IMMUNOFLUORESCENCE TECHNIQUE FOR DETECTION OF MYCOPLASMA HYOPNEUMONIAE IN SWINE LUNGS[1]

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ABSTRACT - Direct and indirect immunofluorescence techniques and a culture method were compared for detection of *Mycoplasma hyopneumoniae* in lungs of experimentally infected swine. Pneumonic lungs were obtained from SPF pigs that had been exposed by contact 6 to 7 weeks previously to other SPF pigs which had been inoculated with the organism. A good association was found between isolation of *M. hyopneumoniae*, positive immunofluorescence test results and presence of macroscopic or microscopic lesions typical of mycoplasmal pneumonia of swine. Direct and indirect immunofluorescence tests were equally efficient for detection of *M. hyopneumoniae* in sections of lung. An azo dye counterstain reduced nonspecific fluorescence and provided a good color contrast without reducing sensitivity of the indirect immunofluorescence test.

Index terms: mycoplasmal pneumonia, enzootic pneumonia, diagnosis.

TÉCNICAS DE IMUNOFLUORESCÊNCIA PARA DETECÇÃO DE MYCOPLASMA HYOPNEUMONIAE EM PULMÕES DE SUÍNOS

RESUMO - Compararam-se as técnicas de imunofluorescência direta e indireta e de cultivo na detecção de *Mycoplasma hyopneumoniae* em pulmões de suínos infectados experimentalmente. Estes pulmões foram obtidos de animais SPF após seis a sete semanas de início de um período de contato com outros animais SPF inoculados experimentalmente com o agente. Uma boa associação foi encontrada entre o isolamento de *M. hyopneumoniae*, resultados positivos em ambas as técnicas de imunofluorescência e lesões macroscópicas e microscópicas típicas de pneumonia micoplásmica dos suínos. Tanto a imunofluorescência direta como indireta foram igualmente eficientes na detecção de *M. hyopneumoniae* em secções de pulmão. Uma coloração azo de fundo reduziu a fluorescência inespecífica e possibilitou um bom contraste de cor sem reduzir a sensibilidade do método indireto de imunofluorescência.

Termos para indexação: pneumonia micoplásmica, pneumonia enzoótica, diagnóstico.

INTRODUCTION

Diagnosis of mycoplasmal pneumonia of swine (MPS) or enzootic pneumonia of swine has been achieved for many years mainly by gross and microscopic evaluation of lungs. In recent years improved methods have been described for isolation of the etiologic agent; however, isolation of *M. hyopneumoniae* is hampered because a) the organism is very fastidious and grows slowly during early passages, b) other more rapidly growing mycoplasmas, especially *Mycoplasma hyorhinis*, are common also in swine lungs and c) some strains of *M. hyopneumoniae* are difficult to isolate even under ideal conditions (Whittlestone 1979).

Work published from several different laboratories has indicated that immunofluorescence techniques provide a rapid and specific diagnosis of MPS thus circumventing the limitations posed by culture methods yet providing a more specific diagnosis than may be obtained by morphologic observations. Although both the direct fluorescent antibody (DFA) technique (L'Ecuyer & Boulanger 1970, Meyling 1971 and Amanfu et al. 1984), and the indirect fluorescent antibody (IFA) technique (Gois et al. 1975) have been utilized for detection of *M. hyopneumoniae* in swine lungs, their relative usefulness with respect to each other and to other diagnostic methods has not been thoroughly ascertained. In the work reported here, we evaluated the DFA and IFA using lungs of experimentally infected swine and found them to be equally effective in detecting the organism and to compare favorably with morphologic observations and a culture method for detection of the organism.

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MATERIALS AND METHODS

Swine

All experimental pigs were obtained from a respiratory--disease-free herd maitained at Iowa State University. The herd contained Yorkshire and Hampshire bloodlines and was established by surgical procurement in 1978 in a manner similar to that used for procurement of specific pathogen free (SPF) swine. Adult swine and progeny have been consistently negative for culture and serological evidence of swine mycoplasmas and other swine respiratory disease agents.

Experiment design

In experiment A, three groups of pigs of different ages were utilized; group 1 consisted of six 3-to-4-week old pigs, group 2 of six 6-to 7-week old pigs and group 3 of six 12-to 13-week old pigs. Each group was exposed by co-mingling for 27 days with "seeder" pigs that had been inoculated with *M. hyopneumoniae* 18-21 days previously. The pigs were necropsied 41-43 days after the beginning of contact-exposure.

In experiment B, two groups of six pigs, each containing three 3-week old pigs and three 11-to 12-week olg pigs were used. Each group was exposed by contact to three "seeders" for 20 days. Following 20 days contact--exposure, each pig was moved to an individual hog house. Twenty-one to 20 days after the contact-exposed pigs had been placed in the hog houses they were necropsied.

Infection with *M. hyopneumoniae*

"Seeders" consisted of 6-to 9-week old pigs that had been inoculated endotracheally (Experiment A) or intranassaly (Experiment B) with a 10% suspension of pneumonic lung containing only *M. hyopneumoniae* strain 11 (L'Ecuyer & Switzer 1963 and Mare & Switzer 1965).

Evaluation of gross lesions

Lungs were evaluated at necropsy according to criteria described by Pullar (1948).

Histological technique and evaluations

Pieces of lung taken from right and left cardiac lobes of pigs in experiment B were fixed in 10% buffered formalin for 48 hours, processed according to standard technique and stained with hematoxylin and eosin. Assessment of microscopic lesions was done as described by Whittlestone (1972).

Isolation of *M. hyopneumoniae*

Approximately 1 gm pieces of right and left cardiac lobes of lungs from pigs in experiment B were collected aseptically at necropsy. Sections were taken to include bronchus and several small bronchioles. Tissue was homogenized in a Ten-Broeck tissue grinder using 5 ml of Friis mycoplasma medium without antibiotics as diluent. Lung suspensions were inoculated in 10 fold dilutions (10^{-1} to 10^{-7}) in Friis medium with antibacterial agents added (Friis 1975, 1979). Incubation was carried out at 35-37 C in a roller drum. Cultures with acid shift and/or turbidity were subcultured 3-5 times and inoculated on Friis agar. Plates were incubated in a candle jar and observed for colony development. Colonies were identified as *M. hyopneumoniae* by epi-immunofluorescence technique (Del Giudice et al. 1967) using antiserum produced in rabbits with a sodium deoxycholate extract (Ross & Karmon 1970) of the organism.

Immunofluorescence

In pigs from experiment A, both cardiac lobes as well as any other lobes with mycoplasmal pneumonia were examined using both DFA and IFA. In experiment B, both cardiac lobes from each pig were examined using DFA and IFA. Samples for immunofluorescence testing were taken at the junction between pneumonic lung and normal lung and included a bronchus and several small bronchioles. Samples were embedded in OCT fluid medium (Lab-Tek Division, Miles Laboratories Inc, Naperville, Illinois), frozen in dry ice and stored at -70C (Amanfu et al. 1984). Sections were cut with a cryostat and fixed with absolute methanol. The DFA test was done as described by Amanfu et al. (1984), including use of an azo-dye counterstain (Potgieter & Ross 1972).

For the IFA, we used fluorescein-conjugated goat IgG antibodies against rabbit IgG (Cappel Laboratories, Cochranville, PA, U.S.A.). Antiserum against *M. hyorhinis* was prepared using several inoculations of rabbits with whole formalinized cells of *M hyorhinis* and antiserum against *M. hyopneumoniae* was prepared using a similar schedule with a sodium deoxycholate extract of that organism (Ross & Karmon 1970).

Titration and determination of specificity of fluorescein-conjugated antirabbit IgG and of antisera against *M. hyopneumoniae* and *M. hyorhinis* was accomplished by checkerboard titration of each antiserum using lung sections from pigs experimentally infected with *M. hyopneumoniae*, pigs naturally infected with *M. hyorhinis* or from noninfected pigs. Two-fold dilutions of sera ranging from 1:2 to 1:1024 were tested. The homologous system included sections of *M. hyopneumoniae* and *M. hyorhinis* infected lung treated, respectively, with antisera against *M. hyopneumoniae* and *M. hyorhinis* and stained with antirabbit IgG conjugate. The heterologous system was similar except that mycoplasma antisera were applied to tissues infected with the heterologous species. In addition normal rabbit serum was applied to similar sections to determine the specificity of the reaction. Lung sections from normal, noninfected pigs were stained also with both antisera and normal rabbit serum to further confirm the specificity of the test. The end point for a given antiserum was the dilution at which minimum

nonspecific fluorescence and maximum specific fluorescence was seen in the bronchi and bronchioli. Cross reactions encoutered when antiserum against *M. hyorhinis* was reacted with *M. hyopneumoniae* were removed by adsorption of the serum with a deoxycholate extract (Ross & Karmon 1970) of *M. hyopneumoniae* for 24 hours at 4 C. Antiserum to *M. hyopneumoniae* was adsorbed with swine liver powder (Cherry et al. 1961) in order to eliminate a diffuse green nonspecific background fluorescence.

The IFA test was conducted at 37 C according to Cherry et al. (1961) but with washing and timing according to Rosendal & Black (1972). Lung sections were mounted on slides, placed in a dish containing a moist filter paper and covered with a drop of the appropriate dilűtion of antiserum. Dishes were tightly covered and incubated at 37 C for 30 minutes. Excess unbound antiserum was removed by two sequential rinsings in PBS (pH 7.4) for 10 minutes in a glass jar on a magnetic stirrer. The sections were blotted dry and arranged again in the dish where they were covered with a drop of fluorescein-conjugated goat anti rabbit IgG antibody. The dishes were sealed and incubated again at 37 C for 30 minutes. Excess unbound conjugate was removed by two sequential rinsings in PBS (pH 7.4) for 10 minutes and one rinsing in distilled water. One set of slides was counterstained with azo-dye (Potgieter & Ross 1972) for 30 seconds and washed twice in distilled water. The washed sections were blotted dry, mounted in phosphate buffered glycerin (pH 7.4) and examined with a Leitz ortholux fluorescence microscope equipped for vertical fluorescence.

The intensity and location of fluorescence in bronchi and bronchioli were scored in DFA and in IFA tests as follows; 1 = scattered granular green-yellow fluorescent particles lining the bronchi with no fluorescence in bronchioli; 2 = thin coating of yellow-green particles lining the bronchi with no bronchiolar fluorescence; 3 = thin coating of yellow-green particles lining the bronchi but with no green-yellow fluorescent granules lining the bronchioli; and, 4 = continuous thin coating of yellow-green fluorescent lining bronchi and bronchioli.

RESULTS

Three procedures were used to detect *M. hyopneumoniae* in lungs of swine infected with the organism; DFA, IFA with azo-dye counterstain and IFA without the counsterstain. Mycoplasmal antigen was detected primarily on the surfaces of bronchi and bronchioli by means of all 3 procedures as a granular or thin coating of bright yellow or apple green fluorescent particles. Typical apple green fluorescence created by use of fluorescence was seen in preparations without counterstain. In sections stained with the counterstain, the specific fluorescence associated with fluorescence was yellow.

A diffuse green nonspecific background fluorescence was observed in sections prepared without counterstain. This nonspecific fluorescence was eliminated almost entirely by adsorption of the *M. hyopneumoniae* antiserum with swine liver powder and by using azo-dye counterstain (Fig. 1 and 2).

Positive DFA and IFA results were obtained with all lobes from pigs in experiments A and B which had gross lesions of pneumonia. With one exception, all of the lobes with no gross lesions were DFA and IFA negative. One lobe from one pig that had no gross evidence of pneumonia, but which had microscopic lesions typical of MPS, was IFA positive. Specific fluorescence was virtually exclusively in bronchi and bronchioli and not in the alveoli or interstitial tissue of the lungs.

A comparison of results obtained by macroscopic evaluation, microscopic evaluation, culture evaluation and DFA of lungs from pigs in experiment B is presented in Table 1. Although slightly more samples had macroscopic or microscopic lesions of MPS, Chi-square analysis indicated results obtained with the four methods were not different ($P > 0.05$). Direct comparison of culture method with DFA indicated that 3 lobes were culture positive for *M. hyopneumoniae* but FA negative while 2 lobes were culture negative but FA positive.

Table 2 presents a comparison of results obtained with the three immunofluorescence procedures. Analysis indicated that DFA, IFA with counterstain and IFA without counterstain were equally sensitive for detection of *M. hyopneumoniae* antigen ($P > 0.05$). Furthermore, no significant differences were detected among the three techniques in intensity of immunofluorescence ($P > 0.05$).

DISCUSSION

Our detection of *M. hyopneumoniae* in sections of lung primarily on the epithelial surfaces of bronchi and bronchioles is consistent with observations reported by L'Ecuyer & Boulanger

FIG. 1. *Cryostat lung section without counterstain* stained by IFA (notice the thin coating of *M. hyopneumoniae* antigen on bronchial epithelial surface (80 x magnification).

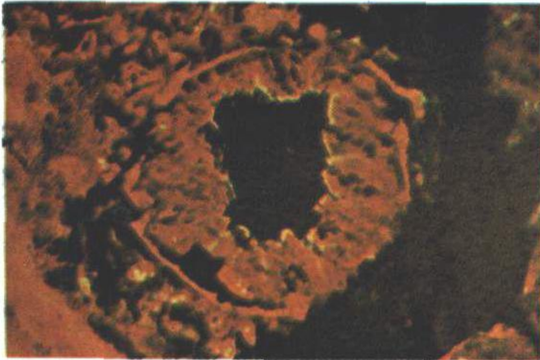

FIG. 2. Cryostat lung section with counterstain, stained by FA (notice the red background provided by the counterstain (80 x magnification).

(1970), Meyling (1971), Livingston et al. (1972), Giger et al. (1977) and Amanfu et al. (1984). The propensity of the mycoplasma to occur primarily in locations with ciliated epithelium is consistent also with the likelihood that it attaches to these cells as do other mycoplasmas such as *Mycoplasma pneumoniae*, a causa of atypical pneumonia in man. Scanning electron microscopy (Mebus & Underdahl 1977) has also provided evidence that *M. hyopneumoniae* attaches primarily to ciliated tracheobronchial epithelial cells in infected swine. The same workers also indicated that they were unable to detect the organism in sections of small bronchioles that were almost devoid of ciliated cells. Livingston et al. (1972) suggested that macrophages may play a role in the clearence of mycoplasmas from the alveoli.

Since no differences were observed between the immunofluorescence techniques utilized, it appears that IFA may be more suitable for less-equipped laboratories because the fluorescein-labelled globulin can be purchased from commercial sources. In addition, such antiserum can often be used at higher dilutions than is possible with a direct antibody conjugate.

Results obtained by means of DFA and IFA tests correspondend closely to those obtained by culture and by macroscopic and microscopic evaluation of the tissues. These results are in agreement with L'Ecuyer & Boulanger (1970), Livingston et al. (1972), Giger et al. (1977) and Amanfu et al. (1984). In spite of this, a few lobes that were culture positive were FA negative while in 2 instances the reverse occured. Others have reported similar discrepancies (Livingston et al. 1972, Gois et al. 1975 and Amanfu et al. 1984). It may be that lower numbers of *M. hyopneumoniae* can be detected better by culture procedure than by FA.

Surely with improvements in the culture medium, this will become even more apparent, especially in the chronic stages of the disease when FA test results tend do be negative (Amanfu et al. 1984). It seems advisable that several techniques should be used for most efficient diagnosis of MPS. For best utilization of FA and culture, lesions could be examined using an FA test; then, if negative, culture technique could be used.

The azo-dye counterstain reduced nonspecific fluorescence and provided a better contrast with no evidence of reduced sensivity. Also, as illustrated in table 2, it was not necessary to use the counterstain. The necessity of using a counterstain could in part depend on the potency of antiserum used. Potent antisera can be used in higher dilutions thus reducing the problem of nonspecific fluorescence. Counterstain does decrease the ambiguity in interpreting weak positive reactions, a factor of importance especially when less experienced personnel are asked to make a determination.

TABLE 1. Comparison between *M. hyopneumoniae* isolation, DFA, MPS - macroscopic lesions and MPS - microscopic lesions from contact exposed pigs.

Number of lobes evaluated	Number of lobes culture-positive for *M. hyopneumoniae*	Number of lobes DFA positive	Number of lobes with macroscopic lesions	Number of lobes with microscopic lesions
24	16	15	17	19

TABLE 2. Comparison of DFA and IFA procedures for detection of *M. hyopneumoniae* antigen in lungs of contact-exposed pigs.

Immunofluorescence procedures	No. of FA-positive lobes	Number of positive lobes in grades of categories of fluorescence[a]			
		1	2	3	4
Experiment A (18 pigs)					
DFA with counterstain	15	2	3	9	1
IFA without counterstain	15	3	4	4	4
IFA with counterstain	14	1	7	5	1
Numbers of lobes examined	29				
Experiment B (12 pigs)					
DFA with counterstain	15	2	0	9	4
IFA without counterstain	16	0	4	8	4
IFA with counterstain	16	2	5	6	3
Number of lobes examined	24				

[a] Intensity of fluorescence was graded 1 = least intense to 4 = most intense.

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