Detection of Mixed Mycoplasma Species

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Immunofluorescence can be used to demonstrate mixed species within a mycoplasma culture; however, it may fail to do so if one species is present in very low numbers. To enhance the detection of minor components in such mixtures, a technique was developed, based on the growth inhibition test, whereby the growth of the major component in a mixed culture was inhibited, thus permitting the minor component to emerge. The method was applied to 67 field isolates from chickens and turkeys, which had been examined by immunofluorescence and were thought to contain only one mycoplasma species. Of these, 26 cultures were found to be mixtures, and in some instances the pathogenic species Mycoplasma gallisepticum and M. meleagridis were revealed.

It is not uncommon to isolate mycoplasmas in mixed culture because several species may share the same tissue tropism within their host. This possibility was first recognized as early as 1939 by Sabin, who reported the recovery of a mixed mycoplasma flora from mice (18), and subsequent publications described the isolation of more than one species from a particular site in other hosts including bovines (13), dogs (11), avians (1, 19), and humans (8).

These earlier findings were based on a variety of criteria such as differences in morphological, biochemical, or pathogenic properties of the isolates, and on the limited range of serological tests available at that time. It was not until the development of fluorescent-antibody techniques and, in particular, methods for staining unfixed mycoplasma colonies in situ on agar, that detection of mixed species could be more readily achieved (2–5, 9, 10, 16).

Successful detection of mycoplasma mixtures by immunofluorescence may depend on the proportions present in the mixture, and it is possible that a fastidious mycoplasma could be masked by the overgrowth of a more prolific species. Barile and Del Giudice (4) remarked on the practical difficulties of applying their direct fluorescent-antibody test to “heavy and mixed mycoplasma growth,” and they referred to incorporation of growth-inhibiting antiserum in their medium.

Immunofluorescence has been used for a number of years in this laboratory for the identification of mycoplasma isolated from poultry, and the detection of mixtures by this technique has been reported (14). On occasion, however, the presence of a pathogenic mycoplasma has been suspected from serological evidence, but culture has revealed only a nonpathogenic mycoplasma. Thus, it was decided to investigate possible ways of revealing mixtures that were not detectable by preliminary immunofluorescence.

This communication describes the use of a modification of the growth inhibition technique for detecting mycoplasma mixtures from avian sources. In conjunction with immunofluorescence, the technique revealed mixtures that were not detectable by immunofluorescence alone.

MATERIALS AND METHODS

Organisms. Reference strains of the recognized chicken and turkey mycoplasmas were used. M. gallinarum DD, M. gallopavonis W1, M. iowae 695, M. pullorum CKK, and M. synoviae WVU 1853 were obtained from the FAO/WHO Collaborating Centre for Animal Mycoplasmas, Aarhus, Denmark. M. gallinarum PG16, M. gallisepticum PG31, M. iners PG30, and M. meleagridis 17529 were obtained from the National Institutes of Health, Bethesda, Md.

Field isolates were from chickens and turkeys from a variety of sources. Most chicken isolates were from small "backyard" flocks, and all turkey isolates were from commercial flocks.

Media. For isolation, routine subculture, and the modified growth inhibition (MGI) technique, mycoplasma broth and agar were as described previously (7). For the MGI technique, 50-mm-diameter petri dishes containing 9 ml of agar medium were prepared. For the production of mycoplasma antigens, broth medium was used to grow mycoplasmas for rabbit inoculation. For this the swine serum in the routine medium was replaced by 1% (vol/vol) PPLO serum fraction (Difco Laboratories, East Molesey, Surrey, England).

Antiserum. Rabbit antisera to M. gallinarum and M. pullorum were obtained from the Collaborating Centre.
for Animal Mycoplasmas, and antisera to the other seven species were produced in this laboratory, using four rabbits for *M. gallisepticum* and *M. synoviae* and two for each of the other species.

Antisera to *M. gallinarum*, *M. gallisepticum*, *M. iners*, *M. meleagridis*, and *M. synoviae* were prepared by a method described previously (15), but for preparation of antisera to *M. gallopavonis*, *M. iowae*, and *M. iners* a different procedure was adopted which involved fewer inoculations and lower doses. Rabbits were given two subcutaneous and two intramuscular inoculations, each containing 5 mg of antigen protein in Freund complete adjuvant, and an intravenous inoculation containing 5 mg of antigen without adjuvant. Blood was taken after 4 weeks, and serum was tested by the growth inhibition test and immunofluorescence. The two rabbits immunized with *M. iners* had sufficiently high titers to be bled out, whereas the rabbits inoculated with *M. gallopavonis* and *M. iowae* were each given an intravenous booster containing 5 mg of antigen protein. These rabbits were bled after an additional 1 to 2 weeks.

**Immunofluorescence.** The indirect fluorescent-antibody (IFA) technique of Rosendal and Black (17) was used with rabbit antisera and fluorescein-labeled horse anti-rabbit immunoglobulin (Netherlands Red Cross Antiserum, Organon Teknika, Huntingdon, England). Appropriate positive and negative controls were included, and all preparations were carefully examined for evidence of mixtures. The method was assessed with artificial mixtures of mycoplasmas before its use on field isolates.

**Mixture detection scheme.** The aim of this procedure was to inhibit the growth of a predominant mycoplasma in a potentially mixed culture and thus allow a minor component to emerge. The method involved four stages, as follows.

(i) **Identification of isolate.** The predominant mycoplasma was identified by applying the IFA test to colony-bearing agar blocks.

(ii) **Preparation of inoculum.** A culture of the isolate was prepared by inoculating a colony-bearing agar block into broth.

(iii) **MGI test.** The MGI test (Fig. 1) was adapted from the method described by Black (6). A running drop of the culture was applied to the plate, and after it had been absorbed, two wells instead of the usual one were cut in the path of the inoculum. Both wells were filled with rabbit antiserum to the already identified mycoplasma. The diameter of the wells was 4 mm, and the distance between them was 5 to 8 mm, depending on the potency of the antiserum, the aim being to inhibit growth of the predominant mycoplasma in the area between the two wells. The plate was incubated for 2 days at 27°C and then at 37°C. It was observed daily, and on the appearance of colonies, or after approximately 1 week if there were no colonies, the agar between the wells was cut out and subcultured onto a fresh plate, which was incubated at 37°C. (In preliminary studies this last step was omitted, and the agar block from between the wells was examined directly and compared with a control block taken from between wells filled with normal serum).

(iv) **Identification.** Resulting colonies were identified by the IFA test. This test always included reexamination for the previously identified predominant mycoplasma.

Note: as with the conventional growth inhibition test (12), it is important to avoid using too large an inoculum in the MGI test. Thus, to achieve a suitable density of colonies, a trial plate can be inoculated with broth culture by the running-drop technique, and the remainder of this broth is frozen until the colonies have grown. If the number of colonies is excessive, then the broth can be thawed and diluted for use in the MGI test.

**Cultures examined for mixtures.**

(i) **Laboratory strains.** The mixture detection scheme described above was tested on mixtures of reference strains of *M. gallisepticum*, *M. synoviae*, *M. gallinarum*, and *M. meleagridis* in various combinations and ratios.

(ii) **Field strains.** Cultures from chickens and turkeys were first examined by the IFA test. Those from chickens were tested initially with antisera to *M. gallisepticum*, *M. synoviae*, *M. gallinarum*, *M. iowae*, and *M. pullorum*, and cultures from turkeys were tested with antisera to *M. meleagridis*, *M. gallisepticum*, *M. synoviae*, *M. gallinarum*, and *M. iowae*. The selection of these species was based on observations of their prevalence by ourselves and colleagues. If unstained colonies were seen, then isolates were tested with antisera to the other avian mycoplasma.

A total of 165 field cultures were examined by the IFA test, and obvious mixtures were recorded.

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**FIG. 1.** MGI test for detecting mixed mycoplasma cultures. (1) Broth culture of original mycoplasma isolate (already identified by immunofluorescence). (2) Agar plate inoculated with running drop of broth culture and containing two wells filled with antiserum to the identified mycoplasma. (3) A fresh plate inoculated with an agar block cut from between the antiserum wells of the first plate. Any resulting colony growth is identified by immunofluorescence.
TABLE 1. IFA test results on mixed cultures of *M. gallinarum* and *M. meleagridis* after application of the MGI test

<table>
<thead>
<tr>
<th>Dilution of mixed culture</th>
<th>Rabbit antiserum used for inhibition</th>
<th>IFA test results with mycoplasma mixture and proportions$^a$:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mgn/Mm (50:50) Mm/Mgn (75:25) Mgn/Mm (90:10)</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>Mgn</td>
<td>+/+/+</td>
</tr>
<tr>
<td></td>
<td>NRS$^b$</td>
<td>+/+/+</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>Mgn</td>
<td>-/+/+</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>+/+/+</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>Mgn</td>
<td>-/+/+(f)</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>+/+/+(f)</td>
</tr>
</tbody>
</table>

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</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>Mm</td>
<td>-/+/+</td>
</tr>
<tr>
<td></td>
<td>NRS$^b$</td>
<td>+(f)/+/+</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>Mm</td>
<td>-/+/+</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>+/+/+</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>Mm</td>
<td>-/+/+(f)</td>
</tr>
<tr>
<td></td>
<td>NRS</td>
<td>+(f)/+/+(f)</td>
</tr>
</tbody>
</table>

$^a$ Mgn, *M. gallinarum*; Mm, *M. meleagridis*; +, fluorescent colonies seen on staining with appropriate antiserum; -, no fluorescence seen; ?, result uncertain; (f), few colonies.

$^b$ NRS, Normal rabbit serum.

In experiments with *M. synoviae* antiserum, it was necessary to move the wells closer together than 5 mm to obtain good inhibition, but all the other antisera could be used in wells between 5 and 8 mm apart.

RESULTS

Laboratory strains. The modified growth inhibition technique was used successfully with artificial mycoplasma mixtures. A typical result with mixtures of *M. gallinarum* and *M. meleagridis* is given in Table 1. In this experiment, cultures of known viability were mixed to give 50:50, 75:25, and 90:10 proportions of colony-forming units. Tenfold dilutions were made to $10^{-3}$, and these were then plated in duplicate. The appropriate rabbit antiserum (or normal serum control) was dispensed into the wells, as indicated in Table 1. It can be seen that total or partial inhibition of the homologous mycoplasma was achieved, leaving the second mycoplasma component readily detectable.

In experiments with *M. synoviae* antiserum, it was necessary to move the wells closer together than 5 mm to obtain good inhibition, but all the other antisera could be used in wells between 5 and 8 mm apart.

Field strains. Of the 165 field strains that were examined by immunofluorescence, 23 were seen to be obvious mixtures and were not examined further. This left 142 cultures which were thought to contain a single species after preliminary IFA tests. Of these, 67 isolates were subjected to the mixture detection scheme with the MGI technique, and an additional 26 mixtures were thus detected.

Tables 2 and 3 show the identification of all the mixtures obtained from chickens and turkeys, respectively. In Table 2 it can be seen that the pathogen *M. gallisepticum* was detected in six isolates from chickens, but in none of these was it revealed on preliminary examination by immunofluorescence. It was only after the major component had been inhibited that *M. gallisepticum* was apparent. Likewise, in the turkey isolates (Table 3), the pathogen *M. meleagridis* was masked on 5 occasions by *M. iowae*, and in 11 isolates the presence of *M. iowae* was undetected until the *M. meleagridis* component had been inhibited.

TABLE 2. Mixtures of mycoplasma species isolated from chickens

<table>
<thead>
<tr>
<th>No. of cultures</th>
<th>Species detected by preliminary IFA test</th>
<th>Additional species found by mixture detection scheme</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td><em>M. gallinarum</em>, <em>M. pullorum</em></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td><em>M. gallinarum</em></td>
<td><em>M. pullorum</em></td>
</tr>
<tr>
<td>4 me, <em>M. gallinarum</em></td>
<td><em>M. gallisepticum, M. pullorum</em></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>M. pullorum</em></td>
<td><em>M. gallisepticum</em>, <em>M. gallinarum</em></td>
</tr>
</tbody>
</table>

DISCUSSION

The use of the MGI test in conjunction with immunofluorescence was found to reveal mixtures that were not detectable by IFA testing alone. Of 67 isolates that appeared to consist of a
single species on preliminary IFA tests, 26 (39%) proved to contain mixtures.

The detection of a minor component by immunofluorescence may depend upon whether its homologous serum is actually used, because it is probably easier to detect a minor component as small areas of fluorescence in an unstained mass of colonies than as a few unstained colonies in a mass of fluorescing ones (Fig. 2 and 3). In these experiments, however, all the mycoplasma species that were revealed after the application of the MGI technique had actually been tested for in the preliminary IFA tests. Thus, the pathogenic *M. gallisepticum* in chickens and *M. meleagridis* in some turkeys gave no indication of their presence until the major component had been inhibited.

It seems likely that this technique could be applied to mycoplasma isolates from other host species, and although the method is costly in terms of time and antiserum, it may well be justified in cases in which a particular pathogen is being sought. It may also be of value for checking the purity of cultures. This point was emphasized during the handling of an isolate of *M. iowae* that was contaminated with *M. meleagridis* in very small numbers. The isolate was passed through a 450-nm-pore filter, and a single colony which was subcultured appeared to be pure *M. iowae* on IFA testing. After application of the MGI technique, *M. meleagridis* was again detected in small amounts in the cloned culture.

ACKNOWLEDGMENTS

We thank Janet McCarthy for valuable technical assistance and the Overseas Development Administration for financial support.

LITERATURE CITED


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TABLE 3. Mixtures of mycoplasma species isolated from turkeys

<table>
<thead>
<tr>
<th>No. of cultures</th>
<th>Species detected by preliminary IFA test</th>
<th>Additional species found by mixture detection scheme</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td><em>M. meleagridis, M. iowae</em></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>M. meleagridis, M. synoviae</em></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>M. meleagridis</em></td>
<td><em>M. iowae</em></td>
</tr>
<tr>
<td>5</td>
<td><em>M. iowae</em></td>
<td><em>M. meleagridis</em></td>
</tr>
</tbody>
</table>

FIG. 2. Isolated fluorescent colonies of *M. iowae* among numerous nonfluorescent colonies of *M. meleagridis*.


