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Comparison of Chalquest and Hayflick Media, with and without Ammonium Reineckate, for Isolating *Mycoplasma pulmonis* from Rats

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Chalquest and Hayflick media with and without ammonium reineckate were compared for isolation of *Mycoplasma pulmonis* from the nasopharyngeal ducts, tracheobronchial trees, and middle ears of 66 naturally infected rats. The results show that 92% (366 of 396) of the samples were positive for *M. pulmonis* in Chalquest medium with and without ammonium reineckate and 66% (260 of 396) were positive in Hayflick medium with and without ammonium reineckate ($P < 0.001$). An enhancing effect of ammonium reineckate on *M. pulmonis* isolation was observed only in Hayflick medium; the isolation rate was 76% (151 of 198) in Hayflick medium with ammonium reineckate as compared with 55% (109 of 198) in Hayflick medium without ammonium reineckate ($P < 0.03$). No significant differences in isolation rates were observed between Chalquest medium with and without ammonium reineckate. The mean growth time of *M. pulmonis* on Chalquest medium was 3.4 days as compared with 5.1 days in Hayflick medium, indicating that *M. pulmonis* can be detected earlier on Chalquest medium than on Hayflick medium. These data indicate that Chalquest medium is superior to Hayflick medium for *M. pulmonis* isolation from rats.

Mycoplasmosis caused by *Mycoplasma pulmonis* is a major disease of laboratory rats. Clinically, infection is manifested primarily as a respiratory disease; rats with advanced cases have significant pulmonary lesions. Although overt disease can greatly compromise studies with rats, the most insidious effects on research are due to the more common subclinical form.

Diagnosis of the inapparent form of the disease is fraught with many problems and uncertainties. The most common methods used for diagnosis of rodent mycoplasmosis are culture (5), indirect immunofluorescence (5), enzyme-linked immunosorbent assay (2), and histologic examination of tissues for characteristic lesions (9). The use of combinations of methods increases the rate of detection of *M. pulmonis*. Culture is the most definitive method for determining infection and should be used as one of the methods for diagnosis.

Primary concerns when using cultural methods for detecting the presence or absence of *M. pulmonis* include the use of media optimal for in vitro isolation and the site or sites selected for culture. The most commonly used medium for isolating *M. pulmonis* is Hayflick medium, although Chalquest medium is also used. Chalquest medium was developed for isolating avian *Mycoplasma* species (3). It differs basically from Hayflick medium in that it contains Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.) in addition to the ingredient common to both, Bacto-Peptone (Difco Laboratories, Detroit, Mich.) and swine serum instead of horse serum.

There are other factors which are important in the isolation of *M. pulmonis* from tissues, but we feel that these have not been addressed adequately. *Mycoplasma* spp. isolation rates are lower when tissues are homogenized than when left intact (17). It is thought that when mononuclear and polymorphonuclear inflammatory cells are disrupted, lecithin released from the cell membranes and lecithinase (phospholipase) released from lysosomal components (4) react to form lysolecithin, which is a powerful inhibitor of *Mycoplasma* spp. (7, 10). In vitro studies have shown that adding ammonium reineckate (AR) or lysophospholipase to mycoplasma medium counteracts the inhibitory effect of lysolecithin on *M. pulmonis*, apparently by forming an insoluble complex with lysolecithin (11).

The objectives of this study were to compare the effectiveness of Hayflick and Chalquest media for isolating *M. pulmonis* from rats with naturally occurring mycoplasmosis and to test the effect of AR on isolation rates.

MATERIALS AND METHODS

Animals. From a commercial conventional colony we obtained 66 Sprague-Dawley and Fischer 344 rats with a long history of confirmed respiratory mycoplasmosis. The rats used in this study were 45 Sprague-Dawley female rats (1 to 2 years old), 10 Sprague-Dawley male rats (1 to 2 years old), 5 Fischer 344 female rats (1 year old) and 6 Fischer 344 male rats (1 year old). All rats had clinical signs of respiratory mycoplasmosis at necropsy.

Culture media. Hayflick agar medium was prepared as previously described (6). Hayflick agar medium with AR was prepared as follows: 1 g of AR (J.T. Baker Chemical Co., Phillipsburg, N.J.) was dissolved in 100 ml of phosphate-buffered saline, continually stirred with a magnetic stirrer, and then filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). One milliliter of this solution was added into 99 ml of Hayflick agar medium just before pouring into small petri dishes. The AR solution was prepared once a month because it is not stable over a month at 4°C.

Chalquest agar medium modified as described previously (8, 14) was prepared as follows: 10.5 g of PPLO broth without crystal violet, 5.8 g of special agar Noble, 2.5 g of soluble starch, and 2.5 g of glucose were added to 450 ml of deionized water, and the mixture was boiled briefly. Five

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TABLE 1. Rates of isolation of *M. pulmonis* in Chalquest and Hayflick media with and without AR[a]

Medium	Addition to medium	No. of isolates (% of total) in samples from the following:			
		Nasopharyngeal duct	Trachobronchial tree	Middle ear	Total
Chalquest	AR	62 (94)	63 (95)	62 (94)	187 (94)
	No AR	59 (89)	63 (95)	57 (86)	179 (90)
	Total	121 (92)	126 (95)	119 (90)	366 (92)
Hayflick	AR	55 (83)	53 (80)	43 (65)	151 (76)
	No AR	44 (67)	40 (61)	25 (38)	109 (55)
	Total	99 (75)	93 (70)	68 (50)	260 (66)

[a] A total of 198 samples were taken from 66 rats. Regardless of sample site and the presence or absence of AR, isolation rates with Chalquest medium were significantly higher than those with Hayflick medium ($P < 0.001$; partitioned chi-square analysis). Regardless of sample site, isolation rates with Hayflick medium with AR were significantly higher than those with Hayflick medium without AR ($P < 0.03$; partitioned chi-square analysis).

milliliters of a 5% Trypticase-peptone solution, made by adding 0.25 g of Trypticase-peptone to 5 ml of deionized water and 1.25 ml of 10% thallium acetate, was added. The solution was autoclaved and allowed to cool in a 56°C water bath. The final ingredients were then added in the following order: 5 ml of 1% NAD (Eastman Kodak Co., Rochester, N.Y.), 50 ml of sterile mycoplasma-free swine serum which had been inactivated by heating at 56°C for 30 min, and 1 ml of potassium penicillin at 100,000 U/ml. Samples (6 to 8 ml) of the medium were poured into small petri dishes (60 by 15 mm). Chalquest agar medium with AR was prepared by adding 1 ml of 1% (wt/vol) of AR into 99 ml of medium, mixed, and poured into small petri dishes.

Collection of samples. (i) Tracheobronchial lavage. Primary cultures were collected from rats killed with an overdose of sodium pentobarbital administered intraperitoneally. The lower jaw and the skin of the ventral cervical area were cut to expose the trachea. The trachea was clamped immediately caudal to the larynx, and 1 ml of sterile saline was injected into the trachea and bronchi with a 23-gauge needle. The saline was withdrawn and reintroduced three to five times. The final aspirate was discharged into a sterile test tube.

(ii) Nasopharyngeal duct swab. The mandible was completely removed. A sterile urethro-calcium alginate applicator swab (Inolex, American Co., Glenwood, Ill.) was introduced into the nasopharyngeal duct immediately above the soft palate. The swab was vigorously rubbed back and forth and placed into a sterile test tube containing 1 ml of sterile saline.

(iii) Middle ear lavage. The tissue overlaying the tympanic bulla was scraped away with a scalpel. A gas flame was used to sterilize the area. A 23-gauge needle was inserted through the tympanic bullae, and 0.2 to 0.3 ml of sterile saline was injected into the middle ear cavity. The saline was withdrawn and reintroduced three to five times. The final aspirate was placed in a sterile tube.

Culture. A 0.1-ml amount of each sample was inoculated onto four agar plates: Chalquest agar with and without AR and Hayflick agar with and without AR. The samples were spread by gentle rotation of the plates. The agar plates were sealed partially with masking tape and incubated at 37°C and 95% humidity. Plates were examined daily for colony formation for a maximum of 14 days before being discarded as negative. Isolation rates were calculated by dividing the number of positive samples by the number of samples cultured. Growth time was defined as the first time *M. pulmonis* colonies were positively identified.

Identification of *M. pulmonis* colonies. Agar plates were examined for mycoplasmal colonies by using a dissecting scope with low illumination. Mycoplasmal colonies were identified initially by morphologic characteristics. Presumptive colonies were transferred into Hayflick broth medium containing no bacterial inhibitors. *M. pulmonis* colonies were identified positively by using the growth inhibition test described by Stanbridge and Hayflick (16).

Lysolecithin determination. Phospholipids were extracted from samples by a modification of the method of Bligh and Dyer (1). Extract was dried under nitrogen, dissolved in a small quantity of chloroform, and applied to thin-layer chromatographic plates of 0.25-mm-thick silica gel HR (Merck & Co., Cincinnati, Ohio) together with lysolecithin standards. Plates were developed in chloroform-methanol-water (65:25:4, vol/vol), and separate lipid spots were demonstrated by iodine vapors. Sample spots containing lysolecithin were scraped from the plate and assayed for phosphate after degradation (12). Several glass beads and 1.4 ml of 70% perchloric acid were added to each sample of scraped silica gel. Each test tube was covered with a glass marble to allow reflux. The tubes were heated on an electric digestor and maintained at approximately 180°C for 3 h to ensure digestion of the lipid. After cooling, ammonium molybdate and aminonaphtholsulfonic acid reagent were added as described by Rouser et al. (15). The tubes were placed in boiling water for exactly 10 min and allowed to cool, and the silica gel was spun down. The optical densities were determined at 660 nm. Standards of sodium phosphate dibasic (1 μmol/ml) were used to calculate the amount of phosphate liberated by acid degradation. A known concentration of lysolecithin was always included with the samples to quantify the loss of lysolecithin at all stages of the extraction. The overall recovery was found to be about 25% due to losses during extraction, evaporation, transfer, thin-layer chromatography, degradation, and assay.

Statistical analyses. Partitioned chi-square analysis with log-likelihood ratio chi-square statistics (18) was employed to compare isolation rates between and among groups, the chi-square test with the Yates correction factor was used to

TABLE 2. Comparison of samples from which *M. pulmonis* was cultured by one medium but not the other without AR[a]

Medium	No. of isolates in samples from the following:		
	Nasopharyngeal duct	Tracheobronchial tree	Middle ear
Chalquest	19	24	36
Hayflick	2	1	2

[a] In the chi-square test with the Yates correction factor, P was <0.01 for all comparisons between the two media.

TABLE 3. Comparison of samples from which *M. pulmonis* was cultured by Hayflick medium with and without AR[a]

Addition to medium	No. of isolates in samples from the following:		
	Nasopharyngeal duct	Tracheobronchial tree	Middle ear
AR	11	12	20
No AR	0	0	1

[a] In the chi-square test with the Yates correction factor, P was <0.01 for all comparisons between Hayflick medium with and without AR.

compare positive and negative cultures in the two media, and the Wilcoxon matched-pairs signed-rank test was used to analyze growth times.

RESULTS

Comparison of Chalquest and Hayflick media without AR. *M. pulmonis* isolation rates with Chalquest medium without AR were significantly higher than those with Hayflick medium without AR for each culture site ($P < 0.001$) (Table 1). From 198 total samples collected from the three sites, *M. pulmonis* was recovered from 179 samples (90% recovery) with Chalquest medium compared with 109 samples (55%) with Hayflick medium. Table 2 displays the number of samples from which *M. pulmonis* was cultured from the three sites with one medium but not the other. Disagreement between the results with the two media occurred in 84 samples. *M. pulmonis* was isolated on Chalquest medium but not on Hayflick medium from 79 samples, whereas the reverse was noted in 5 samples. Regardless of the sample site, Chalquest medium was significantly more reliable than Hayflick medium in recovering *M. pulmonis* ($P < 0.01$).

Effect of adding AR to Chalquest and Hayflick media. The addition of AR to Chalquest medium did not significantly increase isolation rates from any of the three culture sites over rates of Chalquest medium without AR (Table 1). In contrast, the addition of AR to Hayflick medium significantly increased ($P < 0.03$) the isolation rates from each of the three culture sites when compared with Hayflick medium without AR (Table 1). Of the total 260 positive Hayflick medium cultures, disagreement between Hayflick medium with and without AR occurred in 44 samples; 43 were positive in Hayflick medium with AR and negative in Hayflick medium without AR, whereas the reverse was observed once (Table 3). In all instances, isolation rates with Chalquest medium with AR were significantly higher than those with Hayflick medium with AR ($P < 0.01$) (Table 1).

Comparison of Chalquest medium without AR with Hayflick medium with AR. The isolation rates of Chalquest medium without AR (90%) were greater than those of Hayflick medium with AR (76%) when the data of all three sites were combined ($P < 0.01$) (Table 1). These differences were also significant for the tracheobronchial and middle ear sites ($P < 0.01$) but not for the nasopharyngeal duct site.

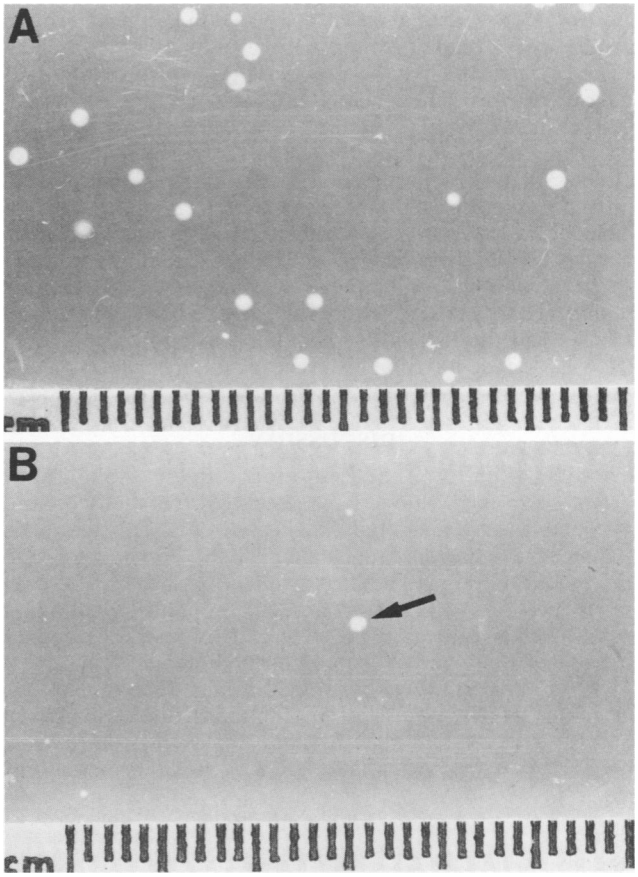

FIG. 1. *M. pulmonis* colonies at day 8 postinoculation. Colonies in Chalquest medium (A) were more numerous, grew faster, and were larger than colonies (arrow) in Hayflick medium (B). (×2.5)

Colony growth times and morphology. The mean number of days when *M. pulmonis* colonies were first detected was calculated for each of the study groups, and analysis by the Wilcoxin matched-pairs signed-rank test was conducted to determine whether one of the media alone or with AR provided an advantage for earlier diagnosis (Table 4). Colonies were detected earlier in Chalquest medium with or without AR (mean, 3.4 days) when compared with Hayflick medium with or without AR (mean, 5.1 days) ($P < 0.01$).

TABLE 4. Mean growth time of *M. pulmonis* in Chalquest and Hayflick media with and without AR[a]

Medium	Addition to medium	Mean growth time (days) ± SD in samples from the following:			
		Nasopharyngeal duct	Tracheobronchial tree	Middle ear	Total
Chalquest	No AR	3.5 ± 1.4	3.1 ± 1.1	3.4 ± 1.1	3.3 ± 1.1[b]
	AR	3.8 ± 1.4	3.1 ± 1.0	3.8 ± 1.2	3.6 ± 1.2[c]
Hayflick	No AR	5.1 ± 2.7	4.8 ± 2.5	5.2 ± 2.7	5.0 ± 2.5[b]
	AR	4.9 ± 2.5	4.9 ± 2.0	5.7 ± 2.8	5.1 ± 2.4[c]

[a] The number of replicate samples ranged from 25 to 63 (see Table 1).
[b,c] In the Wilcoxon matched-pairs signed-rank test, P was <0.01 for all comparisons between the two media with or without AR.

There was no statistical difference noted in the mean growth time for either medium when AR was added.

The colonies in Chalquest medium grew faster and larger and were more homogeneous and regular than colonies in Hayflick medium (Fig. 1 and 2).

Blocking effect of AR on lysolecithin. The concentration of lysolecithin was determined for selected tracheobronchial and middle ear lavage samples and related to cultural results. Table 5 indicates that six of nine (67%) samples were positive for *M. pulmonis* with Chalquest medium without AR, whereas one of nine (11%) was positive with Hayflick medium without AR. The three negative Chalquest medium samples had the highest lysolecithin concentrations. When AR was added, all samples were positive in both media, confirming the potent neutralizing action of AR.

TABLE 5. Blocking effect of AR on lysolecithin inhibition of *M. pulmonis*

Lavage sample	Lysolecithin concn (μg/ml)	Growth in culture medium			
		Chalquest		Hayflick	
		No AR	AR	No AR	AR
Tracheolung	615	−	+	−	+
Tracheolung	410	+	+	+	+
Tracheolung	310	+	+	−	+
Tracheolung	460	+	+	−	+
Tracheolung	515	+	+	−	+
Tracheolung	260	+	+	−	+
Tympanic bullae	515	−	+	−	+
Tympanic bullae	360	+	+	−	+
Tympanic bullae	555	−	+	−	+

DISCUSSION

The best cultural methods for reliable isolation of *Mycoplasma* spp. have been debated for many years. Mycoplasmas are fastidious organisms, and their isolation is influenced by many factors including the composition and enrichment of the media, site of collection, and characteristics of the sample. Our studies focus on culture media that provide quick, accurate identification of the presence or absence of *M. pulmonis* from naturally infected rats.

Hayflick medium was developed to isolate mycoplasmas from humans (6). Since its introduction in 1965 it has been used extensively to isolate *Mycoplasma* spp. from several animal species. Hayflick's original formula or variations thereof are used frequently to isolate *M. pulmonis* from rodents, although diagnostic laboratories that support laboratory animal resources are turning to other formulations which better support the growth of rodent mycoplasmas. Chalquest medium (3) or variations (8, 14) have been used with increasing frequency in laboratory animal medicine but have never been adequately compared with other media.

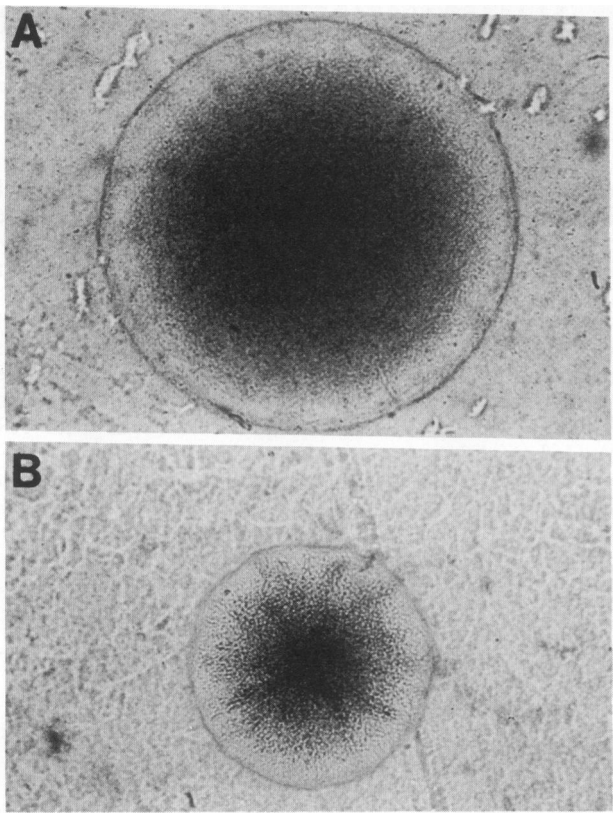

FIG. 2. *M. pulmonis* colonies at day 8 postinoculation. *M. pulmonis* colonies in Chalquest medium (A) were larger and generally more homogeneous and regular than colonies in Hayflick medium (B). (×193)

Our studies indicate that Chalquest medium is superior to Hayflick medium for isolating *M. pulmonis* from rats. Isolation rates were always significantly higher from each of the three culture sites. Rarely was *M. pulmonis* cultured from any site with Hayflick medium when there was no growth with Chalquest medium. In Hayflick medium, *M. pulmonis* colonies generally grew slower, were smaller, and were more irregular and granular, sometimes making it difficult to distinguish the organisms from tissue fragments morphologically.

Another important advantage of using Chalquest medium for culturing *M. pulmonis* from rats is the earlier detection of colonies. *M. pulmonis* colonies could be detected about 2 days earlier with Chalquest medium than with Hayflick medium (Table 4). Earlier detection provides more timely information for making clinical decisions, such as treatment regimens, and for making decisions regarding the use of infected animals in research projects.

The major differences in the formulation of Chalquest and Hayflick media are that Chalquest medium contains swine serum instead of horse serum and Bacto-Peptone in addition to Trypticase-peptone. Swine serum is richer in sterols, which better supports growth of several mycoplasmas, including *M. pulmonis* (13). Bacto-Peptone may also provide more of the nutrients that support luxurious *M. pulmonis* growth.

Clearly, our studies show that the addition of AR to Hayflick medium greatly increases isolation rates (Tables 1 and 5). Overall, isolation rates increased about 20%. The addition of AR to Chalquest medium did not result in significant increases in isolation rates. The enrichment qualities of Chalquest medium may transcend the mechanisms of action of AR. In fact, the isolation rates for Chalquest medium without AR exceeded those for Hayflick medium with AR, again supporting the importance of the additional enrichment that Chalquest medium provides. We cannot rule out the possibility that Chalquest medium may contain an ingredient that neutralizes the lysolecithin effect.

The addition of AR to either medium did not affect detection time of colonies. This is not an unexpected observation, since colony growth time is influenced primarily by

medium enrichment rather than by the number of organisms present. Unpublished data from in vitro studies conducted in our laboratory indicate that detection times were not influenced by the number of *M. pulmonis* organisms in samples.

Mardh and Taylor-Robinson (11) have shown in vitro that the inhibitory effect of lysolecithin on *M. pulmonis* is concentration dependent. The results of this study (Table 5) support their observation in that samples with higher lysolecithin concentration were negative in Chalquest medium.

We conclude that Chalquest medium provides a quick, reliable method for isolating *M. pulmonis* from rats and recommend its use in laboratory animal diagnostic laboratories. However, if Hayflick medium must be used for *M. pulmonis* isolation from rodent tissues, the addition of AR will increase isolation rates significantly.

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