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Protein A of *Staphylococcus hyicus* subsp *hyicus* isolated from pigs

Do-kyoung Kim, Sang-geon Yeo
College of Veterinary Medicine, Gyeongsang National University
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돼지에서 분리한 *Staphylococcus hyicus* subsp *hyicus*의 protein A

김 도 경·여 상 건
경상대학교 수의과대학
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초록 : 돼지로부터 분리한 *Staphylococcus hyicus* subsp *hyicus* 489주의 protein A 존재여부와 함량을 indirect hemagglutination 및 enzyme-linked immunosorbent assay(ELISA)법으로 조사하였다

Indirect hemagglutination test에 의하여 cell-bound protein A 및 extracellular protein A 보유균은 489주 중 각각 87.7% 및 36.0%로 나타났다. ELISA법에 의한 이들 균의 protein A함량 측정에서 전균주의 extracellular protein A는 1ng/ml미만이었으며, cell-bound protein A함량은 대부분의 균주에서 1ng/ml 미만이었고 11주가 25~108ng/ml 수준이었다.

Key words: pigs, *Staphylococcus hyicus* subsp *hyicus*, protein A, indirect hemagglutination, enzyme-linked immunosorbent assay.

Introduction

Since protein A was demonstrated in *Staphylococcus aureus* by Jensen,[1,2] it had been recognized as an antigenic component uniquely in cell wall of this organism.[3-6] Recently, however, the protein A was also found in some strains of *Staphylococcus intermedius* and *Staphylococcus hyicus* subsp *hyicus* cell-bound or extracellular state.[7-11] Protein A has capacity of nonspecific immune binding to the Fc portion of IgG from various mammals.[12] The protein A would, therefore, interfere with host's specific immune response to *Staphylococcal* infections and in vitro serological reactions.[13-16] In addition, the protein A exhibits activation of complement, induction of chemotaxis and hypersensitivity, and lymphocyte activation which are of importance in immunological research.[17-20]

A variety of techniques, such as double immunodiffusion, immunofluorescence, indirect hemagglutination and enzyme-linked immunosorbent assay, are available to determine cell-bound and extracellular protein A of *Staphylococci*.[3,7,9-11,21-24] There are, however, some variations of reactivity from different methods and little informations concerning the quantification of the protein A in *Staphylococci*. The purpose of the present study was to determine protein A of *S hyicus* subsp *hyicus* strains isolated from pigs.

Materials and Methods

Bacterial strains: The 489 strains of *S hyicus* subsp *hyicus* isolated from pigs in Chinju area were examined. These consisted of 216 strains from healthy adult pigs, 248 strains from healthy piglets and 25 strains from piglets affected with exudative epidermitis. The strains were previously identified cultur-

ally and biochemically by the methods of Devriese[25] and Park and Kang.[26]

Detection of protein A by indirect hemagglutination test: Protein A of *S hyicus* subsp *hyicus* was detected by the indirect hemagglutination method of Winblad and Ericson.[27]

Preparation of rabbit anti sheep red blood cells (SRBC) serum: Rabbits were immunized with 2ml of 25% SRBCsolution intravenously, twice per week during five weeks. Blood was collected from the rabbits 7 days after the final injection and serum was kept at −20°C.

Preparation of sensitized SRBC: Half of the minimum hemagglutinating dose of rabbit anti SRBC serum was used as sensitizing dose. The 100ml of the serum was mixed with equal volume of 1% SRBC and reacted for 30 minutes at ambient temperature. The sensitized SRBC was washed three times with sterile veronal buffered saline (pH 7.2) and finally suspended in veronal buffered saline up to 5%.

Detection of cell-bound protein A by slide hemagglution: A loop of culture on brain heart infusion agar at 37°C for 24 hours was emulsified with a drop of 5% sensitized SRBC. When marked hemagglutination occurred in a few seconds, the strains were evaluated to have protein A in their cell walls. For a weak positive reaction the observation was continued for at least 2 minutes. *S aureus* strain Cowan 1 offered by Dr. Takeuchi, S. (National Institute of Animal Health, Japan) was used as positive control.

Detection of extracellular protein A by microplate hemagglutination: Supernatants of the cultures in brain heart infusion broth at 37°C for 20 hours were twofold-diluted with sterile saline containing 0.1% bovine serum albumin in microplate. Equal volume of 1% sensitized SRBC was added to each well of the microplate. The plate was gently shakened and incubated at 37°C for 2 hours. Hemagglutinating titer was expressed as the reciprocal of the highest dilution in which complete agglutination of sensitized SRBC was observed.

Determination of protein A by enzyme-linked immunosorbent assay(ELISA): Protein A contents of the strains shown presence of protein A in indirect hemagglutination test were quantified by the indirect ELISA method of Weiss et al.[9]

Antigen preparation: Antigens for detection of cell-bound protein A were prepared from cultures on 10% sheep blood supplemented tryptic soy agar at 37°C for 24 hours. Cultures were suspended in sterile 0.9M NaCl containing 0.5% formalin for 1 hour at 37°C to inactivate. Cells were centrifuged at 3,000rpm for 15 minutes, twice with 0.9M NaCl and resuspended in 0.9M NaCl up to the concentration of standard barium sulfate turbidity (9×10^8 cells/ml). Antigens for detection of extracellular protein A were prepared from culture supernatants in 5ml of brain heart infusion broth containing 0.3% yeast extract and 0.1% malt extract at 37°C for 20 hours. Cultures were centrifuged at 13,000 rpm for 5 minutes and supernatants were decanted into tubes. These antigen solutions were stored at −20°C until use.

ELISA procedure: Optimal dilutions of reagents were otbained by checkerboard titrations. Antigens were suspended in 0.05M carbonate-bicarbonate buffer (pH 9.6). The 200μl aliquots of the antigens were coated to wells in 96 well-flat bottomed polystyrene EIA plates(Linbro/Titertek Co., U.S.A.) and incubated in a humidified chamber at 37°C for 15 hours. Thereafter plates were washed with 1M NaCl containing 0.05% Tween 20 for 5 minutes, 3 times. The 200μl aliquots of rabbit anti bovine IgG peroxidase conjugate, diluted to 1,000-fold in phosphate buffered saline solution containing 0.05% Tween 20 (pH 7.4), were added to each well. Plates were incubated in a humidified chamber for 3 hours at ambient temperature, and then washed. The ortho-phenylendiamine substrate dissolved in citrate-phosphate buffer (pH 5.0) was added up to 200μl per well. The 100μl aliquots of 2.5M H_2SO_4 were added to each well 30 minutes after substrate reaction. Optical density(OD) was measurred at 490nm by ELISA reader(Dynatech MR-700, U.S.A.). Protein A-positive values were dicided as OD values 5 times higher than protein A-negative control values. *S aureus* strain Cowan 1 was used as protein A-positive control. *S epidermidis*, isolated undesignedly in present study and determined as protein A deficient strain in indirect

hemagglutination test, was used as protein A-negative control.

Linear regression analysis of protein A quantity: ELISA was carried out for commercial purified protein A(Sigma P6650) in quantities of 20, 40, 60, and 80ng/ml as described previously. From the linear regression analysis of the OD values obtained from 18 times-aliquots, standard curve was prepared for quantification of protein A in strains of *S hyicus* subsp *hyicus*.

RESULTS

Presence of protein A in *S hyicus* subsp *hyicus* by indirect hemagglutination test: Cell-bound and extracellular protein A were detected in 87.7% and 36.0% of 489 isolates of *S hyicus* subsp *hyicus*, respectively. Cell-bound protein A was demonstrated in 82.9%, 91.1% and 96.0% of from healthy adult pigs, healthy piglets and affected piglets, respectively. The presence of extracellular protein A was demonstrated in 31.0%, 35.5% and 84.0% of the isolates from each of the above animal groups (Table 1).

Table 1. Presence of cell-bound and extracellular protein A in isolates of *S hyicus* subsp *hyicus* by indirect hemagglutination test

Sources	No. of isolates	No. (%) of protein A-positive isolates	
		Cell-bound[a]	Extracellular[b]
Healthy pigs	216	179(82.9)	67(31.0)
Healthy piglets	248	226(91.1)	88(35.5)
Diseased piglets*	25	24(96.0)	21(84.0)
Total	489	429(87.7)	176(36.0)

a : Determined by slide hemagglutination test.
b : Determined by microplate hemagglutination test.
* : Piglets affected with exudative epidermitis.

Table 2. ELISA values of protein A in *S hyicus* subsp *hyicus* isolates shown presence of protein A in indirect hemagglutination test

Sources	No. of tested isolates	No. (%) of ELISA positive isolates	OD values	
			Min~Max	Average
Cell-bound	429	203(47.3)	0.018~0.587	0.128
Extracellular	176	6 (3.4)	0.011~0.146	0.054

When contents of the extracellular protein A were determined semi-quantitatively by microplate hemagglutination test (Fig 1), the hemagglutinating titer of all of 176 isolates ranged from 4 to 128. The isolates having endpoint titer 32 were most predominant.

Protein A determination by ELISA: Protein A of *S hyicus* subsp *hyicus* was determined by the ELISA (Table 2). The presence of cell-bound protein A was demonstrated in 203(47.3%) of 429 isolates which were protein A-positive in indirect hemagglutination test. The average OD value of these strains was 0.128. The presence of extracellular protein A was demonstrated in 6(3.4%) of 176 isolates that were

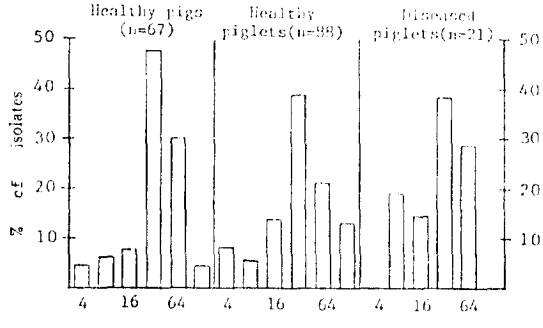

Fig 1. Semi-quantitative determination of extracellular protein A in 176 isolates of *S hyicus* subsp *hyicus* shown presence of protein A in indirect microplate hemagglutination test (HA).

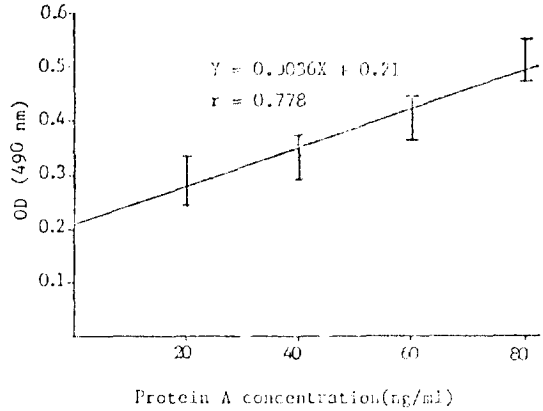

Fig 2. Linear regression analysis of results of enzyme-linked immunosorbent assay for purified protein A.

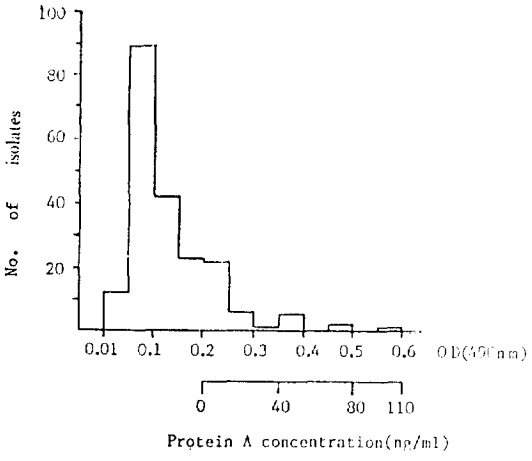

Fig 3. Distribution of OD values obtained for cell-bound protein A of 203 isolates of *S hyicus* subsp *hyicus* and concentration of protein A estimated by linear regression analysis.

protein A-positive in hemagglutination test. The average OD value was 0.054. Linear regression analysis of ELISA values of purified protein A resulted in Fig 2. There was correlation between concentrations of protein A and their OD values. The line of regression was $Y=0.0036X+0.21$ ($r=0.778$).

The contents ofc ell-bound protein A in 203 isolates of *S hyicus* subsp *hyicus* were calculated using the standard curve (Fig 3). Most of the strains produced protein A of less than 1ng/ml. Twenty eight(13.8%) isolates produced 1 to 25ng/ml and 11(5.4%) isolates produced 25 to 108ng/ml.

Discussion

Protein A had been demonstrated as an antigenic substance in cell wall of *S aureus*, *S intermedius* and *S hyicus* subsp *hyicus*.[5,20,21] Cox et al.[10] supposed that the pathogenicity of *S intermedius* could be contributed by the protein A. By the indirect hemagglutination test in the present study, cell-bound protein A was detected from 82.9%, 91.1% and 96.0% of *S hyicus* subsp *hyicus* isolates originated respectively from healthy adult pigs, healthy piglets and piglets with exudative epidermitis. Extracellular protein A was also demonstrated in 31.0%, 35.5% and 84.0% of strains from each of the above pig groups. Rates of protein A-positive strains in *S hyricus* subsb *hyicus* were higher except extracellular protein A of strains of healthy pig origin when compared with the findings of Takeuchi et al.[11] They reported 79.5% of strains from healthy pigs and 89.4% of strains from diseased pigs as cell-bound protein A-positive strains. In case of extracellular protein A, the 81.1% of strains from healthy pigs and 72.3% of strains from diseased pigs were positive.

In order to quantify the protein A of *S hyicus* subsp *hyicus*, ELISA was undertaken for the strains shown the presence of cell-bound or extracellular protein A in indirect hemagglutination test (Table 2). Cell-bound protein A was confirmed from 47.3% of 429 isolates and average OD value was 0.128, whereas extracellular protein A was determined from only 3.4% of 176 isolates of which average OD value was 0.054. Presence of extracellular protein A and OD value were detected greatly lower than those of cell-bound protein A. This phenomenon would explain that extracellular protein A was originated mainly from cell-bound protein A, which was released into the culture medium.[11] Forsgren[3] also noted cell-bound protein A was detected two six times more then extracellular protein A.

Using standard curve from ELISA values for purified protein A, the contents of cell-bound protein A in only 39 of 203 strains were estimated as 1 to 108ng/ml. Protein A contents of the rest were less than 1ng/ml which was undetectable concentration (Fig 3). As the contents of extracelluar protein A of 176 strains shown the presence of protein A in indirect hemagglutination test were unable to be measurred by ELISA (Table 2), endpoints of hemagglutination titers were evaluated. The hemagglutination titers of these strains ranged from 4 to 128 and strains with endpoint 32 were predominant (Fig 1). These were higher than the values of Takeuchi et al.[11] in which the endpoints ranged from 2 to 16 and the most predominant titer was 4.

Summary

The Presence and quantity of protein A in *Staphylococcus hyicus* subsp *hyicus* isolates from pigs were determined by indirect hemagglutination test

and enzyme-linked immunosorbent assay(ELISA). Cell-bound and extracellular protein A was demonstrated in 87.7% and 36.0% of 489 isolates, respectively, by indirect hemagglutination test. When contents of the protein A were estimated by ELISA method, all of the isolates that were positive to protein A in indirect hemagglutination test produced extracellular protein A of less than 1ng/ml. Most of the isolates produced cell-bound protein A of less than 1ng/ml, whereas 28 isolates produced 1 to 25ng/ml and 11 isolates produced 25 to 108ng/ml.

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