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A Solid-Phase Enzyme Immunoassay Using Guinea Pig C3 to Detect Anti-*Mycoplasma pulmonis* Antibody in the Sera of Infected Rats

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A solid-phase enzyme immunoassay to detect antibodies by the measurement of guinea pig C3 bound to antigen–antibody complex has been developed. Briefly, antibodies in heat-inactivated sera are reacted with antigens coated on the surface of microtiter plates. Guinea pig C3 is bound to the antigen–antibody complexes, and then the amount of C3 is measured using horseradish peroxidase-labeled anti-C3.

When this enzyme immunoassay was applied to the diagnosis of *Mycoplasma pulmonis* infection in rats, high sensitivity and specificity were observed: 95% or more of 20 sera from *M. pulmonis*-infected rats confirmed by cultivation were positive regardless of the antigen strains used, while no positive reaction was detected in 20 normal rat sera. In the complement fixation test, only 12 were positive out of the above-mentioned 20 positive sera, despite the use of the solid-phase antigen which gave the maximal binding.

The present assay is simple, highly sensitive and specific, and is considered to be a useful tool for the diagnosis of *M. pulmonis* infection in rats.

Keywords——enzyme immunoassay; *Mycoplasma pulmonis*; antibody titer; rat

Introduction

Mycoplasma pulmonis (Mp) is an important causative microorganism of chronic respiratory disease and otitis media in rats. For diagnosis of the infection, isolation of this organism from the affected tissue is commonly used. This test is highly reliable since the organism can be detected throughout the infection and even at the early stage of the infection when the specific antibody has not yet been elicited.[1] The test is also time-consuming and requires complicated selective media. A complement fixation test to detect antibodies in the sera has been developed to overcome these defects, but the test does not show sufficient sensitivity and specificity for diagnosis of this infection.[2]

Enzyme immunoassay (EIA) is now widely used for the diagnosis of various diseases because of its high sensitivity and specificity and absence of any requirement for special facilities (*e.g.* radioactive materials in radioimmunoassay). In conventional EIA, direct detection of specific antibodies using enzyme-conjugated anti-immunoglobulin (anti-Ig) has been mainly used.

In this paper, we introduce a new solid-phase enzyme immunoassay to detect anti-Mp antibody indirectly by the measurement of guinea pig C3 (GP-C3) combined with antigen–antibody complexes using enzyme-labeled anti-GP-C3.

Materials and Methods

Antigen Strains of Mp——Strains M-3, M-4 and M-5 originating from mice and R-1, R-2 and R-4 from rats were kindly provided by Dr. M. Nakagawa, Department of Veterinary Sciences, National Institute of Health, Japan. Strain M-53, originating from a mouse, was also used. These strains were cultured in Chanock's[3] PPLO broth and subjected to ultrasonication, and the supernatants (20000 *g*, 30 min) of the ultrasonicated organisms were used as antigens. The protein concentration of the antigens was measured by the method of Lowry *et al.*[4]

Coating of Mp Antigens on the Microtiter Plate——Two hundred microliters of 2 μg/ml antigens in 0.1 M $Na_2CO_3/NaHCO_3$, pH 9.8, was added to a 96-well EIA plate (Coster, Cambridge, MA). The wells were allowed to stand for 120 min, washed three times with washing buffer (0.1 M Tris–HCl, pH 7.6, containing 0.85% NaCl), and then filled with 250 μl of 0.1% bovine serum in 1.0 M Tris–HCl, pH 7.6, for 60 min to coat the active sites left on the wells.

Procedure of EIA——The diluted sera taken from Mp-isolated rats and from non-infected normal control rats [1 : 40 in 0.1 M Tris–HCl, pH 7.6, containing 0.01 M ethylenediaminetetraacetic acid disodium salt and 1% bovine serum albumin (BSA)] were added to the wells. The wells were incubated for 120 min at 4 °C and then for 60 min at room temperature. The wells were washed three times with 250 μl of 0.005 M veronal buffer containing 0.005 M $MgCl_2$ and 0.002 M $CaCl_2$, pH 7.4, and 200 μl of appropriately diluted guinea pig serum as a source of C3 was added to the wells. After incubation for 180 min at 4 °C, the wells were washed twice with veronal buffer. Peroxidase-conjugated IgG fraction of goat anti-Gp-C3 (Capell, U.S.A.) was diluted 200 times with veronal buffer containing 1% BSA, and 200 μl of the above solution was added to each well. After incubation for 60 min at 4 °C, the wells were washed three times with the washing buffer, and 200 μl of substrate solution was added to each well. The substrate solution included 0.01 M *o*-phenylenediamine–HCl in 0.1 M phosphate buffer, pH 6.3, supplemented with 0.012% H_2O_2 shortly before use. After incubation of the plate for 10 min at room temperature, 50 μl of 4 N H_2SO_4 was added to terminate the reaction. The mixture was kept for 10 min at room temperature, and the optical density at 492 nm was measured with a microplate photometer (Corona Electric Co., Tokyo, Japan). The specific optical densities were obtained after subtracting optical densities in non-antigen-coated wells from those in antigen-coated wells, and these values were used as the antigen titer.

Rat Sera——A total of 40 sera, 20 from rats showing positive for Mp by cultivation (infected rats) and 20 from Mp-free rats confirmed by periodically performed microbiological monitoring including cultivation and serology (normal rats), were subjected to EIA and the complement fixation (CF) test. All of the sera were diluted 10 times with saline and then inactivated by heating for 20 min at 60 °C. A reference Mp-positive serum was prepared from rats experimentally infected with M-53 strain.

Positive Value for Mp Infection——The Mp values exceeding the value of the mean of 20 normal rat sera plus 3 standard deviations were regarded as positive in EIA.

CF Test——The CF test was carried out by a modification of the CDC standard method.[5] Antibody titers of 1 : 10 or higher were regarded as positive.

Cultivation——Swab samples taken from the pharynx and tracheal mucosa were streaked onto Chanock's[3] PPLO agar and cultivated at 37 °C for 7 d according to the textbook procedures.

Results

Solid-Phase Enzyme Immunoassay for Anti-Mp Antibodies

A preliminary experiment of determine the concentration of antigens which gives the maximal absorption on the microplate was performed. A range of up to 100 μg/ml of antigens was examined for this purpose, and 1 μg/ml of antigen was considered to give the maximal absorption on the microplate regardless of the strain difference among antigen preparations (data not shown), so that 2 μg/ml was used in practice in the following experiments.

The reference Mp-positive serum, which was serially 2-fold diluted, was subjected to the assay of anti-Mp antibody. An example of the results using R-4 antigen is shown in Fig. 1. Higher absorbances at 492 nm were observed in antigen-coated wells, while those in noncoated wells were less than 0.05 even in 20-fold diluted sera. This indicates that this EIA measures Mp-antigen-associated antibodies.

Since the present assay was designed to detect antibodies by measurement of the amount of Gp-C3 combined with antigen–antibody complexes on the microplate surface, the optical densities in the absence of Gp serum containing C3 were measured. The optical densities in Gp-C3 non-added wells were only 5—8% of those in Gp-C3-added wells (Fig. 2). Thus, this

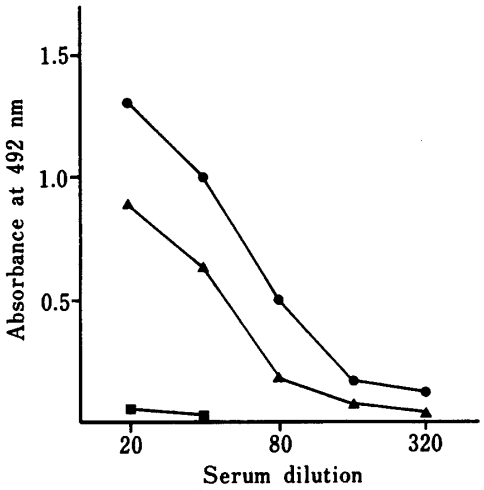

Fig. 1. Standard Curve for EIA of Gp-C3 to Detect Anti-Mp Antibodies

Serial 2-fold dilutions of the reference serum were assayed using R-4 antigen (●, 2 μg/ml; ▼, 0.5 μg/ml; ■, saline) coated on the microplate.

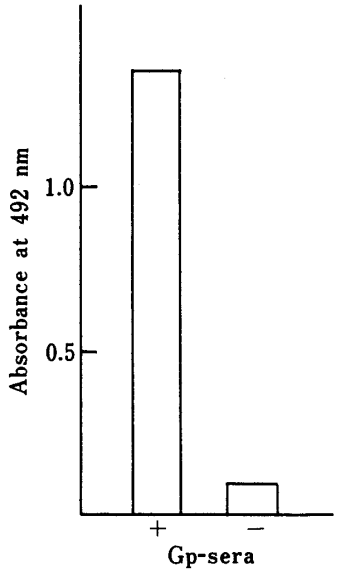

Fig. 2. Specificity of EIA for Gp-C3

EIA using the reference Mp-positive sera was performed in the presence or absence of Gp sera.

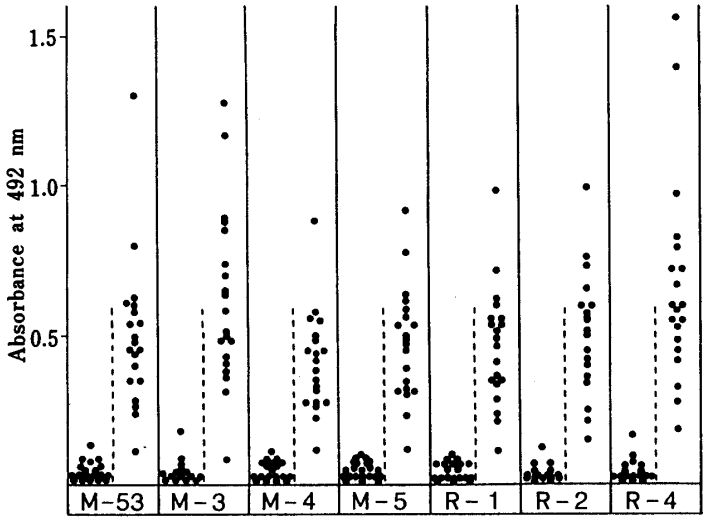

Fig. 3. Results of the EIA Using Seven Solid-Phase Antigens

Twenty sera from Mp-positive rats defined by the cultivation test and twenty normal control sera were subjected to the EIA. In the above columns, the left and right sides indicate the negative and positive rat sera in the cultivation test, respectively.

assay directly measures the combined Gp-C3, but not antigens, antibodies and the complexes themselves. In the present study, rat C3 was completely inactivated by preliminary heating.

Twenty sera from infected rats and 20 from normal rats were subjected to EIA. As shown in Fig. 3, the contents of Mp-antibody in infected rat sera were remarkably higher than those in normal sera although the intensity of absorbance was not consistent among different strains of the antigen.

Comparison between the EIA and CF Test

Antibodies against Mp in each of 20 infected and normal rat sera using 7 antigens were measured by the EIA and CF test. The results are shown in Table I. At least 19 infected rats were positive with all of the 7 antigen strains and no antibodies were detected in 20 normal sera in EIA. In the CF test, only 12 out of 20 positive sera showed positive against M-3 while no antibodies were detected using M-5 and R-4 antigens. These results indicate that EIA is much more sensitive than the CF test.

TABLE I. The Results of EIA and CF Tests in Twenty Mp-Positive Sera Using Seven Mp-Antigens

Antigen	Cultivation test Mp-positive sera 20	
	CF test	EIA
M-53	6	19
M-3	12	19
M-4	4	20
M-5	0	19
R-1	5	20
R-2	7	20
R-4	0	20

Twenty sera from rats defined as Mp-positive by the cultivation test were subjected to these assays.

Although the diagnosis of Mp infection has mainly depended on the cultivation of Mp from the respiratory tract and on the CF test for serum antibody against Mp, these tests seem to have some defects. The cultivation method has the advantage of affording a high detection rate, but it takes 6 to 10 d to evaluate the results, namely, it is time-consuming. The CF test has the advantage of giving the results in a relatively short time, but its sensitivity and specificity are not always satisfactory.[2,6] Recently, enzyme-linked immunosorbent assay (ELISA) has been developed, and the detection rate obtained by ELISA is reported to accord with that by the cultivation test. However, the values measured by ELISA do not always show a good correlation with those by the CF test.[2,7]

Thus, the above conventional tests were not necessarily satisfactory, and hence we undertook the elaboration of a new method, which would exhibit a good accordance with the detection rate by the cultivation test and a good correlation with the values by the CF test.

The solid-phase enzyme immunoassay introduced in the present report is different from the conventional enzyme immunoassay in which specific antibodies reacted with antigens are directly detected. The present assay was designed to detect specific antibodies by measuring C3 newly bound to antibody–antigen complexes on the microplate using enzyme-labeled anti-Gp-C3. The principle is essentially similar to the complement fixation test in which antibodies were indirectly detected by measuring the degree of hemolysis of antierythrocyte-coated erythrocytes by binding to Gp-C3. Therefore, the present assay is expected to be effective in the diagnosis of some diseases for which the CF test is routinely used.

Indeed, when this EIA was performed for the diagnosis of *M. pulmonis* infection in rats, high sensitivity and specificity were observed (Fig. 3). The present assay was more sensitive and specific than the CF test (Table I). The higher sensitivity of our method may be due to direct detection of C3-combined antigen–antibody complexes using enzyme-labeled anti-C3, whereas indirect detection is performed in the CF test.

In conclusion, the present solid-phase enzyme immunoassay in which antibodies were detected by measuring C3 bound to antigen–antibody complex can be applied as a sensitive and specific serological procedure for the diagnosis and seromonitoring of Mp infection in rats. In comparison with EIA, which does not use the complement fixation test, the present method has the advantage of detecting antibodies of IgM and IgG classes simultaneously, and hence this method is expected to be useful for monitoring Mp infection.

References and Notes

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