PRIMARY ISOLATION OF *MYCOPLASMA* ORGANISMS (PPLO) FROM MAMMALIAN SOURCES[1]

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ABSTRACT

BARBER, T. L. (Cornell University, Ithaca, N.Y.) AND J. FABRICANT. Primary isolation of *Mycoplasma* organisms (PPLO) from mammalian sources. J. Bacteriol. **83**:1268–1273. 1962.—*Mycoplasma* (PPLO) were isolated from cattle, pigs, sheep, rats, and dogs. A total of 39 isolates was obtained from 307 samples from 166 animals. Of these, 24 isolates were from semen of bulls in an artificial breeding establishment; the other 15 were from various tissues of the animals mentioned.

Variations of two basal media together with chicken embryos were used in primary isolation attempts. PPLO enrichment broth (Difco) was supplemented with either horse or swine serum. The broth was used as overlay with a horse blood agar slant. *Viande foie* (VF) medium was usually enriched with horse or swine serum and was used both with and without 1% yeast hydrolyzate. Chicken embryos (5 days old) were inoculated via the yolk sac.

Of the 39 isolations made, 22 were obtained on a single variation of one basal medium or only after a passage through chicken embryos. The 22 were isolated as follows: 4 in modified PPLO enrichment broth; 13 in variations of VF medium; and 5 were found only after growth in chicken embryos. The other 17 isolates grew, on primary culture, in a combination of media. Only three of these grew in all media and in embryos. Eight grew in two variations of VF basal, two in both VF and PPLO enrichment broths, one in embryos and PPLO enrichment broth, and three in embryos, and VF basal medium. If either basal medium or chicken embryos had been omitted from the trial, many isolations would not have been made.

The prevalence of mycoplasmas in a variety of animals was reviewed by Morton (1960). Certain primary isolation attempts from mammalian sources have employed only a single variation of one basal medium (Olson et al., 1960; Klieneberger-Nobel, 1959; Edward, Hancock, and Hignett, 1947). Studies have demonstrated that the primary isolation of pleuropneumonia-like organisms (PPLO) from avian tissue is limited by the inadequacy of presently available culture media (Fabricant, 1958; Chalquest and Fabricant, 1960). The approach of this study was to culture samples of tissue suspensions from a variety of mammalian sources on a number of different media, and to compare the numbers of primary isolations of *Mycoplasma* on each medium.

MATERIALS AND METHODS

Media. Basal media used in primary isolation trials were *viande foie* (VF) and Bacto PPLO enrichment broth (Difco).

The VF medium was prepared as described by Turner, Campbell, and Dick (1935). Beef muscle and liver, 100 g of each, and 120 g of pig stomach were sliced and ground in a blender. Water (1 liter) and concentrated hydrochloric acid (10 ml) were added and the suspension incubated at 50 C for 24 hr. Then the suspension was heated to 80 C and filtered through clarifying paper filters. The filtrate was again heated to 80 C and held at 5 to 10 C overnight. The cold solution was filtered through paper filters and the pH adjusted to 7.5 with 10% sodium hydroxide. The solution was held for 15 min at 80 C, after which 1% of a dry buffer-salt mixture (anhydrous disodium phosphate, 379.0 g, and potassium dihydrogen phosphate, 90.8 g) was added. The buffered medium was held overnight at 18 to 19 C, filtered through

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paper filters, checked for pH 7.5, and sterilized by Seitz filtration. The medium was then dispensed into 300-ml lots which were tested for bacterial sterility and held at 5 to 10 C until used.

The buffered VF broth was supplemented and designated as follows: VFS, with 10% swine serum; VYS, with 10% swine serum and 1% yeast hydrolyzate (Nutritional Biochemicals Corp.); VFH, with 10% horse serum; VYH, with 10% horse serum and 1% yeast hydrolyzate; and VFB, with 10% bovine serum.

Dehydrated PPLO enrichment broth was dissolved in distilled water and autoclaved for 15 min at 15 lb pressure. The ingredients of this medium were infusion from beef heart, peptone, and sodium chloride. The broth, when enriched with 10% horse serum, was designated A (Adler, Yamamoto, and Bankowski, 1954); when enriched with 10% swine serum, it was designated SA (Fabricant, 1959). Each formula was used over a horse blood agar slant (Adler et al., 1954). The agar slant was prepared by rehydrating and sterilizing Bacto blood agar base (Difco), and adding 5% defibrinated horse blood and 1,000 units potassium penicillin G per ml. The blood agar was dispensed into tubes (0.75 ml each) and overlaid with the appropriate broth.

Thallium acetate (1:1,000) and penicillin (1,000 units per ml) were included in all broth media. For agar plates the only change was the addition of thallium acetate, 1:2,000. All media were dispensed in 13 by 100 mm glass tubes, 2 ml per tube, with stainless steel closures.

Agar plates were made to correspond to each of the broths. For A and SA, Bacto PPLO agar (Difco) was used with appropriate serum. The plates for A had one ingredient not present in the broth: yeast hydrolyzate (1%). For the *viande foie* series, Bacto-agar (Difco) was prepared as a 6% solution in distilled water and autoclaved to dissolve and sterilize. The broth to be used was prepared with serum and inhibitors, heated to 45 C in a water bath, and added to the agar solution at about the same temperature. The final concentration of agar was 1.2%. Approximately 15 ml were poured for all plates.

All seven variations of the two basal media were not used in each primary-isolation attempt. A and SA were used in all trials. VFB was eliminated after a few samples were run, since it grew only one isolate which also grew on other media. VYH was introduced after the trials had started and showed promise in the first isolation attempts from semen. VFS was then replaced with VYS. The four media, A, SA, VYS, and VYH, were used throughout the major part of the study.

TABLE 1. *Sources of test samples and numbers of isolations*

	Animals	Samples	Animals positive	Isolations
Bovine tissues..	36	46	1	1
Bovine semen...	36	82	14	24
Bovine vaginal mucus.....	35	70	0	0
Sheep tissues...	27	48	5	7
Swine tissues...	15	31	3	3
Horse tissues...	5	5	0	0
Dog tissues.....	5	14	2	2
Rat tissues.....	2	4	2	2
Miscellaneous ..	5	7	0	0
Totals.....	166	307	27	39

Chicken embryos (5 days old) were used in all primary-isolation trials. These were from a flock of chickens believed to be free of *Mycoplasma* organisms, on the basis of serological and cultural examinations.

Bacto nutrient broth (Difco) was used as a diluent in preparation of source material for inoculation, and in making serial dilutions of cultures for growth studies.

Test samples. Table 1 shows the sources of materials. Of the test samples, 155 were tissues collected from animals at necropsy. Tissues were collected at random, some from normal-appearing organs and some from those organs involved in the pathological condition present. Samples were taken with sterilized equipment and were immediately frozen. Undiluted semen from normal bulls (with no history of low fertility) in an artificial breeding establishment was held in refrigeration up to 1 week before testing. Samples of vaginal mucus from virgin heifers were also refrigerated up to 1 week.

Primary isolation. A small portion (0.1 to 0.5 g) of each tissue sample was macerated in a Ten-Broeck glass tissue grinder with 3 ml of diluent. Samples of undiluted semen (0.1 to 1.5 ml) were diluted to a total of 2 to 3 ml. Samples of mucus from cows were mixed with an equal volume of diluent to reduce their viscosity. Each suspension (0.2 ml) was inoculated into a tube of broth medium.

After 3 days of incubation at 37 C, a loopful

of each broth culture was streaked on an agar plate of corresponding media. The plates were incubated in a candle jar with a moistened pledget of cotton at 37 C for 6 days. They were examined at 2, 4, and 6 days at 20× magnification. If colonies appeared, some were removed on the sixth day by cutting out a block of agar. The colonies were placed in broth medium, and the cultures were subsequently transferred twice weekly.

The test samples were prepared for inoculation into chicken embryos by adding 50 μg of dihydrostreptomycin sulfate and 10,000 units of penicillin per ml. After 30 min at room temperature, 0.2 ml of each suspension was inoculated into the yolk sac of each of three embryos.

The inoculated chicken embryos were candled daily, and the yolk was harvested from all dead embryos after the first 2 days. Yolk material was harvested from one embryo in each set of three at 6 days whether the embryos were alive or dead. The harvested yolks (0.2 ml) were cultured in VYS, VYH, or SA medium. After 3 days of incubation, the liquid media were plated and observed as described previously. Each harvested yolk was checked for bacterial sterility on tryptose agar slants.

Growth studies. The ability of isolates to grow in several different media after initial cultivation in vitro was determined. Each organism was plated directly from the broth in which it was growing onto six types of agar plates. Plates used were those corresponding to A, SA, VFH, VYH, VFS, and VYS. The strains were checked for ability to grow after one passage through each of a number of broth media. A, SA, VFS, and VYS were used. After 3 days in the broth, each culture was inoculated on an agar plate corresponding to the broth. Plates were observed for 6 days.

It was determined whether any isolate needed lowered oxygen tension for growth on plates. Each organism was streaked on two sets of plates, and one plate was incubated in a jar with a candle and one in a jar without a candle.

Glucose-fermentation studies were performed in VFS broth. Glucose (1%) and 0.025 g of phenol red per liter were added to the medium. Uninoculated controls were run with the test. Media identical to the above but without the glucose were also inoculated and observed.

Staining. Organisms were taken from 72-hr-old broth cultures for staining. Smears were gently heat-fixed, covered with Bouin's solution for 15

TABLE 2. *Isolates that grew, on primary isolation, in only one medium*

Isolate designation	Directly to medium*							Chicken embryo to media	Source
	A	SA	VFH	VYH	VFS	VYS	VFB		
B-8	N†	N	N	—	N	—	N	G	Sheep lung
B-50	N	N	N	—	N	—	—	G	Rat lung
B-81	N	N	N	N	N	—	—	G	Bull semen
B-292	N	N	N	N	—	N	—	G	Bull semen
B-162	N	N	—	N	—	N	—	G	Pig peritoneum
B-12	G	N	N	—	N	—	N	N	Calf lung
B-35	G	N	N	—	N	—	N	N	Dog lymph node
B-57	N	G	N	—	N	—	—	N	Dog lung
B-169	N	G	—	N	—	N	—	N	Sheep lung
B-9	N	N	N	—	G	—	N	N	Pig pericardium
B-78	N	N	N	G	N	—	—	N	Bull semen
B-105	N	N	—	G	—	N	—	N	Bull semen
B-116, B-184	N	N	—	N	—	G	—	N	Bull semen
B-185, B-187	N	N	—	N	—	G	—	N	Bull semen
B-192, B-194	N	N	—	N	—	G	—	N	Bull semen
B-198, B-200	N	N	—	N	—	G	—	N	Bull semen
B-203, B-204	N	N	—	N	—	G	—	N	Bull semen

* A and SA = variations of PPLO enrichment broth; VFH, VYH, VFS, VYS, and VFB = variations of *viande foie* medium.

† G = growth; N = no growth; — = medium not used.

TABLE 3. *Isolates that grew, on primary isolation, on more than one medium*

Isolate designation	Directly to medium*							Chicken embryo to media	Source
	A	SA	VFH	VYH	VFS	VYS	VFB		
B-90	G†	G	—	G	—	G	—	G	Sheep lung
B-167	G	G	—	G	—	G	—	G	Sheep lung
B-166	G	G	—	G	—	G	—	G	Sheep spleen
B-6	N	N	G	—	G	—	G	G	Sheep lung
B-18	G	G	N	—	N	—	N	G	Pig lung
B-168	N	G	—	G	—	G	—	N	Sheep spleen
B-48	N	G	N	—	G	—	—	N	Rat lung
B-80	N	N	G	G	N	—	—	N	Bull semen
B-112	N	N	—	G	—	—	—	G	Bull semen
B-281	N	N	N	N	—	G	—	G	Bull semen
B-99, B-100	N	N	—	G	—	G	—	N	Bull semen
B-106, B-109	N	N	—	G	—	G	—	N	Bull semen
B-111, B-115	N	N	—	G	—	G	—	N	Bull semen
B-284	N	N	N	G	—	G	—	N	Bull semen

* A and SA = variations of PPLO enrichment broth; VFH, VYH, VFS, VYS, and VFB = variations of *viande foie* medium.

† G = growth; N = no growth; — = medium not used.

min, washed thoroughly with tap water, covered with Giemsa stain for 0.5 hr, rinsed, and dried (Adler et al., 1958).

RESULTS

A total of 24 isolations was derived from bull-semen samples. No mycoplasmas were cultured from samples of heifer mucus. Also, 15 isolates were obtained from tissue samples collected from animals at necropsy. More isolations were made from tissue samples from areas involved in a pathological condition than from normal-appearing tissues.

As shown in Table 2, 22 of a total of 39 primary isolations were obtained on a single medium or only after passage in chicken embryos. The other 17 were acquired on two or more media (Table 3).

The results of the numbers of primary isolations that were made on each medium (Table 4) do not allow the recommendation of one medium for general use. All isolations from bull semen were obtained on variations of VF medium. No organisms from semen were isolated on A and SA, the two variations of PPLO enrichment broth. Isolations from tissue samples, excluding semen samples, were well distributed in most variations of the two basal media and in chicken embryos.

Table 5 shows the growth range of 34 of the isolates after they had been maintained in artificial media for more than eight passages. The organisms would grow on media which failed to support growth on primary culture. More isolates grew when streaked directly on various plates than after 3 days of culture in various broths followed by plating.

All isolates grew as well on plates without lowered oxygen tension as with it. The isolates were also serially passed four times through media containing no inhibitors. When plated, all formed colonies typical of the *Mycoplasma*, and no bacterial contamination of the broth cultures was observable.

TABLE 4. *Summary of primary isolation results giving numbers of primary isolations of Mycoplasma on each medium*

Medium*	Samples tested on medium	All samples/ number growing on primary culture	Excluding semen samples/number growing on primary culture
A	307	6/307	6/225
SA	307	8/307	8/225
VFS	86	3/86	3/82
VYS	221	22/221	4/120
VFH	145	2/145	1/113
VYH	235	15/235	4/153
VFB	35	1/35	1/35
Embryo	307	12/307	8/225

* A and SA = variations of PPLO enrichment broth; VFH, VYH, VFS, VYS, and VFB = variations of *viande foie* medium.

TABLE 5. *Growth results of 34 isolates after plating on various media directly from the broth in which they were being maintained and results of growth on plates after 3 days culture in a broth medium analogous to the plate*

Medium*	Plated directly	Plated after 3 days incubation in analogous broth
A	16†	14
SA	31	14
VFS	34	32
VYS	33	32
VFH	29	N‡
VYH	34	N

* A and SA = variations of PPLO enrichment broth; VFH, VYH, VFS, and VYS = variations of *viande foie* medium.

† Number of cultures growing.

‡ This medium not used here.

Giemsa-stained smears of the organisms revealed small coccoid bodies typical of those described for mycoplasmas. No morphological forms indicative of bacterial contamination were seen. Colony diameters of the various isolates ranged from 230 to 1,000 μ. Two organisms (B-6 and B-8) isolated from sheep lungs fermented glucose.

The effect on VF of autoclaving at 15 lb pressure was investigated. Serum and inhibitors were added after autoclaving. When streaked directly from regular VFH broth to plates prepared from autoclaved VF, 9 out of 16 cultures grew. The same cultures were then passed once through autoclaved broth prior to being plated on similar plates. Only eight isolates grew. All 16 isolates were being passed on VFH medium.

DISCUSSION

The *Mycoplasma* found in this study were not sufficiently characterized to permit classification into species, and their pathogenicity was not determined. However, this further indication of the widespread occurrence of mycoplasmas in mammalian species should encourage more investigation. There is danger in regarding the majority of these agents as nonpathogens. The occurrence of pathogens and nonpathogens in the same animal, as in poultry, must be considered. Mixtures of more than one type of *Mycoplasma* are often found in cases of chronic respiratory disease. Nonpathogenic organisms tend to persist in the affected bird's respiratory tract longer than pathogens. Cultural techniques have favored the isolation of the nonpathogens (Fabricant, 1960). Similar circumstances in cattle, swine, or sheep could mask the mycoplasmal etiology of unexplained pathological conditions.

This is the first report of the isolation of mycoplasmas from bull semen in the United States and adds to the previous reports from other countries (Edward et al., 1947; Nielsen, 1949; Terpstra, 1953; Florent, 1953; Albertsen, 1955; Bakos, Bane, and Thal, 1959; Villemot and Provost, 1959*a*). If these organisms are as numerous in other artificial breeding herds as they were in the one examined, further study is indicated. Rapid dissemination of these organisms through artificial breeding is a definite possibility. The prevalence of *Mycoplasma* in semen should not be disregarded by the artificial breeding industry. It is also important to confirm reports (Provost et al., 1959; Villemot and Provost, 1959*b*) that an agglutinin in common with the etiological agent of contagious bovine pleuropneumonia is found in cattle carrying genital mycoplasmas.

Results of this study indicate the usefulness of a variety of media in the primary isolation of mycoplasmas. Had either of the basal media or chicken embryos been omitted from this study, a number of isolates would have gone undiscovered. The chicken embryo, together with variations of PPLO enrichment broth and *viande foie* basal media, is far from the final answer to the primary isolation problem. The results of this trial emphasize the problem which exists in primary isolation attempts. The primary isolation of mycoplasmas involves the search for organisms whose nutritive requirements are as yet unknown. The only method of supplying these, in light of present knowledge, is the substitution of one relatively crude material for another until media are devised that give accurate results.

Primary isolation of mycoplasmas has been attempted too often with a single medium or with a few variations of one basal medium. Although isolations have been made by such procedures, the variation in the effectiveness of the different isolation methods compared in this study indicates the likelihood that many isolations were missed when only a single basal medium was used.

This study indicated that after initial cultivation in vitro the organisms can be adapted to different media. It is likely that a relatively small

number of media can be formulated, adequate for the passage and study of stock laboratory cultures of many of the mammalian mycoplasmas. The same media may be inadequate for primary isolation work. New media must be tested in primary isolation studies as well as in growth studies of stock laboratory cultures.

The following conclusions were reached. (i) It may be necessary to use a number of different basal media for the primary isolation of mycoplasmas from clinical materials. (ii) The development and use of additional enriched media may improve the efficiency of primary isolation of known *Mycoplasma* species and result in the detection of other species as yet unrecognized. (iii) In this study, 5 isolations would have been missed if chicken embryos had been omitted; 13 if *viande foie* media had been omitted; and 5 if PPLO enrichment broth media had been omitted.

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