COMPARISON OF ANTIGENIC COMPOSITIONS OF DIFFERENT SEROTUPES OF SWINE HAEMOPHILUS PLEUROPNEUMONIAE

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The antigenic components of eight serotypes of Haemophilus pleuropneumoniae (HP) were analyzed by sodium dodecyl sulfate polyacrylamide gel electroghoresis (SDS-PAGE) and Western immunoblotting technique. There were about 38 bands and more than 45 bands of each type revealed in SDS-PAGE with Coomassie blue and silver stain, respectively. However, there was no significant difference among serotypes. The compositions of difference serotypes of HP were very similar. In Western blotting, there were 30, 15 and 13 antigenic bands of each strain, respectively, when the blotting profile reacted with antisera against whole cells, serum from pig naturally exposed to HP and serum against purified lipopolysaccharides (LPS). From the patterns obtained in Western blots, it was suggested that the surface antigens played a major role in provoking immune response. There were two additional antigenic bands, 43K and 70K of serotype 1 showed in Western blots, reacting with antisera against serotype 1. They were concluded to be serotype-specific.

Porcine pleuropneumoniae caused by Haemophilus pleuropneumoniae (HP) is characterized by fibrinous, haemorrhagic, necrotizing pleuropneumoniae[1] It has recently been proposed that there at least twelve serotypes have been identified[2]. Serotyping of HP was successfully done by using different preparations of antigens in tube agglutination, ring precipitation, indirect immunofluorescent, complement fixation, ELISA, and coaggluatination tests etc[3,4,5,6,7,8]. Although serotypespecific antigens of HP have so far not been well defined, it seems that serotype-specific antigen is a lipopolysaccharide-like materials existed in bacterial capsule[9].

In recent years, the electrophoretic patterns obtained by polyacry-

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lamide gel electrophoresis (PAGE) from bacteria were used in taxonomic studies. Colony type, growth medium, time of harvest, and in vivo or in vitro passage had no appreciable effect on the protein profile of the strains examined[10,11] The electrophoretic patterns had also been used in studing the components of HP. Nicolet had examined the protein profiles of SDS-solubiliaed whole cells of HP and found that they were very similar among serotype 1 to 5[10] . However, Rapp had distinguished seven patterns of outer membrane proteins among nine serotypes of HP by SDS-PAGE[11] .

The purposes of the study were to analyze the antigenic components and determine the serotype-specific antigens of different serotypes of HP by SDS-PAGE with different staining methods and Western immunoblotting technique.

MATERIALS AND METHODS

Bacteria

Eight reference strains of serotype 1 to 9 (except 6) of HP were obtained from Dr. C.N. Chang, Department of Vetrinary medicine Taiwan Sugar Cooperation. Bacteria were seperately cultured on chocolate agar (5% defibrinated horse blood in brain heart infusion agar heated at 80°C for ten minutes in two steps) for 18 hours. Harvested bacterial cells were washed three times in Phosphate buffered saline (PBS, pH 7.2). The protein concentration was determined by Lowry method[12] .

SDS-PAGE

Discontineous SDS-PAGE was performed with 10% seperating gel and 3.9 % stacking gel using the Laemmli buffer system[13]. Bacterial samples (30ug) were boiled for three minutes in buffer containing 2% SDS, 5% 2-meceptoethanol and 10% glycerol in 0.0625M Tris-Hcl (pH 6.8). The treated samples were then loaded onto the pre-prepared slab gel and electrophorized at 20mA constant current for about three hours. The slab gels were then stained for protein with Coomassie brilliant blue R-250 and for carbohydrate with silver stain, respectively. Silver stain was done according to the method of Tsai[14] . A molecule weight standard (Pharmacia product) ranging from 14.4k to 94k was used to calculate the molecule weight of the electrophorized bands.

Preparation of antisera used in Western immunoblotting

Three types of antisera against serotype 1 were used in Western blotting. There were hyperimmune serum against formalin-inactivated whole cell antigens, serum from pig that naturally exposed to HP, and hyperimmune serum against phenol-extracted LPS antigen. The LPS antigen was extracted according to the method of Gunnarson[15] . Hyperimmune serum of inactivated whole cells was produced in rabbits intramuscularly immunized three times with formalin-inactivated whole cells

homogenized with Freund's complete adjuvant. The dose of inoculum was 0.5 ml of 10% (v/v) of washed cells. The preparation of hyperimmune serum against LPS was also done in rabbits. The concentration of LPS was diluted to $2.5x10^8$EU/ml in Limulus amoebocyte lysate test. The serum of infected pig was taken from farm where naturally infected with serotype 1.

Western immunoblotting

The slab gels of reference strains of HP after running in SDS-PAGE were electroblotted onto a nitrocellulose paper . The blotted nitrocellulose papers were reacted with HP specific antisera and peroxidase labeled antirabbit immunoglobulin and developed acording to the method of Healey(16) , using 3,3'-diaminobenzidine as substrate.

RESULTS

SDS-PAGE patterns of reference strains of HP: The SDS-PAGE patterns of each strain of HP stained with Coomassie blue and silver nitrate were shown in Figure 1 and 2, respectively. In general, the patterns of each strain were similar either in the number of bands and their electromigrating points or the intensity of the bands. More than 38 bands stained with Coomassie blue in each strain were noticed. The molecule weight of five major bands which showed more intense than others were 14. 4K, 20.1K, 30K, 43K and 55K, respectively. The differences of SDS-PAGE patterns among serotypes were only found in the region between 43K and 64K. Because the differences were so insignificant, therefore it could not differentiate the difference among serotypes by this method. However, Actinobacillus had the distinct pattern as compared to those of serotypes of HP.

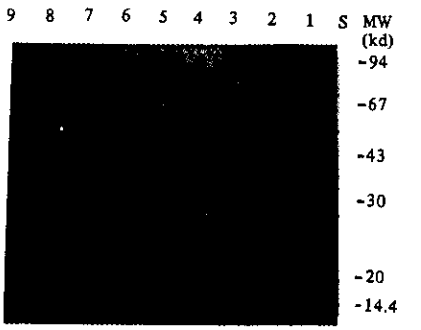

Fig. 1. SDS-PAGE profile of 8 serotypes of HP stained with Commasie blue. Line 1 to 8 are serotypes 1 to 9, without 6. Line 9 is Actinobacillus. The right column is standards for the references of molecular weights.

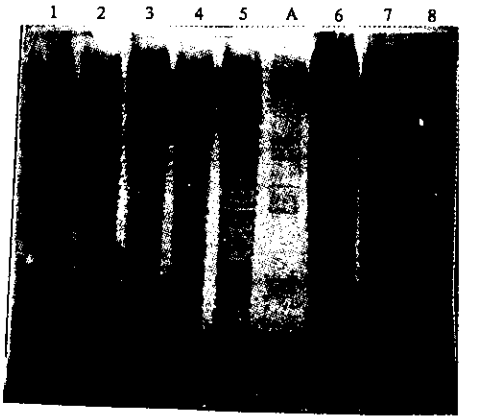

Fig. 2. SDS-PAGE profile of 8 serotypes of Hp stained with silver nitrate. Line 1 to 8 are serotypes 1 to 9, without serotype 6. A is Actinobacillus.

As shown in Figure 2, more than 45 bands were revealed in SDS-PAGE

stained with silver nitrate. The patterns of each strain were similar except the intensity of bands. Because the bands were too many and too close, so it was difficult to numberate the accurate number of bands and estimate the correct molecule weight of each band in each strain. However, the differences in migration patterns between HP and Actinobacillus spp. were significant. Although SDS-PAGE stained with silver nitrate were different from those with Coomassie blue, serotype-specific components were still not identifiable.

Specific antigenic components obtained by Western immunoblotting: Antigenic components on nitrocellulose paper blotted from SDS-PAGE slab gel prepared from 8 serotypes were reacted with three kinds of antisera against serotype 1 of HP as shown in Figure3, 4 and 5. At least 30 bands were obtained by reacting the blotting meterials to antiserum prepared from whole cells of serotype 1. In this preparation

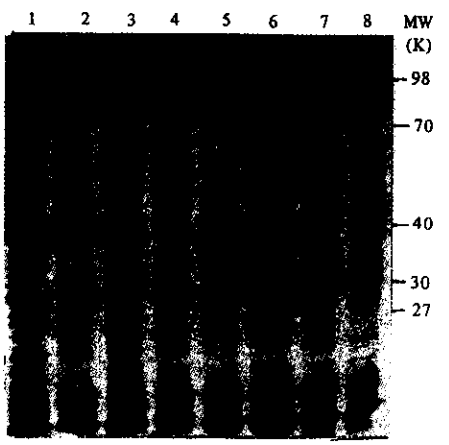

Fig. 3. Immunoblotting pattern of antigenic components of 8 serotypes of HP reacted with antiserum against inactivated whole cell of serotype 1. Lines 1 to 8 are serotypes 1 to 9, without 6.

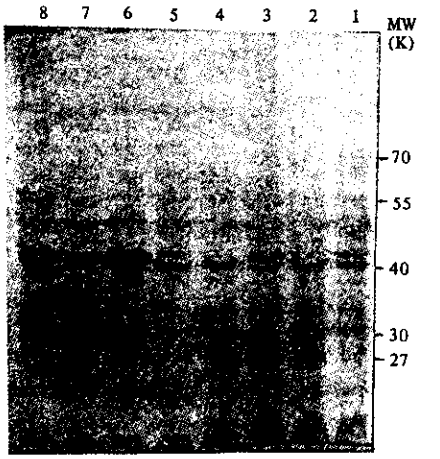

Fig. 4. Immunoblotting pattern of antigenic components of 8 serotypes of HP reacted with antiserum collected from pig naturally infected with serotype 1. Lines 1 to 8 are serotypes 1 to 9, without 6.

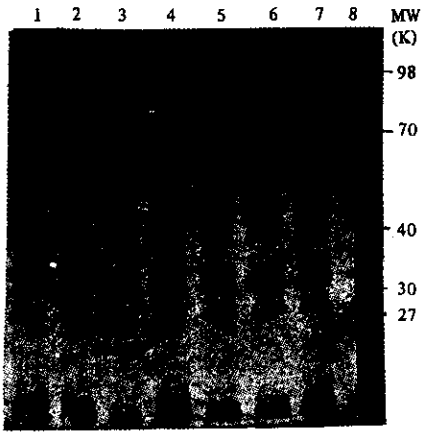

Fig. 5. Immunoblotting pattern of antigenic components of 8 serotypes of HP reacted with antiserum against LPS of serotype 1. Lines 1 to 8 are serotypes 1 to 9, without 6.

hyperimmune serum against serotype 1 reacted similarly as other serotype of HP, indication strongly cross reaction among serotypes.

There were 15 bands obtained by reacting the serum of pig naturally exposed to HP with the immunoblotting antigens on the nitrocellulose paper prepared from serotypes of HP. Gene-

rally, the distribution of antigen-antibody reacting bands were similar among 8 serotypes, except that an additional 70K band was only seen in preparation of serotype 1. In addition to 70K band, slight difference in intensity of bands were also observed for 41K band in serotype 2 and 4.

In the same preparation of immunoblotting antigens of 8 serotypes, but using antiserum prepared from phenol-extracted LPS (serotype 1), 13 reaction bands were observed. The cross reactions among serotypes with anti-LPS serum were also strong. However, two additional bands, 43K and 70K were only observed in serotype 1. Therefore, the 43K and 70K bands seemed to be the serotype-specific for serotype 1. For comparison, a diagram of the immunoblotting profiles of serotype 1 reacting with different preparations of antisera were drawn in Figure 6.

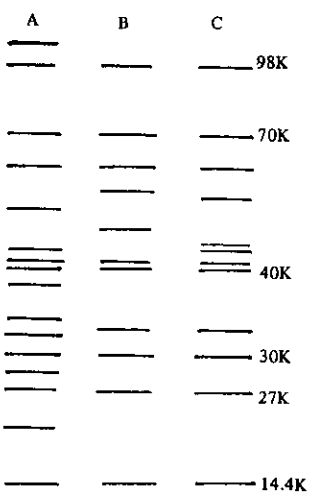

Fig. 6. Comparison of the immunoblotting patterns of antigenic components of serotype 1 of HP reacted with three different antisera against; A: inactivated whole cell; B: naturally infected pig serum; C: phenol-extracted LPS of serotype 1.

DISCUSSION

The Coomassie blue stained SDS-PAGE profiles prepared from different serotypes of HP showed no significant difference among serotypes. However, by using silver stain, which was considered to pick up capsular polysaccharides, the antigenic componints of different serotypes were still indistinguishable in the SDS-PAGE profiles. But it showed significantly different from that of Actinobacillus spp. In Nicolet's study the SDS-PAGE pattern of HP was distinct from those of Haemophilus (H.) parasuis, H. influenzae, H. parahemolyticus, Pasteurella, Actinobacillus, Brucella, Morexella and Bordetella[10]. The SDS-PAGE pattern of HP probably can be used as tool for identifing HP from other bacterial species.

Serum from pig naturally exposed to HP was more suitable to identify the important antigens, because the protective antibodies induced after recovery from infection. However, the immunoblotting patterns of different serotypes of HP using antisera against whole cell, LPS and serum of naturally infected pig gave a fairly similar result. From the result, it also suggested that the cellular surface antigens LPS played a major role in provoking immune response and induced better protective immunity than formalin-inactivated whole cell antigens. The immunoblotting materials of different serotypes reacted with hyperimmune serum against whole cells showed more antigen-antibody reacting

hands, which probably due to the exposure of some hidden inner antigens during the preparation of inactivated whole cells.

The serum against LPS and serum of infected pigs can be used efficiently in serotyping since capsular polysaccharides is thought to be serotype-specific[9]. Supprisingly, antigens revealed in immunoblotting reacting with both sera, respectively, had strong cross reactions among serotypes. There were a lots of common antigens existed in cells of HP. Because the cross protection of serotypes in not present strongly, so the serotype-specific antigens must play an important role in protection, which is needed for further study.

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猪胸膜肺炎嗜血桿菌血清型抗原分析比較

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猪胸膜肺炎嗜血桿菌（Haemophilus pleuropneumoniae, HP)各血清型全菌抗原，分別以丙醯膠電泳（Sodium dodecyl sulfate-polyacrylamide, SDS-PAGE)及西方免疫轉印技術（Western immunoblotting）加以分析。以Coomassie blue及銀染色分別可染到38條及超過45條抗原成份，但是各血清型間並無明顯差異，因此其組成相當類似。在分別以三種不同抗血清與HP各血清型抗原所做的免疫轉印實驗中，與抗血清同型之HP第一型抗原比別的血清型抗原多了兩條抗原成份，分子量分別是70K和43K，這兩條抗原似乎具血清型特異性。並且由免疫轉印圖譜顯示免疫抗體的誘發，主要是由表面抗原所引起。

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