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Comparison of Methods for Detection of *Mycoplasma pulmonis* in Experimentally and Naturally Infected Rats

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Isolation, indirect immunofluorescence, an enzyme-linked immunosorbent assay (ELISA), and histopathological examination of tissues for characteristic lesions were evaluated for their efficiency in detecting *Mycoplasma pulmonis* infection in rats. Whereas all of the methods were efficient in naturally infected Sprague-Dawley rats, none of the methods consistently detected infection in F344 rats experimentally infected with low doses of the organism. In the experimental infections, however, the success rate of any method was directly related ($P < 0.05$) to increasing inoculum dose and time postinoculation. Collectively, the data indicated that isolation of *M. pulmonis* was the most efficient single detection method and the nasopharyngeal duct was the best single site to culture, although sampling of multiple sites within the respiratory tract increased the rate of isolating the organism. The ELISA was understandably the least sensitive method in the low-dose, experimentally infected rats because of the time required for development of a detectable serum antibody response. Although each of the four methods identified a high percentage of naturally infected rats, the ELISA was the most efficient method in these animals as it was uniformly positive. The use of combinations of methods was found to increase the rate of detection of *M. pulmonis* infection in both experimentally and naturally infected rats.

The widespread use of caesarean-derived, barrier-maintained rats and mice has lulled many scientists and rodent breeders into the belief that murine respiratory mycoplasmosis (MRM) due to *Mycoplasma pulmonis* is a disease of the past (27). Although such practices no doubt have reduced prevalence of the infection over the past few decades, recent surveys (32, 34; G. H. Cassell, J. R. Lindsey, J. K. Davis, M. K. Davidson, M. B. Brown, and J. G. Mayo, Lab. Anim. Sci., in press) indicate that it remains a common problem.

MRM is a slowly progressive, often clinically silent disease with slow, cumulative mortality (6, 7, 22, 23). Accordingly, its deleterious effects are especially important in long-term studies such as cancer research and gerontology (22). In addition, *M. pulmonis* genital infections have been reported in rats and are known to decrease breeding efficiency in production colonies (4, 5, 9, 10). Approximately 20 million rats and mice are currently used annually in research projects in the United States alone (36). Therefore, it is both scientifically and economically important to efficiently detect the presence of *M. pulmonis* in both production and research animals.

The objective of this study was to compare the efficiency of available diagnostic methods for detecting *M. pulmonis* in rats. The methodologies presently in use can be divided into four general groups: (i) isolation of *M. pulmonis* in culture media; (ii) detection of *M. pulmonis* antigen in tissues by fluorescein or peroxidase-labeled specific antibody; (iii) detection of specific serum antibody to *M. pulmonis* by serological methods; and (iv) histopathological examination of tissues for lesions compatible with those seen in MRM. Although histopathology is not generally regarded as a detection method per se, it is widely used as an adjunct to the other detection methodologies. A technique from each of these four general categories was selected and evaluated for efficiency of detection of *M. pulmonis* infections in F344 rats infected with low doses of organisms and in naturally infected Sprague-Dawley rats. Each technique tested failed to consistently detect *M. pulmonis* in short-term, low-dose experimental infections, but certain combinations proved moderately effective. All of the methods gave good results in naturally infected rats, but the enzyme-linked immunosorbent assay (ELISA) was superior to

other methods because it identified 100% of naturally infected rats.

MATERIALS AND METHODS

Experimentally infected rats. F344 rats reared and maintained in Trexler plastic film isolators to exclude detectable pathogens were used in all experimental infections (22). When the rats were 3 to 4 weeks old, they were removed from the isolators, housed in polycarbonate shoebox cages equipped with filter top covers, fed an autoclaved diet formulated for axenic rodents (Wayne sterilizable Lab-Blox, Allied Mills, Inc., Chicago, Ill.) and provided with sterile distilled water ad libitum. Sterile hardwood chip bedding (Beta-Chip, Northeastern Products, Warrenburg, N.Y.) was changed daily to keep intracage ammonia at trace levels; ammonia concentrations were measured and recorded daily as described previously (3). Negative control rats were housed in the same manner. One hundred forty rats were assigned randomly to experimental groups and infected intranasally with *M. pulmonis* during sedation with a combination of fentanyl and droperidol (Innovar-Vet, Pitman-Moore, Inc., Washingtons Crossing, N.J.).

Naturally infected rats. Sprague-Dawley rats were obtained from a commercial breeding colony with a long record of enzootic MRM. Intracage ammonia concentrations in the naturally infected colony frequently exceeded 100 ppm. Three hundred sixty-three rats, aged 1 week to 18 months, were purchased during an 18-month period. After arrival, all rats were given sterile distilled water ad libitum and necropsied within 24 h of receipt. In the 1- to 6-week-old groups, complete litters were obtained with their dams.

Necropsy procedures. All rats were euthanized using sodium pentabarbital (Nembutal, Abbott Pharmaceuticals, North Chicago, Ill.) and exsanguinated before necropsy. Specimens were taken, as follows, for bacterial and mycoplasmal cultures, histopathology, immunofluorescence, and serological tests.

Bacterial cultures. Nasal lavages (0.1 ml) from each rat were inoculated into 10 ml of brain heart infusion broth with *para*-aminobenzoic acid and 1% agar (BBL Microbiology Systems, Cockeysville, Md.) and incubated aerobically at 37°C. If growth occurred after 18 to 24 h of incubation, the broth was Gram stained and subcultured to 5% sheep blood agar and eosin-methylene blue agar plates (BBL). The genus of all isolates was determined, and possible pathogens were identified to species using the Minitek system (BBL).

Mycoplasma cultures. Culture samples taken at necropsy included: (i) a laryngeal swab, (ii) tracheobronchial lavage, (iii) nasopharyngeal duct swab, (iv) middle ear lavage, and (v) nasal passage lavage. (The nasopharyngeal duct is the long tube-like passage between the nasal cavity and the oropharynx in rats [12, 21, 29]). Nasopharyngeal duct samples were collected using the method of Hill (12). Nasopharyngeal/urethral swabs (Inolex, Glenwood, Ill.) were used for swab samples. Swabs were placed in 0.4 ml of phosphate-buffered saline (pH 7.4). A tuberculin syringe with a 22-gauge needle and 0.4 ml of phosphate-buffered saline was used for all lavages. Samples were refrigerated immediately after collection and, within 1 h, serially diluted in mycoplasma broth and plated on mycoplasma agar. Cultures were examined after incubation for 7 days at 35°C. Negative cultures were reincubated for 14 days before being discarded as negative.

Mycoplasma media. The following medium was used for all cultures (formulation for 200 ml): mycoplasma broth base (Frey), 4.5 g (GIBCO Diagnostics, Madison, Wis.); glass-distilled water, 132 ml; 1% aqueous phenol red, 0.4 ml (Fisher Scientific Co., Pittsburgh, Pa.); sterile agamma horse serum, 40 ml (GIBCO Laboratories, Grand Island, N.Y.); sterile 25% yeast extract, 20 ml (GIBCO Laboratories); sterile penicillin G, 1 ml (100,000 U/ml); 2% (wt/vol aqueous) thallous acetate, 5 ml (Sargent-Welch, Skokie, Ill.); sterile 50% (wt/vol aqueous) dextrose, 2 ml. For preparation of agar plates, Noble agar (1% final concentration; Difco Laboratories, Detroit, Mich.) was added to the above medium. Each lot of medium was pretested for its ability to support growth of stock strains of *M. pulmonis* (ATCC 19612 and UAB 5782A3).

Experimental mycoplasmal infections. The mycoplasmal strain used in these studies was isolated originally at the University of Alabama in Birmingham from the lungs of a rat with natural MRM. The initial culture was cloned twice and identified as a pure culture of *M. pulmonis* by immunofluorescence (R. A. DelGiudice, Frederick Cancer Research Center, Frederick, Md.). The isolate was passed twice in broth medium and then used to prepare a stock culture (UAB 5782A) which contained 1.2×10^8 colony-forming units (CFU) of *M. pulmonis* per ml. A sample of the stock culture was diluted in mycoplasma broth, and rats were inoculated intranasally with either 10^1, 10^3, or 10^6 CFU of *M. pulmonis* in 50 μl of mycoplasma broth. Control rats were inoculated intranasally with 50 μl of sterile mycoplasma broth.

Identification of mycoplasma isolates. At least one isolate from each rat was confirmed as *M. pulmonis* by the hemadsorption test (25). In addition, several randomly selected isolates from the naturally infected rats were identified by immunofluorescence (R. A. DelGiudice).

IMF. The larynx, trachea, and lungs from each rat were removed intact and infused intratracheally with cold 95% ethanol to return the lungs to approximately normal distension. After fixation, lungs were trimmed so as to give a single horizontal section through the major bronchi of the left, right cranial, and right caudal lobes. Separate preparation of a horizontal section through the major bronchi of right middle and azygous lobes completed the standard sample of lung tissue. Tracheas and larynxes were sectioned longitudinally and transversely, respectively. All sections were processed for indirect immunofluorescence (IMF) according to the Sainte-Marie method (31). Three sections for IMF were cut from each paraffin block. Two sections were treated with a 1:40 dilution of rabbit anti-*M. pulmonis* serum which had an IMF titer of >1,028 and did not cross-react with other rodent mycoplasmas. Negative control sections were stained with normal rabbit serum. Finally, all slides were treated with a 1:40 dilution of fluorescein isothi-

ocyanate-labeled goat anti-rabbit immunoglobulin G (IgG) (BBL). Test sections were grouped with the appropriate control section, and the resulting sets of slides were examined microscopically for presence of specific mycoplasmal fluorescence. All sections were examined using blue incident light fluorescence, a BG23 red suppression filter, two KP490 exciting filters, and K510 and S525 interference filters.

Histopathology. Sections of larynx, trachea, and lung were cut from the same paraffin blocks as those used for IMF. The nasal passages and middle ears also were preserved in cold 95% ethanol, but were decalcified (DeCal, Scientific Products, Atlanta, Ga.) before sectioning. All sections were stained with hematoxylin and eosin. Slides for each rat were examined in a random order and subjectively scored on a scale of from 0 to 4 for lesions compatible with those seen in MRM (6, 18, 22, 23).

ELISA. Serum was collected from each rat at the time of necropsy and stored at −70°C. The ELISA for IgM and IgG antibodies to *M. pulmonis* was performed by the method of Horowitz and Cassell (13). Samples of pooled rat serum from pathogen-free rats of different strains served as negative controls. Several samples of the control serum were included in each assay, and the mean ELISA value was calculated. An animal was considered positive if its ELISA value was >2 standard deviations above the mean value obtained for this pooled control serum. The ELISA values from the experimentally infected rats also were compared to the mean value obtained from uninfected control rats included in the present study. Multiple samples of hyperimmune rat anti-*M. pulmonis* serum served as internal controls to insure within-run and between-run reproducibility.

Viral serology. Approximately one-fourth of the rats included in the study were examined for evidence of infection with common rat viruses. Randomly selected serum samples from all dosage groups of the experimentally infected rats and all age groups of the naturally infected rats were tested for antibodies to Sendai virus, rat coronavirus, Kilham rat virus, H-1 virus, and pneumonia virus of mice (Microbiological Associates, Bethesda, Md.).

Statistical analysis. Non-parametric data were analyzed by a stepwise logistical regression procedure (8) and by chi-square. Parametric data were analyzed by the analysis of variance. When the analysis of variance indicated that a significant difference existed among a set of means, a general linear models procedure and least-squares test were used to determine which were different (8). A probability of 0.05 or less was accepted as significant.

RESULTS

Experimentally infected F344 rats. Six to ten rats of each dosage group were killed at 1, 7, 14, 21, and 28 days postinoculation (p.i.), whereas all negative control rats were killed at 28 days p.i. Control rats were consistently negative for *M. pulmonis* by all detection methods. All rats were negative for bacterial pathogens and were serologically negative for rat viruses.

M. pulmonis was isolated by culture from at least one rat in each dosage group by 7 days p.i. In analyzing the data, it was assumed that all rats that received an *M. pulmonis* inoculum had become infected. Increasing inoculum doses in the range of 10^1 to 10^6 CFU of *M. pulmonis* and time p.i. were directly related ($P < 0.05$) to increased detection of *M. pulmonis* by each method ($P < 0.05$).

The results, irrespective of time p.i., of broth and agar plate cultures for each site sampled are shown in Table 1. Broth and plate cultures gave approximately equal detection rates in the 10^1 CFU and 10^3 CFU dosage groups, but broth cultures yielded markedly higher isolation rates in the 10^6 CFU group at all time points. The nasopharyngeal duct swab detected 99% of the culturally positive animals, followed, in descending order, by: laryngeal swab (98%), nasal passage lavage (97%), middle ear lavage (95%), and tracheobronchial lavage (78%). Inoculum dose significantly influenced ($P < 0.001$) the rate of recovery of *M. pulmonis*, as did time p.i. ($P < 0.02$). For example, samples from the 10^1 CFU dosage group were culturally positive only between days 7 and 21 p.i., whereas samples from the 10^3 and 10^6 CFU groups were culturally positive from day 1 to 28 p.i. Isolation of *M. pulmonis* reached an apparent peak around 21 days p.i. in the 10^3 and 10^6 CFU dosage groups.

The occurrence of lesions compatible with *M. pulmonis* infection is shown in Table 2. This method was not very efficient in detecting experimental *M. pulmonis* infections because early or low-dose infections or both were not always

TABLE 1. *Detection of M. pulmonis in cultures from experimentally infected F344 rats*

Site (method)	Cultures positive (%)					
	10^1 CFU ($n = 44$)[a]		10^3 CFU ($n = 50$)		10^6 CFU ($n = 38$)	
	Broth	Plate	Broth	Plate	Broth	Plate
Nasal passages (lavage)	0	0	11	13	65	38
Nasopharyngeal duct[b] (swab)	0	7	11	16	68	58
Middle ear (lavage)	0	0	11	13	58	37
Larynx (swab)	2	5	11	13	68	39
Tracheobronchial tree (lavage)	0	0	7	9	36	19
% Animals positive (total of 5 sites)	7		18		71	

[a] Inoculum dosage group. *n*, Number of animals per dosage group.

[b] See text for explanation of this anatomical structure.

TABLE 2. *Presence of histological lesions compatible with M. pulmonis infection in experimentally infected F344 rats*

Site	% Positive for lesions		
	10^1 CFU ($n = 44$)[a]	10^3 CFU ($n = 50$)	10^6 CFU ($n = 38$)
Nasal passages	0	2	8
Middle ear	0	0	11
Larynx	2	10	35
Trachea	2	10	19
Lung	0	0	0
% Animals with lesions (total of 5 sites)	5	14	37

[a] Inoculum dosage group. *n*, Number of rats examined.

TABLE 3. *Detection of M. pulmonis by IMF in experimentally infected F344 rats*

Site	% Positive		
	10^1 CFU ($n = 42$)[a]	10^3 CFU ($n = 50$)	10^6 CFU ($n = 38$)
Larynx	26	20	63
Trachea	19	20	53
Lung	9	10	44
% Animals positive (total of 3 sites)	40	34	73

[a] Inoculum dosage group. *n*, Number of rats examined.

accompanied by morphological lesions. No one site was a very sensitive indicator of infection; therefore, examinations of multiple sites were the most suggestive of infection. Inoculum dose and time p.i. were significantly related ($P < 0.0001$) to severity of lesions. In the 10^1 CFU dosage group, mean lesion index scores for each site were never significantly different from the values obtained for negative control rats. In the 10^3 CFU dosage group, the larynx and trachea had significant mean lesion scores only at 14 and 21 days p.i. The mean lesion scores found in the 10^6 CFU dosage group were significantly different from control values for the nasal passages, larynx, and trachea at 21 and 28 days p.i. and for the ears at 28 days p.i. Lung lesions were never found in any of the experimentally infected rats.

Table 3 shows the results obtained using IMF to detect *M. pulmonis* antigen in tissues. As with histology, no one site was particularly sensitive for detection of *M. pulmonis*, but examination of multiple sites increased the efficiency of the method. The larynx was positive in 88% of the rats positive by IMF, whereas trachea and lungs were positive in 84 and 75% of the rats, respectively. The time p.i. did not significantly affect detection of *M. pulmonis* by this method, but inoculum dose did ($P < 0.0001$).

The percent positive rats identified by the ELISA for serum IgM and IgG antibodies to *M. pulmonis* is shown in Table 4. The ELISA values were compared to two different control sera. For purposes of comparison with the other detection methods, pooled normal rat serum served as the negative control. Inoculum dose and time p.i. significantly affected the IgG ELISA values ($P < 0.001$), but only time p.i. affected the IgM ELISA values ($P < 0.001$). The means of the IgG ELISA values for the 10^6 CFU dosage group were statistically different from the normal rat serum control means at 21 and 28 days p.i. However, individual IgG ELISA values were positive as early as 14 days p.i. The means of the IgM ELISA values from any dosage group were never different from the control value.

When the ELISA values were compared to the values obtained from uninfected control rats in the same experiment, the results changed markedly (Table 4). The IgM class was affected more than the IgG antibody class. The increase in positive values was due to the much lower ELISA values for the uninfected control rat sera versus those obtained for the pooled normal rat serum. Although the number of positive animals increased when the ELISA values were compared to values from uninfected control rats, the same trends were seen as when the values were compared to the normal rat serum control, except that there were many animals in the 10^1 and 10^3 dosage groups with a positive IgM but negative IgG value.

The usual method for evaluating a group of techniques is to compare all techniques to the most widely accepted method. Isolation of *M. pulmonis* by culture has been used as the standard in the past. However, these data showed

TABLE 4. *Detection of M. pulmonis by ELISA for serum antibodies in experimentally infected F344 rats*

Serum standard	% Positive					
	10^1 CFU ($n = 42$)[a]		10^3 CFU ($n = 47$)		10^6 CFU ($n = 37$)	
	IgM	IgG	IgM	IgG	IgM	IgG
Pooled normal serum	0	2	0	9	0	41
Uninfected control serum	17	7	24	13	23	46

[a] Inoculum dosage group. *n*, Number of rats examined.

that culture, even when multiple sites were sampled, failed to detect a high percentage of infected rats. Therefore, the methods were compared to each other, and the results were expressed as percentage agreement between methods (Table 5). The percentages of agreement were obtained by dividing the number of rats in which both tests being compared gave the same results, either positive or negative, by the number of rats tested by both methods, and multiplying by 100.

In Table 6, all dosage groups were combined, and the agreements between methods were calculated at 28 days p.i., when all methods were at their maximum efficiency under these conditions. The effect of time p.i. and inoculum dose can be seen more clearly in Table 7, in which the detection rate for each method within each dosage group is related to time p.i. Table 8 shows the overall percent agreement between methods, irrespective of inoculum dose and time p.i. This table shows what one might expect for agreement between methods in a routine screening program for *M. pulmonis* when the inoculum dose and time postinfection were not known.

The stepwise logistical regression procedure and least-squares test showed the relationship between methods to be rather complex. A positive culture from any site in the 10^3 and 10^6 CFU dosage groups was quite likely to be associated with a positive IgG ELISA value from the same rat ($P < 0.001$). However, the IgM ELISA was not related to culture positivity in any dosage group.

Isolation was related ($P < 0.05$) to the IMF results in the 10^3 and 10^6 CFU dosage groups ($P < 0.05$), but no single culture site was specifically related to the IMF results. In fact, any single site often had different cultural and IMF results in the same rat.

Isolation was related to positive histopathology only in the 10^6 CFU dosage group ($P < 0.05$). However, isolation, regardless of site sampled, was related to microscopic lesions in the nasal passages in the 10^3 CFU dosage group ($P < 0.05$). In contrast, isolation from any site was related to microscopic lesions in the larynx in the 10^6 CFU dosage group ($P < 0.01$).

ELISA values and IMF results were not related in any dosage group, because ELISA and IMF tended to identify different individual rats. ELISA and histopathology results were related in the 10^3 and 10^6 CFU dosage groups ($P < 0.05$) because the two tests identified the same individual rats. This relationship held true only for the serum IgG antibodies ($P < 0.05$). IgG positivity was correlated with presence of lesions in the larynx and nasal passages in the 10^3 CFU dosage group ($P < 0.01$), and with lesions in the larynx, nasal passages, and trachea in the 10^6 dosage group ($P < 0.01$).

Certain combinations of methods improved detection of *M. pulmonis*. In general, the combinations which had the lowest percent agreements, and therefore identified different individual rats, gave the best overall detection results. In the 10^1 CFU dosage group, the combinations of culture and IMF, ELISA and IMF, and histopathology and IMF identified about 43% of the infected rats. In the 10^3 CFU dosage group, culture and IMF detected only 39% of the infected rats, whereas the combination of histopathology and IMF identified 45% of the infected animals. In the 10^6 CFU dosage group, the best combinations of methods were culture and IMF (89%), and ELISA and IMF (83%). Culture and histopathology, and IMF and histology, gave approximately 78% detection of *M. pulmonis*. The poorest combination was ELISA and histopathology, which identified from 7 to 53% of the infected rats, depending on the dosage group.

Naturally infected Sprague-Dawley rats. Twenty to fifty rats were killed at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, and 72 weeks of age. The nasal passages of all rats were cultured for the presence of bacterial pathogens, and serum was collected for viral serology. Most rats from this colony had *Pseudomonas aeruginosa* and *Kleb-*

TABLE 5. *Percentage agreement[a] between detection methods for M. pulmonis in experimentally infected F344 rats*

Method	% Agreement								
	10^1 CFU ($n = 44$)[b]			10^3 CFU ($n = 50$)			10^6 CFU ($n = 38$)		
	ELISA	IMF	Histopath	ELISA	IMF	Histopath	ELISA	IMF	Histopath
Culture	95	57	90	96	74	79	70	66	59
ELISA	100	57	93	100	70	85	100	50	75
IMF	57	100	60	70	100	59	50	100	53

[a] Number of rats in which both tests gave the same results, either positive or negative, divided by the total number of rats tested by both methods, and multiplied by 100.

[b] Inoculum dosage group. *n*, Number of rats examined.

TABLE 6. *Percentage agreement[a] between methods in experimentally infected F344 rats at 28 days p.i.*[b]

Methods	% Agreement		
	ELISA	IMF	Histopathology
Culture	92	77	92
IMF	69	100	73
Histopathology	96	73	100

[a] Number of rats in which both tests gave the same results, either positive or negative, divided by the total number of rats tested by both methods, and multiplied by 100.
[b] $n = 26$.

TABLE 7. *Experimentally infected F344 rats: detection methods related to time p.i.*

Method	10^1 CFU	10^3 CFU	10^6 CFU
IMF	1[a]	1	1
Culture	7	1	1
ELISA	28	21	14
Histopathology	>28	28	21

[a] Day when at least one positive animal was detected by each method.

TABLE 8. *Percent agreement[a] between methods in experimentally infected F344 rats*[b]

Method	% Agreement		
	Histopathology	ELISA	IMF
Culture	77	86	69
IMF	61	63	100
ELISA	86	100	63

[a] Number of rats in which both tests gave the same results, either positive or negative, divided by the total number of rats tested by both methods, and multiplied by 100.
[b] All dosage groups and time points p.i. are combined.

siella pneumoniae. These organisms can be opportunistic pathogens in rats, but rarely are primary ones (6). These rats also had serological titers to Sendai virus, an agent that has been shown to enhance MRM in mice (14, 23, 30). Significant serological titers to rat coronavirus, pneumonia virus of mice, and Kilham rat virus also were obtained.

A summary of the detection of *M. pulmonis* in these rats is shown in Table 9. The data agreed with the results obtained from the study of experimentally infected rats. In all age groups, the broth cultures gave consistently higher isolation rates for *M. pulmonis* than did the agar plate cultures. The following individual isolation rates were determined when data were pooled with respect to age: (i) nasopharyngeal duct (90%), (ii) tracheobronchial tree and nasal passages (82%), (iii) larynx (78%), and (iv) middle ears (65%). Age significantly influenced the results ($P < 0.001$). Isolation rates for each site varied considerably in different age groups. All sites in the respiratory tract were positive by 6 weeks of age, and there was general decline in percent positive cultures after 24 weeks of age (M. K. Davidson et al., submitted for publication).

The histopathology results confirmed those in the experimentally infected rats, i.e., evidence of infection was more likely to be found by examinations of multiple sites within the respiratory tract. The nasal passages were most frequently positive (81%), followed by larynx (61%) and trachea (58%). The middle ears and lungs had lesions in only 37% of the rats. The mean lesion score indices reflected these results. Individual animals had microscopic lesions at all sites in the respiratory tract except lung by 1 week of age. The lesions developed more quickly in the nasal passages and larynx, whereas lung lesions appeared more slowly. Interstitial pneumonia, most likely due to Sendai virus, was seen in occasional rats up to 4 weeks of age. Subsequently, lung lesions compatible with those of MRM were present in the majority of rats examined.

IMF was not nearly so efficient at detecting *M. pulmonis* antigen in infected tissues from the naturally infected rats as from the experimentally infected rats. However, results were otherwise similar. The larynx was the best single site to examine by IMF, and the peak of detection at this site occurred in the 4-week-old rats, when 64% were positive at this site. Also, examination of multiple sites was much more suggestive of infection. Once again, the age of rats significantly influenced the results. For example, at 1 week of age, only 10% of the rats were positive by IMF, whereas 80% were positive in the groups sampled from 12 to 72 weeks of age (Davidson et al., submitted for publication).

The ELISA for IgG antibodies to *M. pulmonis* was consistently positive in all age groups of rats. However, serum IgM was not positive, even in individual animals, until 8 weeks of age.

The percent agreement between methods, using both the positive and negative results of all age groups, is presented in Table 10. The stepwise logistical regression procedure and least-squares test showed that the interrelationships between methods were complex. The results obtained from culture and histopathology were related ($P < 0.01$). Rats with isolations usually had lesions at some site ($P < 0.01$). Specifically, isolation of *M. pulmonis* was quite likely ($P < 0.05$) to result in a corresponding microscopic

TABLE 9. *Detection of M. pulmonis in naturally infected Sprague-Dawley rats*[a]

Site (method)	% Positive					
	Culture (n = 219)[b]		IMF (n = 249)	Histopathology (n = 249)	ELISA[c] (n = 363)	
	Broth	Plate			IgM	IgG
Nasal passages (lavage)	64	54	ND[d]	81		
Nasopharyngeal duct (swab)	83	53	ND	ND		
Middle ears (lavage)	45	44	ND	37		
Larynx (swab)	62	50	45	61		
Tracheobronchial tree (lavage)	71	47	37	58		
Lung	ND	ND	16	37		
% Animals positive (any site)	89		57	85	32[e]	100

[a] All ages, from 1 week to 18 months, are combined.
[b] n, Number of rats examined.
[c] Each rat was tested serologically.
[d] ND, Not done.
[e] No positive serum IgM levels were found in rats less than 8 weeks of age.

TABLE 10. *Percentage agreement*[a] *between methods for detection of M. pulmonis in 363 naturally infected Sprague-Dawley rats*[b]

Method	% Agreement		
	ELISA	IMF	Histopathology
Culture	88	63	85
IMF	55	100	61
ELISA	100	55	86

[a] Number of rats in which both tests gave the same results, either positive or negative, divided by the total number of rats tested by both methods, and multiplied by 100.
[b] All ages, from 1 week to 18 months, are combined.

lesion at that same site in the same rat. Because lungs per se were not cultured, only positive tracheobronchial lavage cultures were directly related to occurrence of lung lesions ($P < 0.05$).

Culture and IMF were also related ($P < 0.001$) especially for the larynx and trachea ($P < 0.01$). Furthermore, a positive culture at any site was closely related to a positive IMF result in at least one site ($P < 0.01$). However, only positive middle ear culture was related to positive IMF results from the lung ($P < 0.05$).

Culture results and ELISA results were related ($P < 0.05$), specifically with the serum IgG results ($P < 0.05$). However, even the serum IgM results were related to the cultural isolations from middle ears and nasal passages ($P < 0.01$). The IMF and histopathology results also were related ($P < 0.01$), particularly for the trachea.

When data were pooled with respect to age, the IgG ELISA and IMF results were not related. However, the IgM ELISA was related to the IMF results from the trachea and lungs ($P < 0.05$). Both the IgM and IgG ELISA results were related to microscopic lesions at every site sampled ($P < 0.05$).

In the naturally infected rats, the IgG ELISA was uniformly positive for antibodies to *M. pulmonis*. Therefore, any combination of methods with ELISA gave 100% detection. Other combinations of methods which gave good detection rates were: (i) culture and histopathology (95%), (ii) IMF and histopathology (90%), and (iii) culture and IMF (87%).

DISCUSSION

The rat populations in this study were purposely selected because they represent two extremes seen in MRM and, thus, the extremes for testing efficiency of detection methods. The experimentally infected F344 rats represented the best known conditions for rats, i.e., low-dose infections in uncrowded weanling rats, with no intercurrent infections and housing in an environment virtually free of ammonia. In addition, the *M. pulmonis* infections were studied only to day 28 p.i. This population, with conditions least favorable to growth of the mycoplasmas and development of MRM (3, 6, 23, 24, 37), posed an extremely rigorous test of sensitivity for each of the methods evaluated. In contrast, the naturally infected Sprague-Dawley rats had several intercurrent infections, intracage ammonia levels often exceeded 100 ppm and, most likely, the rats had received higher-dose initial infections. Therefore, these rats provided an opportunity to evaluate the reliability of each test under natural field conditions where several factors known to enhance MRM existed (3, 6, 14, 30, 32). Any of the above factors, plus rat strain differences (J. K. Davis and G. H. Cassell, Vet. Pathol., in press; J. K. Davis, R. B. Thorp, P. A.

Maddox, M. B. Brown, and G. H. Cassell, Infect. Immun., in press), could have influenced the results found in the two separate rat populations in the present study. Thus, it is remarkable that the results from the two populations strongly support the same general conclusions.

The nasopharyngeal duct (21, 29) swab, collected by the method of Hill (12), was the single best sample for isolation of *M. pulmonis* by culture, especially in the naturally infected population. This result is in general agreement with the work of Lemcke (19) and Kohn (17). Ganaway et al. (9) did not sample the nasopharynx, but reported obtaining highest isolation rates from the oropharynx (or throat). From the descriptions of their methods (9), this site most closely resembles the larynx in the present study. The present study is in direct contrast to recent studies by Lentsch et al. (20) and Owens et al. (28) in which the tracheobronchial lavage yielded the highest percent positive cultures. However, inasmuch as even the nasopharyngeal swab sample failed to detect 10% of the culture-positive, naturally infected rats, multiple sampling of different sites within the respiratory tract is indicated for optimal recovery of *M. pulmonis*.

Although broth cultures usually gave higher isolation rates than did agar plate cultures (Tables 1 and 9), it should be noted that occasional isolations were made only on agar plates, especially from the naturally infected rats. In addition, the broth cultures were frequently positive only in the 10^{-3} to 10^{-5} dilutions. This can be explained by the presence of mycoplasmacidal substances in many culture samples (1, 2, 16, 26, 35). It strongly reaffirms the value of either testing dilutions of samples in mycoplasma broth (1, 2, 35) or adding ammonium reinechate or lysophospholipase (26) to the medium.

The results obtained from histological examination of experimentally infected and naturally infected rats differed as the naturally infected rats had far more severe lesions, particularly in the distal airways (Tables 2 and 9). However, this can be explained by the concurrent Sendai virus infection, high intracage ammonia concentrations, and possibly other factors affecting the naturally infected rats. Broderson et al. (3) and Lindsey and Conner (24) showed that ammonia levels as low as 25 ppm increased the progression and severity of MRM. In addition, recent studies in our laboratory have shown that environmental ammonia significantly increases the growth of *M. pulmonis* in the nasal passages and that this event precedes the growth of the organism in more distal airways (T. R. Schoeb, Ph.D. thesis, University of Alabama, Birmingham, 1981). Since it takes time for such lesions to develop (Table 7) and no lung lesions were seen in any of the experimentally infected rats during the 28-day observation period, these results simply emphasize the importance of standardized, rigorous examinations of all parts of the respiratory tract for lesions compatible with MRM.

The IMF results for the naturally infected and experimentally infected rats also were in disagreement (Tables 3 and 9). However, IMF appeared to have one rather clear advantage in the present study. In the low-dose, experimentally infected rats it was successful in detecting the organism in a few animals of all dosage groups by day 1 p.i., the same as culture in the 10^3 and 10^6 dosage groups, but 6 days before a positive culture was obtained for the 10^1 dosage group. Because culture samples were taken from the same sites as those on which IMF was performed, thus removing most of the organisms and any exudate present in the lumens, the results obtained by IMF may have been unduly low.

The ELISA was the least sensitive method studied in the experimentally infected rats. However, the inoculum dose and time p.i. significantly ($P < 0.0001$) affected detection of the infection by this method. As would be expected, it takes time to develop an immune response (Table 8) and, depending on the infecting dose and possibly other factors, that time can exceed a month. When these factors were taken into consideration (Table 6), the ELISA compared favorably with other detection methods.

When individual ELISA values were compared to the uninfected control rats included in the experiment, a marked increase in percent positive animals was noted (Table 4). This probably represents seroconversion. The uniform positivity of ELISA results from the naturally infected rats could be explained easily by the high levels of IgG found in the young naturally infected rats. Since rats and mice absorb IgG but not IgM from colostrum and milk (11, 15, 33), the high IgG levels in nursing pups probably represented passively transferred maternal antibody to *M. pulmonis*.

In overt MRM, typified by the naturally infected rats in the present study, diagnosis of *M. pulmonis* infection and disease is not a serious problem because any of the methods described will detect a high percentage of infected animals. However, the data from the low-dose experimental infections showed that the infection can be difficult to detect. This is particularly pertinent because the experimental infections more closely approximated those conditions found in the better rodent facilities where intercurrent infections

are kept to a minimum and good environmental conditions are maintained. Therefore, since the time p.i. and inoculum dose cannot be known in naturally occurring *M. pulmonis* infections, and latent infections are known to occur (9), a single screening of animals within a colony, using just one detection method, may not be conclusive. However, if animals 2 to 3 months old, or older, are screened, the ELISA may prove to be the method of choice for natural infections because of its simplicity, low cost, rapidity, and high sensitivity.

Finally, the conclusions to be drawn from this study are: (i) all of the methods presently in use are valuable in the detection of *M. pulmonis* infection and MRM, but none alone is optimal under all circumstances, particularly in detection of low-dose infections; (ii) combinations of methods should be used whenever possible to optimize detection; (iii) isolation of *M. pulmonis* should be used as one of the methods since it is one of the most efficient methods available; (iv) multiple sites should be cultured, but of individual sites, a swab of nasopharyngeal tube gave the best isolation rate; and (v) although the inoculum dose of organism was an important factor in detection, the time p.i. was also important for detection of *M. pulmonis* in rats with any of the methods used.

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