cm of mercury). This was followed by 50 ul of 1% α-casein (Sigma, St. Louis, MO) to block unoccupied protein-binding sites on the membrane. After 15 min. incubation at 22° C, the casein was removed by vacuum filtration.

Thirty microliters of the monoclonal mouse primary antiserum (Bethesda Research laboratories, Gaithersburg, MD) were added, typically at a 1:10 dilution in CMF-PBS. Antisera were specific for *A. laidlawii*, *M. hyorhinis*, *M. arginini*, *M. orale*, and *M. salivarium*. Specificity was verified by using each antiserum against stock cultures of ATCC-originated pure strains of mycoplasmas. Each ELISA assay incorporated all five antisera. Incubation was at 22° C for 30 minutes. Excess

antibody was removed under vacuum, and wells were washed 3× (2–30 second washes and 1–10 minute wash before vacuum) with CMF-PBS containing 0.05% Tween 20. Thirty microliters of the secondary, biotinylated goat anti-mouse IgG (1:10 in CMF-PBS with 1% α-casein) were next added to each well. The solution on the cells was incubated 30 minutes at 22° C, vacuum filtered, and wells were washed 3× as described previously. Streptavidin-horseradish peroxidase (Bethesda Research Laboratories) diluted 1:10 in 1% α-casein was added and incubated for 30 minutes at 22° C. Wells were washed 3× again with CMF-PBS (without Tween 20). The reaction was developed with the selected substrate (50 ul of 4-chloro-1-naphthol (4-CN), Kirkegaard and Perry Laboratories, Gaithersburg, MD; o-phenylene diamine dihydrochloride, OPD, Sigma). Incubation was for 10 minutes at 22° C using 4-CN and 30 minutes with OPD, in the dark. The reaction was stopped with distilled water and the wells were decanted. Membranes were removed and air-dried. Each test always included multiple sets of wells of negative controls, to include medium blank without cells and heterologous antisera and/or an ascites control when monoclonal antibodies were used. The degree of reactivity of coloration in these wells were used to establish the background. Reactions in other wells which clearly had color development greater than the background were deemed positive.

Densitometry. Scans of peroxidase-labelled reaction sites were done with a video-densitometer developed by Bionique Laboratories, Inc. (Saranac Lake, NY). Basically, the sheets were illuminated by reflected white light and televised. The video image was electronically scanned utilizing a proprietary process (patent pending) without transporting the specimen. The light-dark
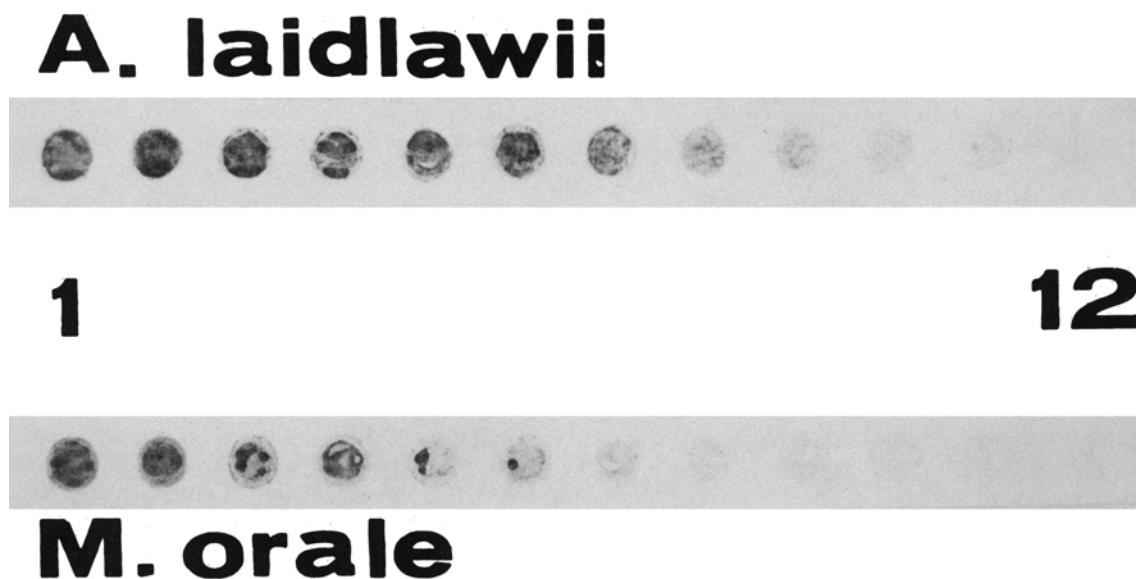

FIG. 1. Strips of NA-49 ion exchange membrane from a 96-well manifold used in a BAMM-ELISA reaction with an OPD substrate. Each row of wells received a mycoplasma of broth culture origin and its specific antiserum. Well #1 received 1.5 × 10⁷ cfu of *Acholeplasma laidlawii* or 2.7 × 10⁶ cfu of *Mycoplasma orale*. The next ten wells received 2-fold dilutions. Well #12 was the blank (no cells) control.

variations were converted to an analog voltage signal which was translated into a densitometric profile on a strip-chart recorder.

RESULTS

ELISA Protocol and Sensitivity. The assay protocol which we developed employed a biotinylated secondary antibody in order to maximize the signal. Preliminary experiments indicated that the use of Protein A and polyclonal antibodies generated non-specific reactions. Hence, the final protocol in the biotin-avidin microporous membrane (BAMM-ELISA) employed vacuum deposition of organisms on the membrane filter, followed by monoclonal primary antibody (mouse) and a polyclonal secondary antibody (goat) which was biotinylated. Streptavidin horseradish peroxidase and a dye substrate solution (e.g., 4-chloro-1-naphthol and hydrogen peroxide or OPD-hydrogen peroxide) completed the reaction sequence to generate a visually-detectable signal.

Studies done with 1:10 dilutions of the primary antisera indicated that the BAMM-ELISA test could detect mycoplasma when present at approximately 10^5 to 10^6 cfu/ml. Since the actual input sample was 50 ul, a level of 5×10^3 to 5×10^4 organisms could generate a clearly visible reaction. Typical reactions are shown in Figure 1. From left to right, each well in the manifold (96-well microtiter format) received 50 ul of two-fold dilutions of *Acholeplasma laidlawii* or *Mycoplasma orale* suspensions prepared from broth cultures. The blocking agent was 1% α-casein in CMF-PBS, and the membrane was cation exchange (S&S NA49). Reactions were clear and visibly distinct from approximately 10^7 cfu/ml through 8 dilutions (10^5 to 10^6 cfu/ml). The negative control well (extreme right in each row) had minimal staining. The technique required only small amounts of reagents (e.g., 30 ul of dilute antisera), and was complete within 3 hours.

The BAMM-ELISA can be conducted either in 96-well manifolds or slot-blot devices. The slot-blot system presents a somewhat smaller surface area with the same volume of reagents. This tends to concentrate the colored precipitates formed by the peroxidase. As a consequence, sensitivity is slightly enhanced in such a format.

Specificity. The BAMM-ELISA technique, if it is to be used to identify as well as detect mycoplasmas, has to be specific. Our data (Figure 2) show that if high quality antisera are available, species of mycoplasmas can be identified. The sample membranes (cation exchange, S&S NA49) have received *A. laidlawii* cell suspensions, and either anti-*A. laidlawii*, anti-*M. hyorhinis*, or anti-*M. orale sera*. At high levels of organisms, some color developed with heterologous sera due to non-specific entrapment in the membrane pores. However, careful examination with either the unaided eye or a dissecting microscope clearly revealed discrete particulate colored zones (clusters of mycolpasma cells) in the reaction of identity, down to the 11th column (5×10^3 cfu).

The use of a slot-blot device (Figure 3) reflected a similar reaction (i.e., strongest reactions with the homologous antiserum, particularly at higher dilutions). These slot-type reactions were particularly suited to densitometric analysis and quantitation. The first four wells from each column in Figure 3 were scanned with a videodensitometer in order to document the specificity of the reaction. The data (Figure 4) show the significant differences in intensity of the homologous versus the heterologous reactions. Wells #3 and 4 (containing 1.4×10^6 and 7.0×10^5 cfu/well, respectively), had very clear peaks in the densitometer profile when treated with serum specific for Acholeplasma (row A), whereas the anti-*M. hyorhinis* (row B) and anti-*M. orale* (row C) had negligible peaks.

Mycoplasma Speciation. A clear advantage of the BAMM-ELISA is that it can be used both as a detection system and as a speciation protocol. Randomly selected, unknown mycoplasms isolated from actual cell culture samples were grown in fortified commercial broth. They were concentrated 5-fold by centrifugation, and 50 ul of each of 3 separate isolates (plus an *M. hyorhinis* positive control) were placed in quadruplicate wells (Figure 5). When each of the 5 standard antisera (common cell culture contaminants), plus an ascites control, were added in the BAMM-ELISA protocol, two unknowns plus the control reacted with the anti-*M. hyorhinis* antiserum. This tentative *M. hyorhinis* identification was verified by growth inhibition assays with antiserum. The third
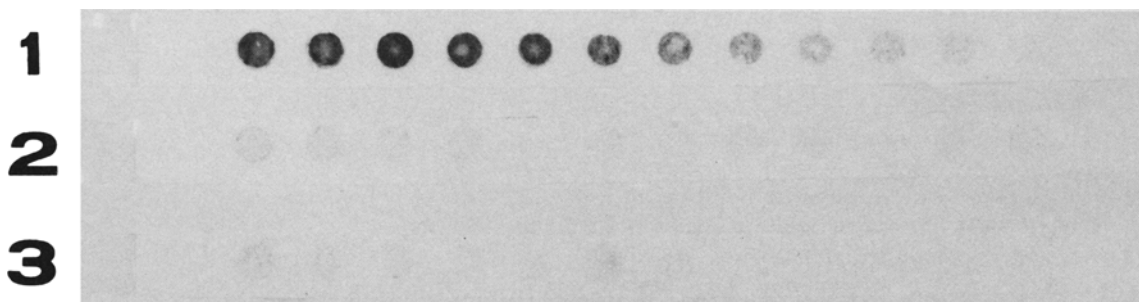

FIG. 2. BAMM-ELISA reaction as in Figure 1, but used to speciate a mycoplasma culture. *Acholeplasma* organisms were added to each well (left column received 5.6×10^6 cfu/well; the next ten wells received 2-fold dilutions, while well #12 received no organisms). Row #1 was treated with anti-*A. laidlawii*; row #2 received anti-*M. hyorhinis*; row #3 received anti-*M. orale*.
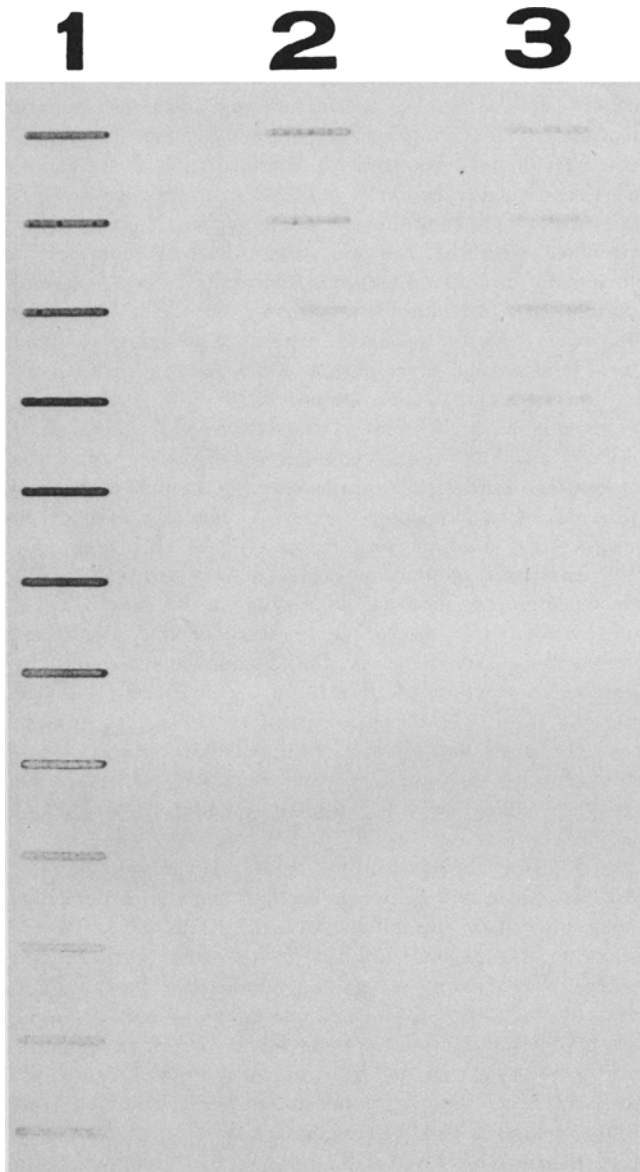
FIG. 3. BAMM-ELISA reaction as in Figure 2, but using an S&S slot manifold. *A. laidlawii* organisms were used in all wells, with concentrates in the top row (5.6 $\times$ 10^6 cfu/well) and 2-fold dilutions in the next ten rows. The bottom row of wells had no organisms (blank). Column #1 received anti-*Acholeplasma* serum; column #2 had anti-*M. hyorhinis*; column #3 received anti-*M. orale*.

standard antisera were combined into a single "cocktail." This was further diluted 1:10 in CMF-PBS and used to screen for mycoplasmas commonly associated with cell cultures. The results (Figure 6) show that this mixture was capable of indicating the presence of mycoplasmas at levels of approximately 10^5 cfu/ml. Thus, one could use the BAMM-ELISA approach in a one-step method using a mixture of antisera to detect contamination, or with individual, specific antisera to determine species identity.

Effect of Membranes and Enzyme Substrates. Several types of microporous membranes can be used in the BAMM-ELISA assay. Polycarbonate, mixed cellulose ester, cellulose acetate, cation exchange, and nitrocellulose are all suitable. However, the latter two membrane types provide firm attachment for proteins and low background staining.

The nature of the optimal enzyme and substrate depend upon the particles being studied. We found that the mycoplasma antigen is clearly delineated by either alkaline phosphatase or horseradish peroxidase. The latter has the advantage of being compatible with a wide variety of commercially available substrates-dye complexes. Optimal results were observed with OPD (o-phenylene diamine dihydrochloride) or 4-CN (4-chloro-1-naphthol), the latter of which gave somewhat darker, more easily visible reactions.

isolate, which did not react in this system, was not one of the five mycoplasmas for which we had monoclonal antibodies. In any type of ELISA assay, a negative, or non-reaction, simply means no match with the antisera tested. It does not mean that mycoplasmas are absent. Since the five strains studied account for more than 95% of cell culture contamination, we do earn a degree of confidence with this approach, though it is not absolute.

Detection of Contamination with Antiserum "Cocktail." In order to increase the efficiency of contamination detection with the BAMM-ELISA technique, all 5
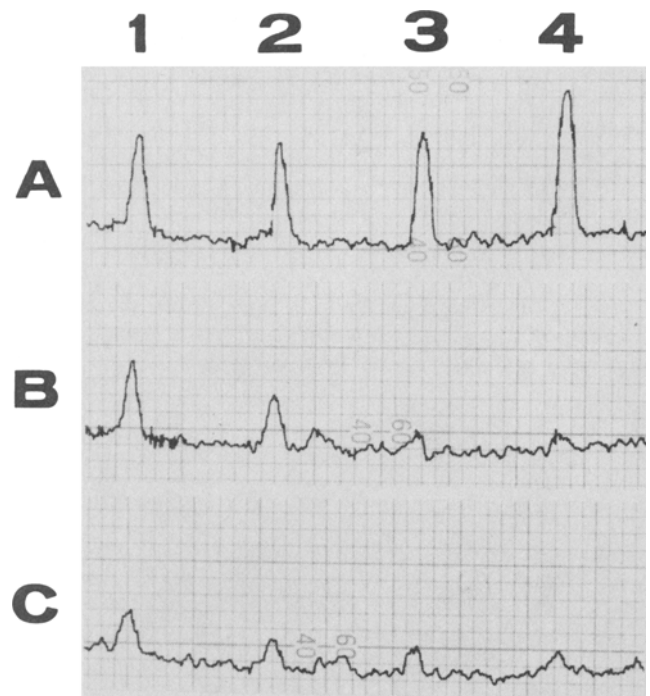

FIG. 4. Densitometric analysis of the first 4 rows of the BAMM-ELISA reactions from Figure 3. Wells were treated with: anti-*A. laidlawii* (A); anti-*M. hyorhinis* (B); or anti-*M. orale* (C). Peak height reflects intensity of darkness in reflected yellow light; numbers refer to wells (i.e., #1 on left received 5.6 $\times$ 10^6 cfu of *Acholeplasma* organisms; wells 2, 3, 4 were 2-fold dilutions).

DISCUSSION

We have developed an ELISA system into an assay capable of mycoplasma detection and/or speciation, with a special application for cell culture contamination. The unique features of our assay include the biotin-avidin amplification reaction, plus a microporous membrane support. This latter approach (i.e., depositing particulate antigen on a filter-type support) greatly simplifies the critical, multiple washing steps in the ELISA reaction chain. More importantly, it also minimizes any risk of washing off and losing the antigenic particles which are more subject to physical dislodgement by shear forces than are soluble antigens.

All available contamination-detection methods, including our BAMM-ELISA, have both advantages and disadvantages. Direct culture is the most sensitive since it can theoretically detect the presence of a single viable mycoplasma in the inoculum. It suffers from a long incubation period (one to three weeks). Also, some mycoplasmas (e.g., several strains of M. hyorhinis) often will not grow on artificial media, particularly those containing the yeast extract required by most other species. Agar and broth culture appear equally efficient. Biphasic culture is at least as effective as simple agar or broth, and has the advantage of multiple media in a single vessel.

The DNA-fluorochrome assay (e.g., Hoechst stain) is both sensitive and rapid. While it is subjective and suffers from artifacts such as the degenerating nucleii in hybridoma cultures; it can be standardized if known positive and negative controls are available. Both DNA and RNA probes are reasonably effective at detecting mycoplasmas, but sensitivity is only fair (i.e., 10^4 to 10^5 cfu/ml).

Isotope labels on the RNA probe are helpful in generating an easily detectable signal, but generate radioactive waste, hazardous solubilizers, and scintillant waste. Radioisotope labels also require the use of a scintillation counter and strict adherance to protocols.

We have developed a modified ELISA which is sufficiently sensitive to detect typical levels of mycoplasma contamination in cell cultures, and is rapid and economical. More importantly, it also can yield data on the species of the contaminant to aid in contamination control.

The most useful techniques to date for the identification or speciation of mycoplasma isolates have been serological. Batteries of specific antisera exist, and these
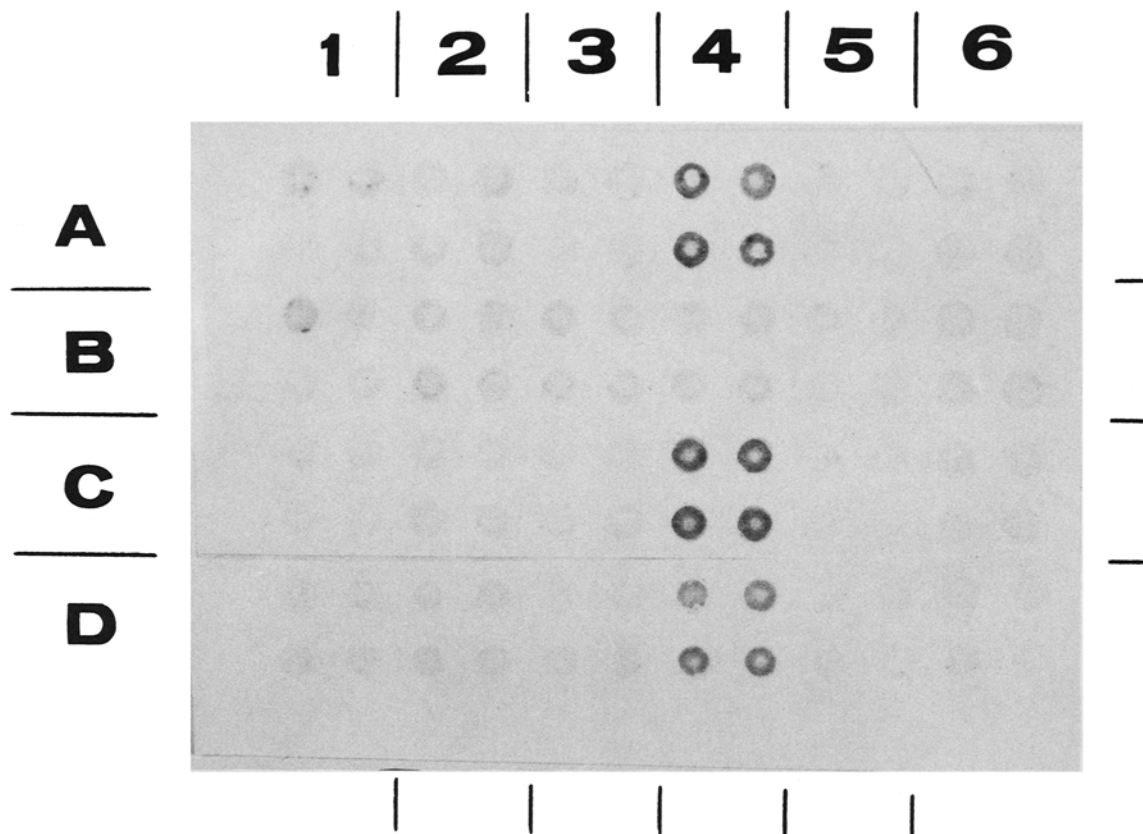

FIG. 5. BAMM-ELISA assay in a dot-blot manifold used to speciate unknowns isolated from cell cultures. Wells were used in quadruplicate replicates. Rows A, B, and C were the unknown isolates; row D was a known M. hyorhinis control. Columns received various primary antisera (1, anti-Acholeplasma; 2, anti-M. arginini; 3, anti-M. orale; 4, anti-M. hyorhinis; 5, anti-M. salivarium; 6, ascites control). Note reactions of identity (homologous antigen-antibody reactions) in A4, C4, and D4.

can be used to establish the identification through relatively rapid antigen-antibody reactions. One of the oldest and most popular assays is the growth inhibition method of Clyde (12). The presence of a zone of inhibition around an antiserum-impregnated filter paper disk indicates a reaction of identity. Several modifications (15,28) of the assay have been made, but it still suffers from a relative lack of sensitivity and long (3 to 14 day) incubation periods. The broth-growth inhibition assay, often using tetrazolium reduction as an indicator (18,43), is a popular variation on this theme but suffers from the same problem.

The introduction of the agar immunofluorescence assays by Del Guidice, et al. (13) vastly improved the mycoplasma identification system. This assay uses antiserum tagged with fluorescein, and it is applied directly to the mycoplasma colonies. A positive match is indicated by colony fluorescence (1,42). While the test is more rapid and sensitive than the metabolic/growth inhibition assay, the fluorescent antisera are very expensive, and a UV fluorescence microscope is required. The response is difficult to quantitate and hence the results are subjective. In addition, some agar and many colonies autofluoresce and cause interference on the interpretation of a positive response. Improvements have been attempted (e.g., through the use of a peroxidase label instead of fluorescein). However, these immunoperoxidase systems still suffer from relatively low sensitivity and non-specific cross reactions (20) and some groups (22) report very low correlation between such results and those obtained with complement fixation and metabolic inhibition assays.

One of the more recent developments/improvements in immuno-based detection systems is the microtiter plate, ELISA, or enzyme-linked immunosorbent assay. This test, introduced in the early 1970's, is very widely used and is the subject of numerous reviews and critiques (38,47). The initial application to mycoplasmas was led by Cassell and Brown, et al. who developed ELISA tests to detect antibody to *M. hominis* and *M. pulmonis* (7,9,19,27). It has been applied to *Ureaplasma* (45), *M. bovis* (5), and *M. bovigenitalium* (32) serology, and since 1980 has been used in the serological diagnosis of *M. pneumoniae* infections (8). In terms of actual identification of organisms in colonies through enzyme-linked assays, this approach has been used with some success with *Ureaplasmas* (36), *Acholeplasmas*, and several *Mycoplasmas (35)*.

These types of immunoperoxidase reactions have some advantage over certain other assays since they have a theoretical sensitivity 1,000-fold greater than can be obtained with complement fixation or neutralization assays. However, standard microtiter plate ELISA tests in general also have some problems. These include both false negative reactions and false positives (6,14,40,41). Investigators have also noted insensitivity and inability to
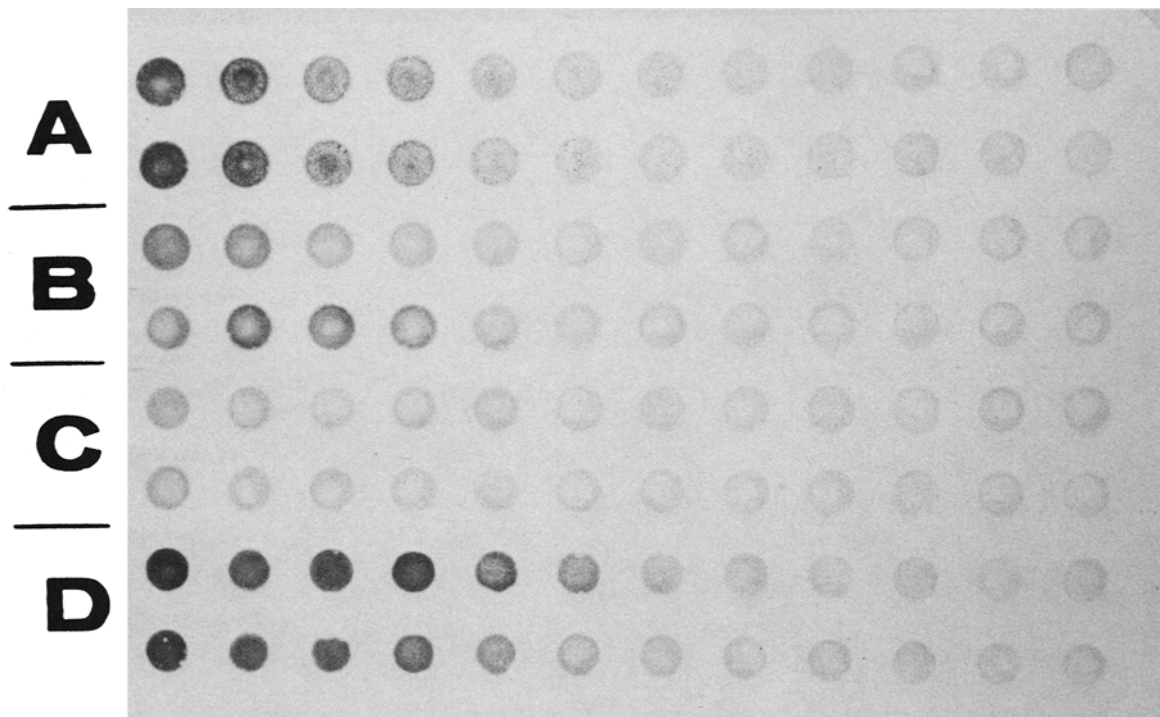

FIG. 6. Use of the BAMM-ELISA assay to detect mycoplasmas in a one-step (antiserum cocktail) approach. Wells were loaded in duplicate with *A. laidlawii* (A), *M. hyorhinis* (B), *M. arginini* (C), or *M. salivarium* (D). Column #1 wells (left) received a 5-fold concentrate of broth cultures containing approximately 5×10^6 cfu/well; the next 10 wells (left to right) received 2-fold dilutions of organisms, while column #12 wells had no organisms. All wells were treated with a 1:10 dilution of a mixture of equal amounts of the 5 standard antisera described in Materials and Methods.

differentiate virus strains (34), plus confusing cross-reactivity (23,25). There are also reports of technical problems such as loss of material during the repeated wash cycles (30), non-specific adsorption of the immuno-reagents (17), and edge effects (thermal gradients causing erroneous reactions) in the microtitration plates (31). These problems are compounded by the relatively high cost of the washing and quantitating devices.

One of the most exciting new developments in antigen detection techniques since immunoenzymes is the biotin/avidin approach. Biotin and avidin are two naturally occurring biological compounds with a remarkably strong affinity for binding to each other. The phenomenon was originally discovered when animals which were fed large quantities of egg white (containing avidin) developed a vitamin H, or biotin, deficiency. Unique features of the association include the fact that avidin is a 68 000 mol. wt. protein with 4 separate binding sites for biotin (mol. wt. 274). The small size of biotin allows many molecules of it to be bound to proteins without any reduction in biological activity, and each biotin can react with a separate avidin molecule. Even more significant is the nature of the binding. The affinity constant is approximately 10^{15} M^{-1}, that is, a thousand to a million times stronger than the typical antigen-antibody reaction.

Proteins (e.g., antibody) can be "biotinylated" and allowed to bind with avidin which has been tagged with an enzyme label (or even radioactive or fluorescent label). Some cells have a natural, non-specific interaction with avidin, however, and various attempts have been made to improve the system.

The advantages of this approach are many. The high binding constant gives superb sensitivity with little loss during the washing steps in the reaction sequence. The multiple steps for biotin binding give an amplification of the reaction, which becomes even further enhanced by using an enzyme as the marker. Steric hindrance as seen with large fluorescein molecules is non-existent, and no special fluorescence optical equipment is required to detect a positive response since it is detectable visually. Background fluorescence is eliminated, and non-specific binding is minimal if one begins the reaction sequence by blocking any nonspecific binding sites with excess protein such as the purified casein used here, and uses antibody molecules specific for the antigen in question.

The original ELISA system developed for microtiter plates and used as the basis in our test has already given rise to numerous permutations on the immunoenzyme theme. For example, the solid substrate can be in gels (DIG-ELISA, 3), on polystyrene beads (11), polyethylene sticks (39), nitrocellulose disks (29), cuvettes (46) or plastic Terasaki plates (33). Each has both advantages and disadvantages. In the standard ELISA assay in tubes or microtiter plates, or even dot-blot or immunospot assays (29) in which reactant is simply dropped onto sheets of absorptive material, there is an element of risk in the numerous rinsing steps since antigen and/or antibody may be lost. In the BAMM-ELISA described here, the combined use of manifold wells, microporous membrane, and vacuum precludes this danger. Any par-

ticulate antigen larger than the pores in the membrane will be retained on the upper, visible surface. This configuration also permits one to add several successive applications of initial reactant, and in effect, concentrate antigen in situ from dilute samples.

One very recent development in mycoplasma detection assays is the dot-blot system developed by Kotani and McGarrity (Rapid and Simple Identification of Mycoplasmas by Immunobinding, J. Imm. Methods, in press, 1985). They used direct application of cell culture supernatants, broth cultures, and clinical specimens to nitrocellulose sheets with an enzyme-linked secondary antibody. Their approach proved to be sensitive, and capable of detecting approximately 10^4 cfu/ml, depending on the species.

Unlike many other assays (e.g., direct culture, RNA probes, or DNA-fluorochrome stains) for detecting mycoplasmas in cell cultures, the BAMM-ELISA appears to be suitable for both mycoplasma detection and speciation. Since the manifold wells are perfectly aligned, it is instrument compatible (e.g., the reaction can be quantitated by densitometry) so it can be either visually or machine scored. Since the new BAMM-ELISA uses a microporous membrane to capture mycoplasmas in a suspension, it should prove applicable for all manner of other microbes and particulate antigens. As such, it should be useful not only for cell culture contamination control efforts but also clinical diagnostics.

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