Comparison of Techniques for Primary Isolation of Respiratory *Mycoplasma pulmonis* from Rats

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Studies were performed to determine the optimal sites for primary isolation of *Mycoplasma pulmonis* from the respiratory system of laboratory rats. In a preliminary study, a group of 42 rats was cultured for respiratory *M. pulmonis* at several sites. Tracheal washes yielded the highest number of positive cultures (61.9%) and ground lung tissue yielded the lowest (19.0%), with the nasopharyngeal culture in the middle (52.38%). In a subsequent study, the tracheal wash was again the optimal method since 76.7% of 103 rats were positive for *M. pulmonis*, whereas culture of the nasopharyngeal area resulted in 53.9% positive cultures. In a third study, all 45 rats were positive for *M. pulmonis* when the tracheal wash method was used, whereas only 51% were positive when the nasopharyngeal area was cultured, indicating that the tracheal wash was the most reliable of the commonly used procedures. Other aspects of these experiments demonstrated the tympanic cavity of animals with otitis media to be an excellent source for obtaining material to culture for *M. pulmonis*.

Mycoplasma pulmonis is the sole etiological agent of chronic respiratory disease (7, 10, 11) or murine respiratory mycoplasmosis (2). This disease, described by Lindsey et al. (11), involves the nasal passages, middle ears, trachea, and lungs, producing rhinitis, otitis media, tracheitis, and pneumonia. The seriousness of respiratory *M. pulmonis* infections is more fully appreciated now that its pathogenicity in rodent colonies has been better established. This agent is carried by and readily transmitted to laboratory rats and mice from wild rodents. Thus it threatens nearly all research in mice and rats.

A basic part of a quality assurance program and eventual control or eradication of mycoplasmosis is an efficient culturing technique to detect mycoplasmal carriers and asymptomatic animals. A primary concern is to utilize media optimal for in vitro isolation of *Mycoplasma* spp. Also of concern is the matter of finding the most reliable sites for culturing mycoplasma from infected animals.

Although some investigators (5, 16) have not emphasized the primary source for culture of mycoplasma, others (1, 2, 4, 5, 7, 9, 13, 17) have found advantages or disadvantages among specific sites of culturing. Organick and Lutsky (13) and Kappel et al. (9) each suggest that culturing from the nasopharynx is more reliable than culturing from the trachea or lungs. However, other investigators (8, 16, 17) report mycoplasmal inhibitors within the lungs and trachea and describe methods for reducing or diluting these inhibitors. Clearly, the reliability of various primary culturing techniques needed to be reevaluated with respect to the new methods (8, 12, 15, 17) of isolation from the lower respiratory tract.

This investigation compared commonly used methods for primary isolation of *M. pulmonis* with a tracheal wash method, which allowed thorough sampling of the lower respiratory tract and dilution of inhibitors. Our method, a modification of a lung perfusion technique (15), does not require use of a cannula.

MATERIALS AND METHODS

Animals. Rats, MCR: (SD), of varying ages and of both sexes, were obtained from a conventionally housed commercial colony with a long history of clinical laboratory confirmed murine respiratory mycoplasmosis.

Media. The agar medium, a modification of Chalquest's (3), was prepared as follows: add 16.3 g of PPLO agar without crystal violet (Difco Laboratories) and 2.5 g of soluble starch (Fisher Scientific Co.) to 450 ml of glass-distilled deionized water. After mixing, boil briefly to dissolve. Then add 5 ml of a 5% Trypticase-peptone solution, made by adding 0.25 g of Trypticase-peptone (BBL Microbiology Systems) to 5 ml of glass-distilled water. The solution is autoclaved and allowed to cool to 50°C for 30 min in a water bath. The final ingredients are then added in the following order: 5 ml of 1% nicotinamide adenine dinucleotide (Sigma Chemical Co.); 50 ml of heat-inactivated sterile swine serum (Microbiological Associates); 0.5 ml of potassium penicillin G (100,000 U/ml) (Pfizer Inc.); and 1.25 ml of 10% thallous acetate solution (Fisher).

A 6- to 8-ml amount of the medium is poured into each small petri dish (60 by 15 mm; Scientific Products). Optionally, a phenol red indicator may be used in the agar.

Frey's mycoplasma broth (6) is made in the following manner: 22.5 g of mycoplasma broth base (Pfizer) is dissolved in 850 ml of glass-distilled water. Three grams of dextrose (Difco), 0.1 g of cysteine hydrochloride (Fisher), and 0.025 g of phenol red (Fisher) are added to the mixture and the pH is adjusted to 7.8 with 0.1 N NaOH. The broth is then autoclaved and allowed to cool to 50°C. The final ingredients are then added in the following order: 0.1 g of nicotinamide adenine dinucleotide, 150 ml of sterile swine serum heat inactivated at 56°C, 0.5 g of thallous acetate, and 500,000 U of potassium penicillin G.

Culturing techniques. All primary cultures were taken at necropsy.

(i) **Tympanic bullae.** A transverse cut on each side of the animal's head through the ramus of the mandible was used to remove the mandible and expose the oral cavity. In cases of otitis media, a whitish or yellow-green material was often evident through the transparent tympanic bullae. Affected tympanic bullae were opened with a sterile scalpel, and the exudate was collected by introducing a small sterile bacteriological loop into the tympanic chamber. The material on the loop was then streaked onto a mycoplasma agar plate (alternatively, the tympanic cavity can be cultured through the tympanic membrane).

(ii) **Nasopharynx.** The nasopharynx immediately above the soft palate was adequately exposed for obtaining inocula after the mandible was removed. A small sterile loop was vigorously rubbed in the nasopharynx directly above the soft palate and streaked onto the agar plates.

(iii) **Trachea lung wash.** The trachea was cultured first by inserting a sterile loop into the open end of the trachea and scraping along the entire length of the trachea. The trachea was then clamped off at the larynx. Frey's broth (0.3 to 0.5 ml) was injected into the trachea by using a tuberculin syringe with a 25-gauge needle. The broth was withdrawn and reintroduced five times. The final aspirate was collected in the syringe. Three drops of this fluid was inoculated onto a mycoplasma agar plate.

(iv) **Lungs.** The lungs were cultured by grinding portions of the lung in a Ten Broeck tissue grinder (Thomas) with a small volume of sterile buffered saline and plating the resulting slurry onto mycoplasma agar.

Confirmation as mycoplasma. Agar plates were examined for mycoplasmal colonies under 40 and 100× magnification and low illumination on a light microscope. To distinguish mycoplasmal colonies from L-forms, the colonies were transferred into Frey broth medium containing no bacterial inhibitors. Mycoplasma were identified as to species by the growth inhibition test described by Clyde (4) and modified by Stanbridge and Hayflick (14). *M. pulmonis* was the only species of *Mycoplasma* isolated.

RESULTS

A preliminary study was performed using 42 rats. The nasopharynx, trachea, and a lung of

each rat were cultured. In this study, the trachea was not clamped at the larynx; therefore, broth that entered the lungs was not fully recovered because air entered the syringe via the open trachea. Poor flushing of the lung resulted, and, for the most part, only fluid left in the trachea was withdrawn. The tracheal wash produced 61.9% positive cultures (Table 1). The nasopharyngeal loop yielded 52.38% positive cultures, whereas culture of a ground lung slurry resulted in 19.0% positives.

To further investigate the optimal site for primary isolation of *Mycoplasma* spp. from rodents, mycoplasmal cultures were obtained from two or three different sites, depending upon lesions and symptoms of individual animals. A total of 103 rats were cultured from both the trachea and nasopharyngeal areas; additionally, 74 of these animals had mucopurulent otitis media and were cultured from the middle ear. Positive *M. pulmonis* cultures were obtained from 86.5% of the 74 animals with otitis media, and the tracheal wash produced 76.7% positive cultures. A common site for culturing in many laboratories, the nasopharyngeal area, produced the lowest percentage (53.9%) of positive cultures of the three sites.

Another group of 45 rats was obtained from a colony with a long history of mycoplasmosis to culture *Mycoplasma* as in the previous experiment. The results presented in Table 2 clearly reinforced the trends in Table 3, with the tracheal wash being the optimal source as 100% positives were obtained. With this group, cultures of the middle ear when otitis media was grossly detected produced 91% positive recov-

TABLE 1. *Results of in vitro culture of M. pulmonis from three sites in the respiratory tracts of 42 rats*

Primary source	% Positive	% Negative
Tracheal wash[a]	61.9	28.5
Nasopharyngeal	52.38	47.62
Ground lung	19.0	81.0

[a] 9.6% bacterial overgrowth.

TABLE 2. *Results of attempts to culture M. pulmonis from rats[a]*

Primary source	No. cultured	No. of positive cultures	% Positive
Nasopharyngeal area	45	23	51
Middle ear[b]	33	30	91
Tracheal wash	45	45	100

[a] Cultures were obtained through the use of nasopharyngeal swabs, middle ear swabs, and tracheal washes.

[b] Cultured only when mucopurulent otitis media was observed grossly.

eries of *M. pulmonis.* As in previous trials, the nasopharyngeal area produced a lower percent of positive cultures, i.e., 51%.

Finally, 26 rats were used to determine whether a tracheal wash was necessary or whether sampling of the trachea by an inoculating loop would be just as beneficial. The tracheal wash produced 76.9% positive cultures, whereas 69.2% positive cultures resulted when the loop technique was used. The tracheal wash technique resulted in positive mycoplasma cultures in all animals in which the tracheal loop produced positive cultures.

DISCUSSION

In recent years, there has been a great deal of interest in confirmation of murine mycoplasmosis by in vitro culture. The results of substantial research efforts have emphasized the pathogenic nature of murine *Mycoplasma* spp. This information has stimulated concern among researchers to strive for optimal procedures for isolation. Different sites have been proposed for primary culture of rodent *Mycoplasma* spp. (1, 5, 9, 12, 17). Current and reliable methods were used to collect data for comparisons in an effort to obtain information that could be used in making decisions as to optimal sites for diagnostic culture of *Mycoplasma* spp.

The data in Table 1 indicate that culturing of *M. pulmonis* by a tracheal wash technique is superior to cultures attained from the nasopharynx and far superior to those obtained by culture of ground lung tissue. The 10% overgrowth probably resulted from outside contamination because the trachea was not tied off. The data in Tables 2 and 3 resemble the data of Table 1 and strongly indicate that the tracheal wash procedure is the most reliable. Although middle ear cultures produced the highest percentage of isolates, these figures are misleading because the middle ear was cultured only when *Mycoplasma* infection was suspected by grossly observing otitis media.

TABLE 3. *Comparison of nasopharyngeal swab, middle ear swab, and tracheal wash as methods (sites) optimal for isolation of M. pulmonis in 103 rats*

Primary source	No. cultured	No. of + cultures	% Positive
Nasopharyngeal[a]	102	55	53.9
Middle ear[b]	74	64	86.5
Tracheal wash	103	79	76.7

[a] One culture had bacterial overgrowth.
[b] Cultured only when mucopurulent otitis media was observed grossly.

Finally, the study comparing sampling from the trachea by an inoculating loop and the tracheal wash technique demonstrated the advantage of the tracheal wash procedure.

A simple explanation for why the tracheal wash is the most effective method for obtaining primary cultures is not possible, since several factors may be involved. The tracheal wash technique results in sampling from a large portion of the respiratory tract with aspiration of broth throughout the trachea and the primary air passageways of the lungs. Mycoplasmal inhibitors (1, 3, 12, 17) in the lungs must be considered. Inhibitors in the lungs may have been responsible for the low percent of positive cultures obtained from culture of ground lung (Table 1). Successful cultivation of *M. pulmonis* by the tracheal wash is probably due to the absence or low concentration of inhibitors in the trachea and primary air passageways of the lungs and to dilution of inhibitors by the broth.

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