Korean J. Vet. Res., Vol. 23, No. 2 (1983)

Serologic Studies on Porcine Strains of *Haemophilus parahaemolyticus (pleuropneumoniae)*: Antigenic Specificity and Prevalence of Antibodies to Serotypes

Byong-Kyu Kang, D.V.M., M.S., Ph.D.
Department of Veterinary Medicine, College of Agriculture, Chonnam National University
K. Yamamoto, D.V.M., M.S. and M. Ogata, D.V.M., Ph.D.
Department of Veterinary Microbiology, Faculty of Agriculture, University of Tokyo

Introduction

Haemophilus parahaemolyticus (syn. *Haemophilus pleuropneumoniae*)(HP) has been reported to be one of the causative agents of porcine contagious pleuropneumoniae[2,8~10,12,18,19] causing a significant economic loss.[13,16]

Serologic studies of HP have been reported and five serotypes have been isolated.[5,14,15,17] The differentiation of serotypes on isolates or the determination of the prevalence of antibodies in serum samples is essential for preventing the spread of the disease.

Various serological diagnostic methods, such as agglutination,[4,5] indirect hemagglutination,[17] latex agglutination[11] and complement fixation test,[1,14,15,17,20] have been developed for the determination of serotypes and for the confirmation of the etiologic diagnosis, but the establishment of routine serological method or the specificity on the reaction is not fullly investigated. An application of agglutination and complement fixation tests posed certain limitations such as a least sensitivity or a non-specific reaction and a marked procomplementary activity of swine serum.[21]

The purpose in the present study was to clarify the antigenic relationship among the serotypes of HP in the swine infected experimentally and in the serum obtained from swine herds by serological tests. The ultimate objective was to determine the most rational approaches for serological diagnosis.

Materials and Methods

Organisms: Four serotypes of HP strain 4074 (serova 1), 1536 (serova 2), 1421 (serova 3) and 966 (serova 5) were used for serology mainly in preparation of antigen, and strain HP 8(serova 2) was used for the artificial infection in SPF pigs. The origin of all cultures is shown in Table 1.

Table 1. Designation of Cultures and Isolates Used and Their Origin

Designation	Serotype	Disease or organ	Country	Obtained from
4074	1	Pneumonia	Argentina	J. Nicolet, Bern
1536	2	Pleuropneumonia	Switzerland	J. Nicolet, Bern
1421	3	Periarticular and pulmonary abscess	Switzerland	J. Nicolet, Bern
966	5	Pleuropneumonia	Taiwan	C.N. Chan
HP 8	2	Pleuropneumonia	Japan	Fresh isolate

本 論文의 要旨는 1979年度 大韓獸醫學會에서 발표하였음.

Antigens: Each of the 5 serotypes of HP was grown for 18 hours on blood chocholate agar plate medium(basal medium was trypticase soy agar medium, BBL) from a lyophilized state of stock culture. A mucoid colony was selected and subsequent cultivations were done as follows. Primary seed cultures were prepared in trypticase soy broth (BBL) supplemented with 5% yeast extract (Y-TSB) in amount of 2m*l* and by incubating at 37C for 3 to 5 hours. For secondary cultures, full dosis of this primary cultures were inoculated into 1800m*l* of Y-TSB and after incubating at 37 C for 10 to 19 hours, antigen was harvested in 1 : 10,000 merthiolate-phosphate buffer solution (M-PBS). The antigen was washed twice and was resuspended in M-PBS in a concentration of one two hundredth of the original volume of the medium used. The antigen was stored as a stock at 4C untill used.

The optimum dilution of antigen for each serotype was determined by block titration. The density of antigen for agglutination test was adjusted to the same absorbance value to the tube No. 4 of the McFarland nephlometer scale and for complement fixation test was adjusted to 1 : 4 dilution of the agglutination antigen.

Agglutination Test(AG): The procedure for the AG was essentially described by Gunnarson *et al.*[4,5] with a minor modification. M-PBS was used for the diluent and equal amount of antigen was added to 0.5m*l* of serial two-fold dilution of serum. After incubation at 52C for 2 hours in a waterbath and at room temperature through the night left off, reactions were read optically and it was designated as the routine agglutination test, AG(−).

Agglutination with the serum treated by the kaolin(KIO_4), designated as a kaolin-treated agglutination test, AG(+), was also conducted. Equal volumes of 25% kaolin in M-PBS and serum were incubated at room temperature for one hour and then obtained sera after centrifugation at 2,000∼3,000 rpm for 10 minutes was examined by the same procedure of the routine agglutination described previously.

Complement Fixation Test(CF): A procedure adapted to tube and microtiter methods by Mayer[7] was followed with a minor modification. The diluent was the gelatin-veronal buffered solution (GVB^{2+}) and the volume of each reagent was 0.2 m*l* in the tube method and 0.025m*l* in the microtiter method. Lyophilized guinea pig complement was reconstituted with the diluent containing normal calf serum in a concentration of 2.5% to make the exact 2.5 units as described by Jeon *et al.*[6] All factors of reagent concerned on the test were determined by the box titration, especially on the normal calf serum.

Animals and Blood Serum Samples: Blood samples were taken from the 2-week-old SPF pigs (No. 1,2 and 3) before inoculation with the HP serotype 2, strain HP 8 (10^8 organisms) and afterwards at weekly intervals. The uninoculated control SPF pigs were shown to be free of HP infection throughout the experimental period by pathological, bacteriological and serological examinations.

The field sera from Japan (herd A) and Korea (herd B) were also examined. On the herd A, HP infection has been known to be present, based on the isolation of organism from lung lesions and on herd B, blood samples were collected at random from various swine herds and the incidence state of pneumoniae or HP infection was uncertain.

Results

Comaprisons of Antibody Titers Determind by Agglutination and Complement Fixation Test: Sera obtained from the artificially infected SPF pigs(No. 1,2 and 3) which were inoculated with the strain HP 8 (serova 2) were examined by different serological methods, AG(−), AG(+) and CF tests and antibody titers against its homologous serotype of HP strain 1536 (serova 2) are shown in Fig. 1 and 2.

Fig. 1 shows the development of agglutinating antibodies in each of three SPF pigs. Agglutinating antibodies rose rapidly and it was noted that the titer of 1 : 10 was detected in all three pigs

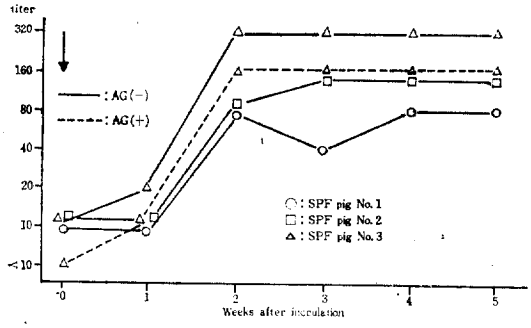

Fig. 1. Agglutinating antibody titers of SPF pigs inoculated with strain HP 8 of *H. pleuropneumoniae*, serotype 2.

AG(−): routine agglutination.
AG(+): agglutination with the serum pretreated with kaolin.

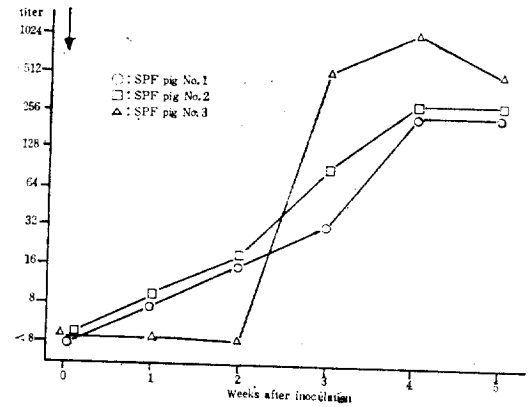

Fig. 2. Complement-fixing antibody titers of SPF pigs inoculated with strain HP 8 of *H. pleuropneumoniae*, serotype 2.

before inoculation. However, this non-specific reaction was eliminated when test was done with the serum pretreated with the kaolin; AG(+).

As shown in Fig. 2, complement-fixing antibodies seemed to rise slowly than agglutinating antibodies and were not detected at all at 1 : 8 dilution of sera obtained before inoculation.

Agglutination titers reached to a peak level at 2 weeks after inoculation and then remained unchanged up to 5 weeks (Fig. 1), while complement-fixation titers reached to the maximum at 2 to 4 weeks after inoculation and persisted at the peak level up to 5 weeks (Fig. 2). The titers of agglutinin and complement-fixing antibodies or the general patterns of antibody development were parallel with each other.

From these results, the sensitivities of the tests were revealed and it can be suggested that the titer of 1 : 10 in agglutination and 1 : 8 or lower in complement fixation tests might be considered

Table 2. Comparison of Antibody Titers Determined by Homologous and Heterogous AG and CF Tests for Sera from an SPF Pig Infected Experimentally with Strain HP 8 of *H. pleuropneumoniae*, Serotype 2

Test serum and weeks after inoculation*	Titers against antigen											
	Serotype 1 (4074)			Serotype 2 (1536)			Serotype 3 (1421)			Serotype 5 (966)		
	AG(−)	AG(+)	CF	AG(−)	AG(+)	CF	AG(−)	AG(+)	CF	AG(−)	AG(+)	CF
0	10**	—***	—	10	—	—	—	—	—	—	—	—
1	20	—	—	20	10	—	—	—	—	—	—	—
2	40	20	64	320	160	16	—	—	—	20	20	8
3	20	20	64	320	160	512	—	—	8	20	—	8
4	40	20	64	320	160	1024	—	—	8	20	—	8
5	20	—	32	320	160	512	—	—	8	20	—	8

*: Sera from the SPF pig No. 3 infected with *H. pleuropneumoniae* serotype 2 (HP 8) were collected 0, 1, 2, 3, 4, and 5 weeks after inoculation.
**: Data(titers) are reciprocals of highest dilution of serum in which positive reaction occurred.
***: − =No agglutination in AG and 70% or more hemolysis at dilution of 1 : 8 in CF.
AG(−): routine agglutination test.
AG(+): agglutination test on the serum treated with the kaolin.
CF: Complement fixation test.

Table 3. Detection of Antibodies against *H. pleuropneumoniae* Serotypes on the Field Blood Samples by Various Serological Methods

Region and No. of serum examined*	Serology	Positive for serotype			
		Serotype 1 (4074)	Serotype 2 (1536)	Serotype 3 (1421)	Serotype 5 (966)
	AG(−)**	17(48.6)***	23(65.7)	0	2(5.7)
A 35	AG(+)	0	17(48.6)	0	0
	CF	0	13(37.1)	0	0
	AG(−)	3(7.7)	5(12.8)	0	3(7.7)
B 39	AG(+)	0	0	0	0
	CF	0	0	0	0

*: Blood samples were collected at random from field swine herds in the region of A(Japan) and B (Korea) during 1978.

**: Positive cross-reactors of each serotypes were included in the test of AG(−).

***: Figures in parenthesis are percentages of swine with positive antibodies(titer 1 : 20 or higher in AG and 1 : 8 or higher in CF). AG(−), AG(+) and CF: Same as in Table 2.

as non-specific.

Cross Serological Examinations: To compare with the sensitivity and specificity between the serotypes cross serological examinations were carried out. Sera from SPF pigs inoculated with the serotype 2, HP 8 were tested against homologous and heterologous antigens by different serological tests, AG(−), AG(+) and CF tests and the results obtained from the SPF pig No. 3 are shown in Table 2.

Weak cross reactions between different types were observed, especially with the serotypes 1 and 5, however, the titers were lower than those of homologous reaction.

Serological Survey of Swine Haemophilus Infection by the Application of Serodiagnostic Tests: Serum samples were collected from field herds for serological survey and the results obtained are shown in Table 3. In herd A, serotype 2 of HP infection was detected in 37.1% by the CF test (titer 1 : 64 to 1 : 256) and 48.6% by AG(+) test (titer 1 : 10 to 1 : 1, 120) of the 35 sera tested. On the other hand, a few cases were presented as positive in serotypes 1, 3 and 5, but the titers were very low in CF test(1 : 32) and AG(+) test (1 : 20).

Discussion

The serotyping of isolated strain and the prevalence of antibodies of HP serotypes are important in the epidemiologic and immunologic studies of HP infection. Although various serological tests have been used for the diagnosis,[1,5,11,14,15,17] a more convenient method, especially on the sensitivity and specificity for the detection of antibodies was desirable. Problems are focused on the swine serum that has the property of procomplementary activity[21] on the CF test and the nonspecific reaction on the agglution test.

Jeon *et al.*[6] reported that the modified complement fixation test of swine erysipelas by employing fresh rabbit or bovine serum factors to the complement system. Nicolet *et al.*[15] has described a CF test which was conducted with fresh bovine serum added in this system. Recently, Shulz *et al.*[20] reported that lyophilized guinea pig complement was reconstituted with normal serum from a 6 to 8 week-old pig and in this way, the procomplementary effect of the swine serum could be avoided in the titration of the guinea pig complement. The results obtained in this experiment revealed that, as shown in Fig. 1 and 2, CF test seemed to be more specific than AG test although the development of CF antibody was slower than AG antibody. And it was also demonstrated that the addition of normal calf serum to guinea pig complement restored the CF activity of heat-inactivated swine serum as obser-

ved by Nicolet *et al.*[15]

It has generally been agreed that serotypes of HP field isolates are antigenically distinct,[5,9,15] but weak cross reactions among serotypes have been indicated.[4,11]

Weak cross reactions were observed in this experiment as shown in Table 2. Some of non-specific reaction was reduced by the KIO_4 treatment to the swine serum(Fig. 1). Nicolet *et al.*[15] suggested that CF titers of 1 : 10 or more are indicative of previous infection and Shulz *et al.*[20] also reported that an animal having CF antibody titer of more than 1 : 4 was positive. Comparison of the present procedure of CF test with that of Nicolet *et al.*[15] and Shulz *et al.*[20] revealed that our 1 : 8 dilution, which was the lowest dilution tested, was the same as their 1 : 10 or 1 : 4 dilution.

As can be seen in Table 3, the major serotype of HP infection in the field was serotype 2. This result was coincident with the result of Chan *et al.*[3] who examined the isolates from the field cases of HP infection.

The application of AG(+) and CF tests described here in is very simple and convenient for the detection and titration of HP antibodies and, in particular, the tests were found to be useful for the large-scale serological suevey of infection.

Conclusions

The development of circulating antibodies and cross-reaction of *Haemophilus parahaemolyticus*(HP) serotypes 1, 2, 3 and 5 were studied in experimentally infected SPF pigs with the freshly isolated strain of HP 8 (serotype 2) and the prevalence of antibodies was also examined for the blood samples collected from field herds. Serodiagnosis was conducted by the routine agglutination, modified agglutination, in which test serum was pretreated with kaolin, and modified complement fixation tests employing normal calf serum as a source of supplementary complement factor.

Agglutinin and complement-fixing antibodies were detectable at the one week following exposure to infection and reached peak values after 2 to 3 weeks. The complement fixation test proved to be more sensitive and specific than the agglutination test. Weak cross-reaction or non-specific reaction between different serotypes was observed. A noticeable finding was that 37.1% of swine blood samples were positive for the serotype 2.

References

1. Bachmann, P.: Beitrag zur Epidemiologie der kontagiosen Pleuropneumonie beim Schwein (Studies on the epidemiology of contagious pleuropneumonia of swine), Schweiz. Arch. Tierheilk. (1972) 114 : 363.
2. Biberstein, E.L. and Cameron, H.S.: The family *Brucellaceae* in veterinary research. Ann. Rev. Microbiol. (1961) 15 : 93.
3. Chan, C.N., Yamamoto, K., Konishi, S. and Ogata, M.: Isolation and antigenic characterization of *Haemophilus parahaemolyticus* from porcine pneumonia. Jap. J. Vet. Sci. (1978) 40 : 103.
4. Gunnarson, A., Biberstein, E.L. and Hurvell, B.: Serologic studies on porcine strains of *Haemophilus parahaemolyticus(pleuropneumoniae)*: agglutination reactions. Am. J. Vet. Res. (1977) 38 : 1111.
5. Gunnarson, A., Hurvell, B. and Biberstein, E.L.: Serologic studies of porcine strains of *Haemophilus parahaemolyticus(pleuropneumoniae)*: antigenic specificity and relationship between serotypes. Am. J. Vet. Res. (1978) 39 : 1286.
6. Jeon, Y.S., Cho, H.J. and Oh, W.T.: Studies on the modified complement fixation test of swine erysipelas. Korean J. Vet. Res. (1967) 7 : 1.
7. Kabat, E.A. and Mayer, M.M.: Experimental immunochemistry. 2nd ed. Charles C. Thomas. (1961) p. 135.
8. Kilian, M.: A taxonomic study of the genus *Haemophilus* with the proposal of a new species. J. Gen. Microbiol. (1976) 93 : 9.
9. Kilian, M., Nicolet, J. and Biberstein, E.L.: Biochemical and serological characterization of *Haemophilus pleuropneumoniae*(Matthews and Pattison 1961) Shope 1964 and proposal of a neotype strain. Int. J. Syst. Bacteriol. (1978)

28 : 20.

10. Little, T.W.A.: Haemophilus infection in pigs. Vet. Rec. (1970) 87 : 399.
11. Mitui, T., Onaga, H., Nagasawa, Y., Nomura, Y. and Kuramasu, S.: Studies on Haemophilus infection in swine. 1. Application of the latex agglutination test to the diagnosis of *Haemophilus pleuropneumoniae* (*H. parahaemolyticus*) infection. Vet. Microbiol. (1981) 6 : 339.
12. Mylrea, P.J., Fraser, G., MacQueen, P. and Lambourne, D.A.: Pleuropneumonia in pigs caused by *Haemophilus parahaemolyticus*. Aust. Vet. J. (1974) 70 : 255.
13. Nicolet, J. and Konig, H.: Zur Haemophilus-pneumoniae beim Schwein. Bacteriologische, pathologisch-anatomische und histologische Befunde. Vorlaufige Mitteilung. Pathol. Microbiol. (1966) 29 : 301.
14. Nicolet, J.: Haemophilus infection in pigs. III. Serological studies on *Haemophilus parahaemolyticus*. Zbl. Bakt. (1971) 216 : 487.
15. Nicolet, J., Meuron, P.A. and Bachmann, P.: Haemophilus infection in pigs. Ⅳ. The use of the complement fixation reaction in the diagnosis of *Haemophilus parahaemolyticus* infections. Schweiz. Arch. Tierheilk. (1971)113 : 191.
16. Nielsen, R.: Pleuropneumoni hos svin, framkaldt af *Haemophilus parahaemolyticus*. Ⅱ. Undergelser over den isolerede bakteriens identitet of pathogenitet. Nord. Vet. Med. (1970) 22 : 246.
17. Nielsen, R.: Serological and immunological studies of pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*, Acta. Vet. Scand. (1974) 15 : 80.
18. Schiefer, B., Moffat, R.E., Greenfield, J., Agor, J.L. and Majka, J.A.: Porcine *Haemophilus parahaemolyticus* pneumonia in Saskatchewan. I. Natural occurence in findings. Can. J. Comp. Med. (1974) 38 : 99.
19. Shope, R.E.: Porcine contagious pleuropneumoniae. I. Experimental transmission, etiology and pathology. J. Exp. Med. (1964) 119 : 357.
20. Schulz, R.A., Young, T.F., Ross, R.F. and Jeske, D.R.: Prevalence of antibodies to *Haemophilus pleuropneumoniae* in Iowa swine. Am. J. Vet. Res. (1982) 43 : 1848.
21. Tizard, I.R.: An introduction to veterinary immunology. W.B. saunders. (1977). p.127.

***Haemophilus parahaemolyticus* (*pleuropneumoniae*)菌의 血淸學的硏究**
특히 血淸型別抗原特異性 및 抗體價의 分布에 대하여

康 炳 奎
全南大學校 農科大學

山本 孝史·尾形 學
東京大學農學部家畜微生物學敎室

抄 錄

Haemophilus parahaemolyticus(*pleuropneumoniae*)菌(HP菌) 血淸 1型(4074株), 2型(1536株), 3型(1421株) 및 5型(966株)의 血淸反應特異性을 검토할 목적으로, 2週齡의 SPF豚에 HP菌 新鮮分離株(HP8株)를 經鼻接種하여 經時的으로 나타나는 抗體價를 주로 凝集反應(AG)과 補體結合反應(CF)으로 비교검토하였다. 아울러 野外에서 채취한 豚血淸에 대하여 각 血淸型別 抗體價의 分布도 AG및 CF反應으로 조사하였다. 얻어진 結果를 요약하면 다음과 같다.

1. 凝集抗體(AG抗體) 및 補體結合抗體(CF抗體)는 接種한 다음 1주일에 나타나기 시작하였고, AG抗體는 接種 2주후에 그리고 CF抗體는 2~3주후에 최고치를 나타내었다. 그리고 CF抗體는 AG抗體보다 약간 늦게 나타나는 경향이 인정되었다.

2. HP菌(HP 8株)血淸 2型感染豚 血淸에 대한 각 血淸抗原間의 反應에서는 抗體價가 낮기는 하나 交叉反應이 인정되었고, 특히 血淸 1型 및 5型抗原에서 이러한 현상이 인정되었다.

3. 野外血淸에 대한 HP菌의 血淸型別抗體는 주로 HP菌2型이 대부분이었고, 그 檢出陽性率은 AG反應에서 48.6%, CF反應에서 37.1%를 나타내고 있어, HP菌血淸 2型菌의 感染이 존재함이 血淸學的으로 확인되었다.

4. HP菌의 血淸反應의 실시에 있어서는 AG反應에서 나타나는 非特異反應은 可檢血淸을 kaolin처리 함으로서 이를 배제할 수 있었고, CF反應에 있어서는 新鮮仔牛血淸을 補體系에 첨가함으로써 효과적인 반응을 실시할 수 있음이 확인되었다.