Comparison of the Enzyme-linked Immunosorbent Assay and the Indirect Hemagglutination and Complement Fixation Tests for Detecting Antibodies to *Mycoplasma hyopneumoniae*

C.H. Armstrong, M.J. Freeman, L. Sands-Freeman, M. Lopez-Osuna, T. Young and L.J. Runnels*

ABSTRACT

Caesarean-derived, colostrum-deprived swine were exposed to a broth culture of a low passage field isolate of *Mycoplasma hyopneumoniae* by intranasal inoculation. The intranasal-inoculated swine subsequently were commingled with their litter-mates to effect transmission via contact-exposure. Sera were collected from the swine at two to four week intervals for approximately one year postexposure and evaluated by the enzyme-linked immunosorbent assay (ELISA), indirect hemagglutination and complement fixation tests.

The intranasal-exposed swine seroconverted earlier, developed higher titers and remained indirect hemagglutination and complement fixation positive longer than the contact-exposed swine. It was concluded that the antibody response of intranasal-exposed swine was artificially high and that sera from such swine were not suitable for evaluating the sensitivity of mycoplasmal pneumonia of swine serodiagnostic tests.

The indirect hemagglutination test was relatively insensitive and technically cumbersome and the least promising as a practical field test. The complement fixation test appeared to be slightly more sensitive in detecting early antibody production (especially in contact-exposed swine) but it was the least sensitive in detecting late antibodies. The ELISA was generally the most sensitive procedure. Individual high ELISA titers were from ten to 32 times greater than maximum complement fixation and indirect hemagglutination titers. The most striking difference among the three tests was the persistence of high ELISA titers late in the study. All swine were ELISA positive at necropsy approximately one year postexposure despite the fact that lungs were devoid of lesions and culturally and immunofluorescent negative for *M. hyopneumoniae.*

Key words: *Mycoplasma hyopneumoniae (suipneumoniae)*, mycoplasmal pneumonia of swine, enzootic pig pneumonia, enzyme-linked immunosorbent assay (ELISA), indirect hemagglutination test, complement fixation test.

RÉSUMÉ

Cette expérience portait sur des porcelets obtenus par césarienne et privés de colostrum; elle consistait à leur inoculer, par la voie intranasale, un bouillon de culture d'une souche de *Mycoplasma hyopneumoniae*, isolée d'un cas clinique et soumise à quelques passages. On retourna ensuite ces porcelets dans leurs portées respectives, dans l'espoir de réaliser la transmission horizontale de cette infection. On préleva des échantillons de sérum, à intervalles de deux à quatre semaines, pendant environ un an, et on les soumit aux épreuves suivantes: ELISA, hémagglutination indirecte et déviation du complément.

Les sujets inoculés par la voie intranasale développèrent plus rapidement des anticorps, lesquels atteignirent un titre plus élevé; ces porcs réagirent aussi de façon positive aux épreuves de l'hémagglutination indirecte et de la déviation du complément, plus longtemps que leurs congénères. Les auteurs en vinrent à la conclusion que la réaction immunitaire des porcs inoculés par la voie intranasale s'avérait artificiellement élevée et que leur sérum se prêtait mal à l'évaluation de la sensibilité des épreuves sérologiques destinées à diagnostiquer la pneumonie porcine à mycoplasme.

*Department of Veterinary Microbiology, Pathology and Public Health (Armstrong, Freeman, Sands-Freeman, Lopez-Osuna), Department of Large Animal Clinics (Runnels), School of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47907 and Veterinary Medical Research Institute, Iowa State University, Ames, Iowa 50010 (Young). Present address of Ms. Lopez-Osuna is el Departamento de Investigacion Biomedica, IMSS Division de Immunologia, Apdo. P73-032, Mexico D.F., Mexico 03020.

Supported in part by the USDA/SEA Research Grants Program.

Submitted as Journal Paper No. 9314 Agricultural Experiment Station, Purdue University, West Lafayette, Indiana 47907.

Submitted January 7, 1983.

L'épreuve de l'hémagglutination indirecte se révéla relativement insensible, techniquement fastidieuse et la moins prometteuse comme épreuve pratique de dépistage. Celle de la déviation du complément s'avéra un peu plus sensible pour détecter la réaction immunitaire précoce, surtout chez les sujets témoins; elle se révéla toutefois moins sensible pour déceler les anticorps tardifs. En général, la technique ELISA manifesta le plus de sensibilité; les titres individuels élevés qu'elle détecta se révélèrent en effet de dix à 32 fois plus élevés que les plus hauts obtenus avec les deux autres techniques. La différence la plus frappante entre ces trois épreuves résidait dans la persistance de titres élevés avec la technique ELISA, même vers la fin de l'expérience. Tous les porcs réagirent de façon positive à l'épreuve ELISA lorsqu'on les sacrifia, au bout d'environ un an, en dépit du fait que leurs poumons n'affichaient aucune lésion et n'arboraient pas *M. hyopneumoniae*, comme le démontrèrent les résultats négatifs de la culture et de l'immunofluorescence.

Mots clés: *Mycoplasma hyopneumoniae (suipneumoniae)*, pneumonie porcine à mycoplasme, pneumonie porcine enzootique, ELISA, épreuve de l'hémagglutination indirecte, épreuve de la déviation du complément.

INTRODUCTION

Mycoplasmal pneumonia of swine (MPS) (1) (enzootic pneumonia of pigs) is an economically important disease of swine throughout the world (2). The disease is extremely difficult to control, due in large part to the lack of a reliable antemortem diagnostic test. The indirect hemagglutination (IHA) test (3,4,5) and the complement fixation (CF) test (3, 6, 7, 8, 9, 10, 11) have been used to diagnose MPS. However, there are questions regarding the sensitivity of these procedures. The IHA test, which is adequate for detecting antibodies to *Mycoplasma hyopneumoniae* in swine infected experimentally with large doses of the agent, may not be sensitive enough to detect antibodies in all naturally infected swine. The sensitivity of the CF test is satisfactory during early and mid phases of infection, but some swine become CF negative during the late stages of infection. The enzyme-linked immunosorbent assay (ELISA) is reported to be very sensitive (12). Results of initial studies indicate that the ELISA is a promising serodiagnostic procedure for MPS (13, 14).

The objectives of the present study were to compare the IHA and CF tests and the ELISA as methods of evaluating the onset, magnitude and duration of antibody production in swine infected experimentally with *M. hyopneumoniae*.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Caesarean-derived, colostrum-deprived swine, maintained under isolation conditions, were infected experimentally as follows. Approximately 10^9 color changing units of a cloned low-passage field isolate of *M. hyopneumoniae* (strain P-5722-3) (recovered by the senior author from the lung of a market swine with lesions typical of MPS) was administered intranasally (IN) to each of four, three day old pigs. At 21 days of age the four IN-exposed pigs were commingled with ten litter-mates to effect contact-transmission. One IN-exposed pig was killed 37 days post-exposure (PE) and three contact-exposed pigs were killed from 45 to 77 days PE. The lungs from these swine were examined grossly for lesions typical of MPS and culturally and by an indirect immunofluorescent (IIF) procedure for the presence of *M. hyopneumoniae*.

Sera were collected from the remaining ten swine (three IN-exposed and seven contact-exposed) at two to four week intervals for approximately one year PE and examined by the ELISA, IHA and CF tests. At the end of the study the swine were killed and their lungs were examined grossly, culturally and by the IIF procedure for evidence of *M. hyopneumoniae* infection.

CULTURAL AND IMMUNOFLUORESCENT EXAMINATION OF LUNG TISSUE

Cultural examination for *M. hyopneumoniae* was conducted as described (15,16,17). The IIF examination was performed as follows. Blocks of lung tissue, approximately 1 cm^3, were collected from the right and left cardiac and apical lobes. When lesions typical of MPS were present, samples were selected from the advancing edge of the lesion. In the absence of lesions, tissues were collected from the ventral margin of each lobe. The tissue blocks were oriented so that the airways would be cut transversely, mounted on an object holder in O.C.T. compound (Lab-Tech Products, Division of Miles Laboratory, Inc., Naperville, Illinois) and sectioned with a freezing microtome. Two serial sections were cut from each tissue block and mounted side by side on a microscope slide. The sections were air dried and fixed in acetone for ten minutes. One section was reacted with a 1:80 dilution of *M. hyopneumoniae* antiserum (prepared in rabbits (15)) and one section was reacted with a 1:10 dilution of normal rabbit serum. The preparations were incubated in a moist chamber at room temperature for 30 minutes, rinsed in 0.01 M phosphate buffered saline, pH 7.4 (PBS), washed in PBS for ten minutes and dried. A 1:80 dilution of goat antirabbit serum conjugated with fluorescein isothiocyanate (Antibodies Incorporated, P.O. Box 442, Davis, California) was applied to the lung sections. The sections were incubated for 30 minutes at room temperature, rinsed, washed in PBS, mounted in buffered glycerin (1 part PBS:9 parts glycerin) and examined with an incident light fluorescence microscope (Ploemopak 2 Dialux, Filter Module H, E. Leitz, Inc., 24

Link Drive, Rockleigh, New Jersey). Two sections of lung tissue known to contain *M. hyopneumoniae* were mounted on a second slide, stained in the same manner and used as additional controls. The presence of fluorescing elements on the epithelial surface of the bronchi and bronchioles was regarded as a positive reaction (Fig. 1).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Mycoplasma hyopneumoniae antigen and antiporcine IgG conjugate were prepared and titrated as described by Armstrong *et al* (18). The ELISA procedure was performed as described (18) except a semi-automated processor-analyzer (PR-50 Processor-Analyzer, Gilford Instruments, Inc., Oberlin, Ohio) was used. Antigen, twofold-dilutions of sera, conjugate and substrate were used in 250 μL volumes. A seven cycle wash in PBS was used between reaction with each reagent.

Reagent controls for the test consisted of multiple wells reacted with: 1) substrate alone, 2) antigen plus substrate, 3) conjugate plus substrate, 4) antigen plus conjugate plus substrate and 5) the lowest dilution of serum tested plus conjugate plus substrate. A standard positive and negative control serum (16) were also included with each assay. The maximum optical density (O.D.) obtained for reagent controls (usually serum plus conjugate plus substrate) was subtracted from the gross O.D. (O.D. at 30 min minus O.D. at 0 min) of each test serum to obtain the net O.D. A net O.D. $\geqslant$ 0.200 was considered a positive reaction since this value was approximately two standard deviations greater than the mean value of all serum controls. Nonlinearity became marked at an O.D. lower than 0.200.

INDIRECT HEMAGGLUTINATION (IHA) TEST

A modification of the procedures described by Lam and Switzer (4) and by Holmgren (19) was used. Sheep red blood cells (SRBC) were collected in Alsever's solution, washed in 0.15 M PBS, pH 7.2 and fixed with glutaraldehyde (20). Ten mL of a 4% suspension of the glutaraldehyde fixed cells were treated with an equal volume of a 1:2000 solution of freshly prepared tannic acid (in 0.85% NaCl solution) for exactly ten minutes at 37°C. The tanned cells were washed in PBS, resuspended in 10 mL of PBS containing 100 μg/mL of the SDS-extracted *M. hyopneumoniae* antigen (the same antigen used in the ELISA) and incubated for 30 minutes at 37°C. Sensitized SRBC were washed twice in PBS and suspended to a 1% concentration in PBS containing 1% normal rabbit serum that had been heat inactivated and absorbed with SRBC (21). Sensitized cells were stored at 4°C and used until their reactions with standard negative and positive control sera became erratic (standard control sera were the same as those used in the ELISA).

Doubling dilutions of sera were tested in 25 μL volumes in a microtiter system using an equal volume of a 1% suspension of sensitized SRBC. Standard positive and negative control sera, diluent controls and tests with tanned, nonsensitized SRBC were included with each titration. Settling patterns were read about two hours after the addition of SRBC, depending on the settling patterns in the wells containing the standard positive and negative control sera. Endpoints were designated as the reciprocal of the highest dilution of serum that gave a classic 2+ reaction (21).

COMPLEMENT FIXATION (CF) TEST

The J strain of *M. hyopneumoniae* was grown in a modified Eagle's medium with 25% acid-treated swine serum (22). Cells were harvested by centrifugation, washed twice in veronal buffered diluent (VBD) (23), heated for 30 minutes at 50-52°C and stored at -70°C (up to several months) until used as antigen. The microtiter CF test was used as described by Casey (23) except that complement was diluted in whole unheated normal swine serum rather than in VBD (10). Titers were recorded as the reciprocal of the highest dilution of serum resulting in 30% or less hemolysis.

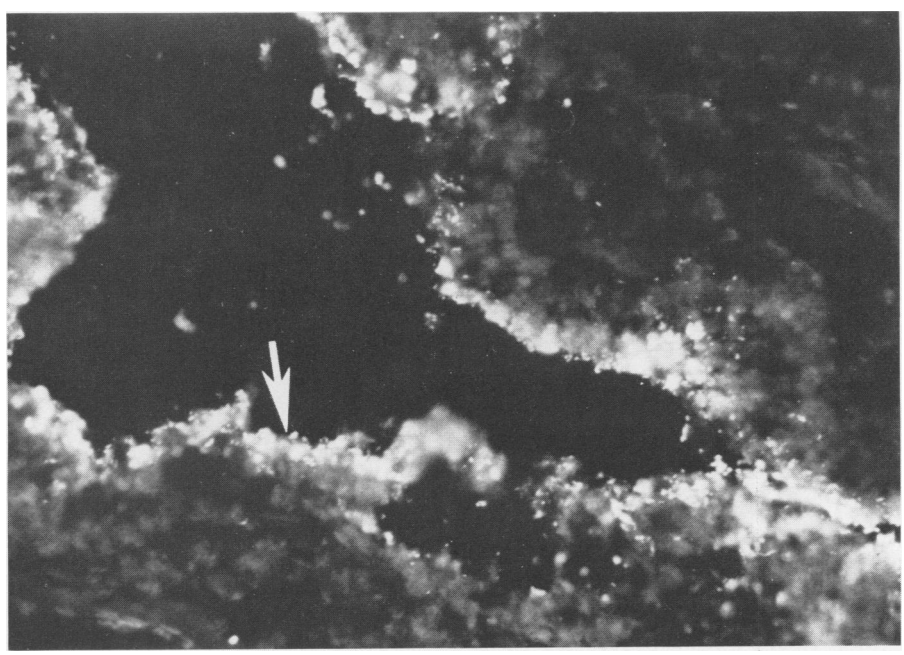

Fig. 1. An immunofluorescent positive bronchiole. The bright particles on the epithelial surface (arrow) are *M. hyopneumoniae* cells.

RESULTS

EXPERIMENTAL TRANSMISSION OF *M. HYOPNEUMONIAE*

One IN-exposed and three

contact-exposed swine were subjected to necropsy 37 to 77 days PE to confirm that *M. hyopneumoniae* infection had been established. Results of examining sera and lungs from these swine are summarized in Table I. The results indicated that infection was readily established by both IN and contact-exposure.

It was assumed that the ten swine that were retained for serological studies also became infected with *M. hyopneumoniae*. The serological data support such an assumption. However, the lungs were devoid of gross lesions and they were IIF and culturally negative when these swine were killed approximately 48 to 52 weeks PE.

SEROLOGY

Sera were collected from three IN and seven contact-exposed swine at two to four week intervals from two weeks to approximately one year PE and examined by the ELISA, IHA and CF tests. ELISA titers ≥80, IHA titers ≥20 and CF titers ≥8 were considered positive.

Response of IN-Exposed Swine — Geometric mean titers (GMT) obtained by the three serological procedures are summarized in Fig. 2. Antibodies were first detected at four weeks PE when one of the three IN-exposed pigs was positive by all three tests. At six weeks PE, all three swine were CF positive and two of three were ELISA and IHA positive. All swine were seropositive to all tests eight weeks PE. Complement fixation titers peaked at eight weeks and began to decline at 12 weeks. One swine was CF negative at 27 weeks, two were CF negative at 36 weeks and all were CF negative at 46 weeks PE. Indirect hemagglutination titers peaked at eight and ten weeks PE and declined thereafter. One swine became IHA negative at 19 weeks and two were IHA negative at 36 weeks. The third pig remained IHA positive at low titer for the duration of the study. Enzyme-linked immunosorbent assay titers peaked at 19 weeks, declined slightly thereafter but remained at least eightfold greater than the minimal positive titer for the duration of the investigation.

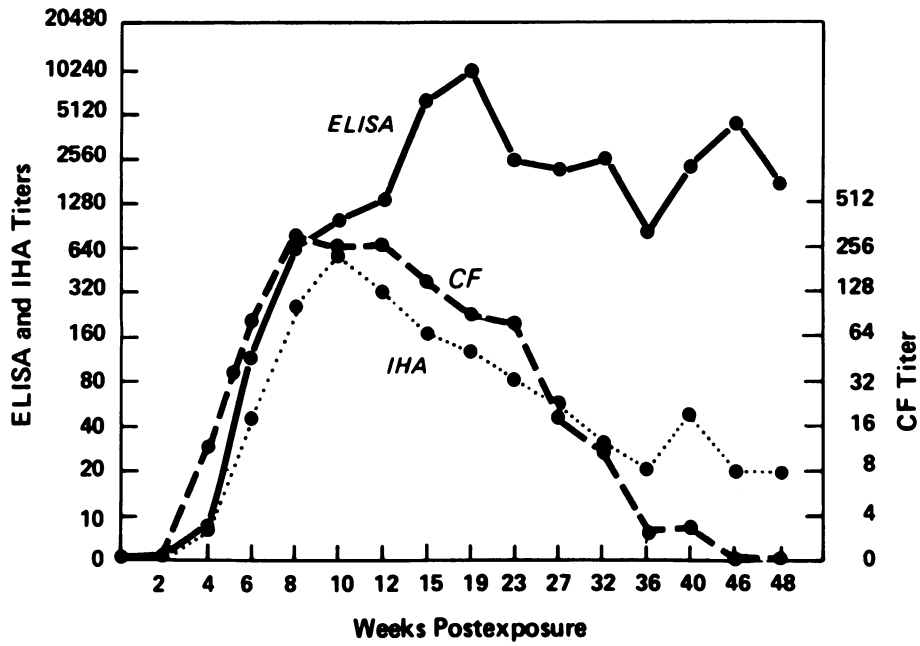

Fig. 2. Comparison of geometric mean ELISA, IHA and CF titers of swine infected with *M. hyopneumoniae* by intranasal inoculation.

Response of Contact-Exposed Swine — The GMT obtained by the ELISA, IHA and CF tests of sera from contact-exposed swine are summarized in Fig. 3. Antibody was first detected at five weeks when one swine was CF positive. At seven weeks, two were ELISA positive, four were IHA positive and six were CF positive. At nine weeks PE all swine were seropositive by all three tests. Maximum CF GMT were observed at nine weeks and individual peak CF titers occurred at 13 and 17 weeks. The CF titers were declining in most swine at 17 weeks PE. Two swine were CF negative at 21 weeks, six were CF negative at 26 weeks and all were seronegative at 30 weeks. Indirect hemagglutination titers peaked at nine weeks and began to decline 11 weeks PE. One swine was IHA negative at 17 weeks and six were negative at 21 weeks. However, some of the latter swine were alternatively seropositive and seronegative on successive bleedings. Two swine were still

TABLE I. Results of Examining Sera and Lungs from Swine Exposed to *M. hyopneumoniae*

Pig Number	Route of Exposure	Postexposure Day Killed	Titer[a] at Necropsy: ELISA	IHA	CF	Gross Lesions	Immunofluorescent Examination	Cultural Examination
15	intranasal	37	160	160	256	11.1%[b]	27.0%[c]	10^{8}[d]
11	contact	45	<80	20	16	24.3%	37.2%	10^{7}
13	contact	45	<80	<20	64	12.2%	29.8%	10^{8}
5	contact	77	80	40	32	16.4%	20.2%	10^{6}

[a]ELISA titer ≥ 80, IHA titer ≥ 20 and CF titer ≥ 8 regarded as positive
[b]Percentage of lung surface occupied by typical MPS lesions
[c]Total number of immunofluorescent positive airways in the four anterior lobes × 100/total number of low power fields examined
[d]Number of color changing units of *M. hyopneumoniae* recovered per gram of lung tissue

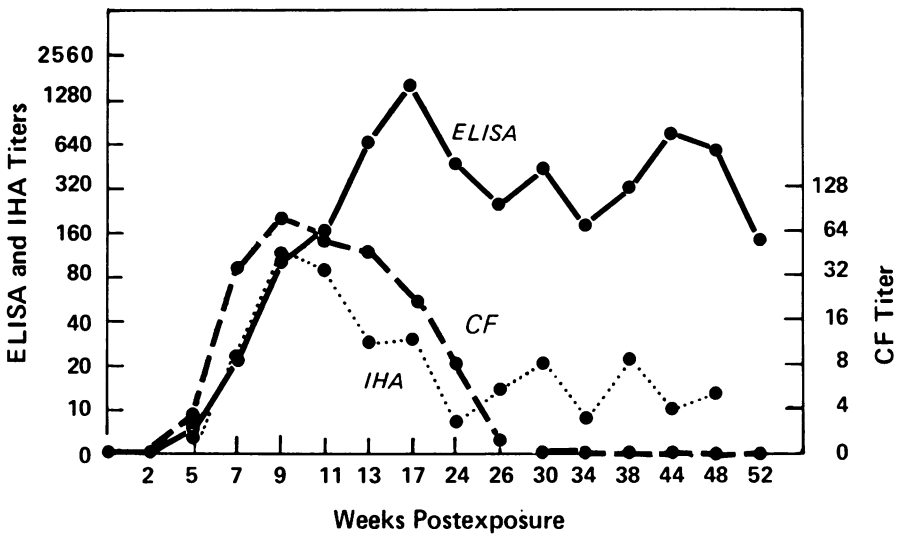

Fig. 3. Comparison of geometric mean ELISA, IHA and CF titers of swine infected with *M. hyopneumoniae* by contact-exposure.

IHA positive 48 weeks PE. Maximum ELISA titers occurred 17 weeks PE. The ELISA titers began to decline at 21 weeks; however, all swine remained ELISA positive for the duration of the study with the exception that one swine was seronegative at one bleeding.

Comparative Antibody Response of IN and Contact-Exposed Swine — Swine infected by IN-inoculation developed significantly higher antibody titers than those infected by contact-exposure (Table II). This difference in response occurred in all three tests.

DISCUSSION

The ELISA, as expected, (12) was substantially more sensitive than either the CF or the IHA test, e.g. among IN-exposed swine the peak ELISA titer was 40,960 whereas maximum CF and IHA titers were 4096 and 1280 respectively. A striking feature of the ELISA was the persistence of high titers late in the course of the investigation, i.e. significant ELISA titers existed at necropsy when all swine were CF negative and two of three were IHA negative. Despite the fact that the IN-exposed swine were all ELISA positive at the time of necropsy, the lungs from these swine were devoid of gross lesions and *M. hyopneumoniae* was not recovered culturally nor visualized by IIF examination. The persistence of ELISA titers may have been due to the continued presence of low (nonculturable) levels of *M. hyopneumoniae* or to the continued presence of *M. hyopneumoniae* antigen or the titers may reflect the sensitivity of the assay in detecting low levels of antibodies.

A comparison of the antibody response of IN-exposed and contact-exposed swine revealed some significant differences. The IN-exposed swine seroconverted earlier and remained seropositive longer. The most marked difference was in the magnitude of the antibody response. Intranasally-exposed swine had fourfold greater IHA titers, eightfold greater ELISA titers and as much as sixteenfold greater CF titers than the contact-exposed swine. These results suggest that the antibody response of IN-exposed swine is artificially high and that sera from such swine are not suitable for evaluating the sensitivity of MPS serodiagnostic procedures.

Swine infected with *M. hyopneumoniae* via contact-exposure probably respond immunologically like naturally-infected pigs and therefore represent a useful model for developing and evaluating serodiagnostic procedures. Contact-exposure is a reliable and reproducible method of transmitting *M. hyopneumoniae*. We have used this technique extensively and have never failed to transmit the agent, i.e. every contact-exposed CD, CD pig killed within 90 days PE has had typical MPS lesion and the lungs of all have been immunofluorescent and culturally positive for *M. hyopneumoniae* (unpublished data, Armstrong *et al* 1981, 1982). Others also have been successful in transmitting MPS via contact-exposure (10, 24, 25, 26).

The ultimate aim of our studies is to develop a reliable immunodiagnostic test for MPS. Information generated during the present study indicated that the IHA test has serious limitations as a practical test. It was technically cumbersome in that preparation of uniformly sensitized SRBC was sometimes difficult and evaluation of endpoints was very subjective due to irregular settling patterns. The IHA test was also relatively insensitive for detecting antibodies in contact-exposed swine.

The CF test is a simple and inexpensive procedure that readily lends itself to large-scale testing. However, results of the present study indicate that swine may become CF negative during the latter period of *M. hyopneumoniae* infection. Data from other studies in our laboratories support this conclusion. One thousand swine (from ten herds) were tested for *M.*

TABLE II. Peak Antibody Titers Obtained in Intranasal (IN) and Contact-exposed Swine as Determined by the ELISA, IHA and CF Tests

Serological Procedure	Peak Individual Titer		Peak Geometric Mean Titer	
	IN-Exposed	Contact-Exposed	IN-Exposed	Contact-Exposed
ELISA	40,960 (19)[a]	5,170 (17)	10,200 (20)	1,413 (17)
IHA Test	1,280 (8 & 10)	320 (9 & 10)	508 (10)	119 (9)
CF Test	4,096 (8 & 10)	256 (13 & 17)	323 (8)	81 (9)

[a]Figures in parenthesis indicate the week postexposure when peak titers occurred

hyopneumoniae antibodies by the ELISA, IHA and CF tests. Selected seropositive swine were killed about one month after the initial test. Sera were collected at necropsy and retested and lungs were examined culturally. Some swine that had been CF positive in the first test were CF negative but culturally positive for *M. hyopneumoniae* at necropsy (unpublished data, Armstrong *et al* 1982).

The ELISA is potentially very attractive as a practical serodiagnostic test for MPS. The present study indicates that it is extremely sensitive for detecting antibodies to *M. hyopneumoniae*. Furthermore, it lends itself to automation and thus would be economical for herd testing. However, the assay is not entirely specific in detecting antibodies to *M. hyopneumoniae* (16, 27). Results of recent studies indicate that the apparent lack of specificity is due to antigenic relatedness among the porcine mycoplasmas, especially between *M. hyopneumoniae* and *M. flocculare* (28, 29). It seems likely that the ELISA could be rendered specific if cross-reactive antibodies could be removed from test sera or if cross-reactive determinants could be removed from the test antigen.

ACKNOWLEDGMENTS

The authors thank Dr. R.F. Ross for permitting the CF tests to be performed in his laboratory and acknowledge the excellent technical support provided by Ms. Janet Bess.

REFERENCES

1. **MARÉ CJ, SWITZER WP.** New species: *Mycoplasma hyopneumoniae*. A causative agent of virus pig pneumoniae. Vet Med 1965; 67: 841-846.
2. **SWITZER WP, ROSS RF.** Mycoplasmal diseases. In: Dunne HW, Leman AD, eds. Diseases of swine. Ames, Iowa: Iowa State University Press, 1975.
3. **GOODWIN RFW, HODGSON RG, WHITTLESTONE P, WOODHAMS RL.** Immunity in experimentally induced enzootic pneumonia of pigs. J Hyg Camb 1969; 67: 193-207.
4. **LAM KM, SWITZER WP.** Mycoplasmal pneumonia of swine: Development of an indirect hemagglutination test. Am J Vet Res 1971; 32: 1731-1736.
5. **HOLMGREN N.** On the immune response in porcine serum and tracheobronchial secretions following experimental infection with *Mycoplasma hyopneumoniae*. Zentralbl Veterinaermed 1974; 21: 188-201.
6. **BOULANGER P, L'ECUYER C.** Enzootic pneumonia of pigs: Complement-fixation tests for the detection of mycoplasma antibodies in the serum of immunized rabbits and infected swine. Can J Comp Med 1968; 32: 547-554.
7. **TAKATORI I, HUHN RG, SWITZER WP.** Demonstration of complement-fixation antibody against *Mycoplasma hyopneumoniae* in the sera of pigs infected with swine enzootic pneumonia. Natl Inst Anim Health Q (Japan) 1968; 8: 195-203.
8. **ROBERTS DH.** Serological diagnosis of *Mycoplasma hyopneumoniae* infection in pigs. Vet Rec 1968; 82: 362-363.
9. **HODGES RT, BETTS AO.** Complement-fixation tests in the diagnosis of enzootic pneumonia of pigs. I. Experimental studies. Vet Rec 1969; 85: 452-454.
10. **SLAVIK MF, SWITZER WP.** Development of a microtitration complement-fixation test for diagnosis of mycoplasmal swine pneumonia. Iowa St J Res 1972; 47: 117-128.
11. **SLAVIK MF, SWITZER WP.** Comparison of a microtitration complement-fixation test and a tube latex agglutination test for diagnosis of *Mycoplasma hyopneumoniae* swine pneumonia. Am J Vet Res 1981; 42: 862-864.
12. **VOLLER MF, BIDWELL D, BARTLETT A.** Microplate enzyme immunoassay for the immunodiagnosis of virus infections. In: Rose NR, Freedman H, eds. Manual of clinical immunology. Washington: ASM, 1976: 506-512.
13. **BRUGGMANN S, KELLER H, BERTSCHINGER HU, ENGBERG B.** A new method for the demonstration of antibodies against *Mycoplasma suipneumoniae* in pigs sera. Vet Rec 1976; 99: 101.
14. **BRUGGMANN S, KELLER H, BERTSCHINGER HU, ENGBERG B.** Quantitative detection of antibodies to *Mycoplasma suipneumoniae* in pigs' sera by an enzyme-linked immunosorbent assay. Vet Rec 1977; 101: 109-111.
15. **ARMSTRONG CH.** A diagnostically practical method of isolating and identifying *Mycoplasma hyopneumoniae*. Proc 1st Int Symposium Vet Lab Diagnost, Guanajuato, Mexico; 1977: 754-771.
16. **ARMSTRONG CH, FREEMAN MJ, LOPEZ-OSUNA M, RUNNELS LJ, SANDS LL.** Mycoplasmal pneumonia of swine: A comparison of the enzyme-linked immunosorbent assay, indirect hemagglutination, indirect immunofluorescence and complement fixation as immunodiagnostic tools. Proc Int Pig Vet Soc Congress, Copenhagen; 1980: 224.
17. **ARMSTRONG CH, FRIIS NF.** Isolation of *Mycoplasma flocculare* from swine in the United States. Am J Vet Res 1981; 42: 1030-1032.
18. **ARMSTRONG CH, FREEMAN MJ, SANDS LL, FARRINGTON DO.** The enzyme-linked immunosorbent assay (ELISA) for diagnosing mycoplasmal pneumonia of swine. Proc Am Assoc Vet Lab Diagnost, Buffalo; 1978: 377-389.
19. **HOLMGREN N.** An indirect hemagglutination test for detection of antibodies against *Mycoplasma hyopneumoniae* using formalinized tanned swine erythrocytes. Res Vet Sci 1974; 16: 341-346.
20. **BING DH, WEYAND JGM, STAVITSKY AB.** Hemagglutination with aldehyde fixed erythrocytes for assay of antigens and antibodies. Proc Soc Exp Biol Med 1967; 124: 1166-1170.
21. **STAVITSKY AB.** Micromethods for the study of proteins and antibodies. I. Procedure and general applications of hemagglutination and hemagglutination inhibition reactions with tannic acid and protein treated red blood cells: J Immunol 1954; 72: 360-367.
22. **SWITZER WP.** Mycoplasmal pneumonia of swine. J Am Vet Med Assoc 1972; 160: 651-653.
23. **CASEY HL.** Standardized complement fixation method and adaptation to microtest. Public Health Monograph 74. Washington: US Dept Health Ed Welfare, 1965.
24. **GOODWIN RFW, WHITTLESTONE P.** The comparative susceptibility of hysterectomy-produced, colostrum-deprived pigs and naturally born, enzootic pneumonia-free pigs to enzootic pneumonia. J Hyg Camb 1971; 69: 391-397.
25. **LAM KM, SWITZER WP.** Mycoplasmal pneumonia of swine: Serologic response in pigs. Am J Vet Res 1972; 33: 1329-1332.
26. **FARRINGTON DO.** Immunization of swine against mycoplasmal pneumonia. Proc Int Pig Vet Soc Congress, Ames, Iowa; 1976: PP4.
27. **NICOLET J, BRUGGMANN S, PAROZ P.** Serological investigations on enzootic pneumonia with an enzyme-linked immunosorbent assay (ELISA). Proc Int Pig Vet Soc Congress, Copenhagen; 1980: 225.
28. **SANDS LL, FREEMAN MJ, ARMSTRONG CH.** Evaluation of the ELISA for diagnosis of mycoplasmal pneumonia of swine (MPS): I. Cross-reactivity between *M. hyopneumoniae* and the other porcine mycoplasmas to reference antisera. Proc Int Pig Vet Soc Congress, Mexico City; 1982: 91.
29. **FREEMAN MJ, ARMSTRONG CH,**

SANDS LL, RUNNELS LJ, SCHEIDT AB, THACKER HL. Evaluation of the ELISA for diagnosis of mycoplasmal pneumonia of swine (MPS): II. Antigenic relationships between *M. hyopneumoniae* and *M. flocculare* of reference antisera and sera of MPS-affected swine. Proc Int Pig Vet Soc Congress, Mexico City; 1982: 92.