Indonesian Journal of Biotechnology, December (2000): 413 - 418

Identification of hemolysin associated with virulence of *Streptococcus suis*

Siti Isrina Oktavia Salasia[1]

1) Department of Clinical Pathology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia

Abstract

This study was designed to identify hemolysin of *Streptococcus suis* isolated from diseased and healthy pigs in Indonesia. Hemolysin of *S. suis* strain D282 has been isolated and purified from culture supernatant by ammonium sulphate precipitation, using filtration steps and Superose-12 column chromatography. All bacterial cultures used in this study were identified as *S. suis*. Serotyping of the *S. suis* isolates revealed capsular types 1, 2, 4, and 11 and several cultures were non typeable. The purified hemolysin of *S. suis* strain D282 had an apparent molecular mass of 31,000-55,000 and exhibited a specific activity of 890 U/ml. Hemolytic activity was reduced after incubation at 37°C for 3 hours and after the addition of proteinase K. Most of the different serotype strains appeared to secrete hemolysin. This study indicated that hemolysin could be found in the different serotypes *of S. suis*. This hemolysin might be used as a protective factor of *S. suis*.

Keywords: *Hemolysin — fast protein liquid chromatography* — Streptococcus suis

Introduction

Streptococcus suis is well known as the causative agent of wide variety of infections in pigs. These include many cases of meningitis, pneumonia and septicaemia (Higgins and Gottschalk, 1990). *Streptococcus suis* can also be isolated from clinically healthy pigs and from human (Vecht *et al.*, 1991; Arends and Zanen, 1988). The latter cases mainly occured among human who handled pigs or pigs products (Arends and Zanen, 1988).

Some *S. suis* cultures share group specific antigens with group D streptococci. This common cell wall antigen was shown to be lipid bond teichoic acid (Elliott *et al.*, 1977). In addition up to 29 present different capsular types of *S. suis*, another type 2 of *S. suis* has been described as the most prevalent capsular type in diseased pigs (Arends and Zanen, 1988; Salasia, 1994; Salasia and Lämmler, 1995).

Streptococcus suis infections cause significant problems in the intensive pig industries. More than 50% of the streptococcal infections in pigs in the Netherlands were due to *S. suis* (Vecht *et al.*, 1985). In May 1994 streptococcosis outbreak of pigs and monkeys occurred in Bali with case fatality rate (CFR) 45% of pigs, indicated that the disease was serious. Dharma *et al.* (1994) isolated *ß-hemolytic Streptococcus* from the infected pigs associated whith consistently meningitis, and the disease was called streptococcal meningitis. Dharma *et al.* (1994) suggested that the streptococcus infection was mixed with *S.*

suis type 2, while the symptoms of meningitis were similar to *S. suis* type 2 infection. Williams and Blackmore (1990) reported that the palatine tonsils were the port of entry for *S. suis*. Adult pigs could carry the streptococci in the tonsils and nasopharynx without showing clinical signs. These carriers could be the main source of transmission of the disease.

Little is known about the pathogenesis of the disease, the virulence factors or protective antigens of *S. suis*. The potential virulence factors, i.e. hemagglutinins and fimbriae have been described, but its roles in pathogenesis have not been fully understood and the respective molecules or proteins were not identified (Gottschalk *et al.*, 1990). The surface hydrophobicity of *S. suis* appeared to be significantly related to hemagglutinating properties of the bacteria (Salasia *et al.*, 1994; Salasia and Lämmler, 1994a). However, not all *S. suis* isolates with hydrophobic surface hemagglutinated the erythrocytes. Gottschalk *et al.* (1992) proposed that a 44,000 protein which was absent in a totally avirulent mutant strain could act as a virulence factor. This mutant had a higher surface hydrophobicity and showed increased adherence to human epithelial cells and to lung macrophages of rabbits as compared to the virulent parent strain (Salasia *et al.*, 1995).

Hemolysin activity is associated with the virulence and pathogenicity of several Gram -positive and Gram-negative bacteria, i.e. *E. Coli, Streptococcus pneumoniae, Listeria monocytogenes, Pasteurella hemolytica* and *Actinobacillosis pleuropneumoniae*. Many *S. suis* colonies are able to produce α-hemolysis and ß-hemolysis on sheep blood agar (Salasia, 1994). Many *S. suis* strains showing narrow zones hemolysin could involved in the pathogenesis of *S. suis* infections. Hemolytic activity of *S. suis* type 2 was decreased after incubation at 40°C and the addition of trypsin or amylase (Feder *et al.*, 1994).

The antigenic proteins that could be produced and identified might help to have better understanding of the pathogenesis of *S. suis*. The main objective of the study is to determine the virulence factors of *S. suis* that might have a significant contribution in the diagnosis, prevention and controle of streptococcosis.

Materials and Methods

Bacterial strains

Streptococcus suis serotypes 1 to 28 were kindly obtained from Ch. Lämmler, Institut für Bakteriologie und Immunologie, Justus Liebig Universität, Giessen, Deutschland. *S. suis* type 2 strains D282 was provided by U. Vecht, DLO-Central Veterinary Institute, Department of Bacteriology, Lelystad, Netherland. The 10 cultures of *Streptococcus suis* were isolated from the tonsils of healthy and bloods of infected pigs in Yogyakarta and West Java. Bacterial strains were streaked on sheep blood agar and cultured for 24 h at 37° C. The cultures were characterized biochemically and serologically as described previously (Salasia, 1994).

Preparation of hemolysin

Bacterial strains were streaked on sheep blood agar and cultured for 24 h at 37°C. For the determination of hemolysin production in batch cultures, a few colonies were inoculated into 100 ml of THB and cultured at 37°C until the end of the exponential growth phase. Subsequently, the cells were removed by centrifugation at 10.000 x g for 10 min, and the supernatant was stored at -20°C until used (Jacobs *et al.*, 1994).

Titration of hemolytic activities

Culture supernatants were added in triplicate to round-bottom, 96 wells titer plates (Nunc) in the following amounts: 2, 5, 10, 15, 20, 30, 50, 70 and 100 ml. None was added to control wells. The volume of each well was standardized to 100 ml with 10 mM Tris buffered saline (TBS; pH 7,4). Subsequently 150 ml of a 2% (washed) sheep erythrocytes suspension in 10 mM TBS was added to each well. After the wells were sealed, the plates were incubated on a coulter mixer for 2 h at 37°C. After unlysed erythrocytes were allowed to pellet overnight at 4°C, 150 ml of the supernatant fluids were transferred to a polystyrene flat bottomed microtiter plates (Nunc) and measured at 540 nm with a micro ELISA reader (Lab. System, Titertek, Multiscan). The titer was defined as the reciprocal of the highest dilution of hemolysin inducing at least 50% lysis of erythrocytes (Feder *et al.*, 1994; Jacobs *et al.*, 1994).

Purification of hemolysin

A 12-liter THB was inoculated with 250 ml of an overnight culture of strain D282 in the same medium. After growing for 6 h at 37°C the culture was cooled to 4°C, and all subsequent steps were carried out at 4°C. The cells were removed by the centrifugation at 10.000 x g for 30 min. The supernatants were precipitated by the addition of 50% $(NH_4)_2SO_4$ for 24 h. After the centrifugation at 10.000 x g for 30 min, the pellet was resuspended in 120 ml TBS pH 7,4, dialysed against 50 mM NaCl, pH 6,0 for 24 h. The preparation was sterilized by filtration through 0,22 µm-pore size filter (Schleicher and Schuell) and 1-ml portions were applied to a Superose-12 gel filtration column (FPLC, Sigma) and eluted in 40 mM phosphate buffered saline (PBS, pH 7,5) supplemented with 0,5 M NaCl. Fraction (0,5 ml) were collected and analysed by SDS-PAGE and immunoblotting (Ding, 1994; Jacobs *et al.*, 1994).

Hemolytic activity after different treatments

For all treatments 0,5 ml portions of purified hemolysin with a titer of 1:4 in 40 mM PBS pH 7,2 were used. The effects of different temperatures were measured in the hemolysin assay after incubation of the hemolysin at 4°, 20°, 37° and 50°C for 3, 6, 8 and 24 h. To test the effects of many kinds of treatment proteinase K (20 µg/ml), β-mercaptoethanol (0,1%), TLCK (N-α-p-Tosyl-L-Lysine chloromethylketone, 0,1%), H_2O_2 (0,1%) and cholesterol (0,1%), each solution were added to 0,5 ml of hemolysin solution and incubated at 20°C for 10 min. Residual activity was determined in the hemolysin assay. Control tests were carried out with duplicate samples from which the respective reagents were left out. Furthermore, any effect of the reagents on erythrocytes in the hemolysin assay was tested by omitting hemolysin from the reaction mixtures (Ding, 1994; Jacobs *et al.*, 1994; Feder *et al.*, 1994).

Results and Discussion

All 10 cultures used in this study displayed almost identical biochemical properties and could be identified as *S. suis*. The capsular antigen belonged to serotype 1 (n=2), 2 (n=2), 4 (n=1), 11 (n=2) and not typeable (n=3) (Table 1). Eight cultures of *S. suis* were isolated from infected pigs and 2 cultures from healthy pigs. These isolates were comparatively investigated for serotype and for the presence of various putative virulence factors. As already described (Estoepangesti and Lämmler, 1993; Salasia and Lämmler, 1995), most of the *S. suis* cultures belonged to classical serotypes 2, 1 and 1/2. However, part of the cultures could

be serotyped with the type specific antisera 4 and 11 and part of the cultures were not typeable.

Table 1. Distribution of serotypes and hemolytic activities of different *S. suis* strains

Isolate Code	Serotype	Hemolysis on BAP	Hemolytic activity[2] (U/ml)
D282[1]	2	α	136
A1	1	α	83
A2	1	β	608
A3	NT	α	ND
A4	2	α	ND
B1	11	α	82
B3	NT	α	82
B4	NT	α	627
C2	2	α	82
C4	11	β	672
C7	4	α	647

[1] Reference strain; NT: not typeable; BAP: sheep blood agar plates; ND: not determined; [2] detected in culture supernatants

After incubation for 24 h at 37°C on sheep blood agar, *S. suis* strain D282 resulted an α-hemolysis. The hemolytic activity of *S. suis* strain D282 are shown in Table 2. Maximum hemolytic activity 890 U/ml was obtained from culture filtrated with Superose 12 in fraction 42.

Table 2. Characteristics of hemolysin *Streptococcus suis* D282 after different purifications

Purifications	Volume (ml)	Hemolyti activity (U*/ml)	Total activity	Protein (mg/ml)
Supernatant	12.000	136	1.632.000	0
Ammonium sulfate	120	820	98.400	9,3
Filter 0,22 µm	100	880	88.000	16,3
Superose 12	30	890	26.700	9,6

* Hemolytic activity was defined as the reciprocal of the highest dilution of hemolysin inducing at least 50% lysis of erythrocytes

The effects of different treatments on hemolytic activity showed that the hemolysin was heat labile and susceptible to proteinase K digestion, oxidation by H_2O_2, and alkylation with TLCK. Furthermore, hemolytic activity was inhibited by cholesterol. The increased activity was found after incubation with β-mercaptoethanol (Table 3). Duplicate samples from which the various reagents or the hemolysin was left out showed no effects of the different treatments on hemolytic activity or effects of the different reagents on erythrocytes, respectively.

Table 3. Hemolytic activity of purified hemolysin after different treatments

Treatment	Treatment time and temperature	Hemolytic activity (U/ml)
Temperature (°C)		
4	3 hours	652
20	3 hours	655
37	3 hours	86
50	3 hours	88
Proteinase K (20 mg/ml)	10 min, 20°C	142
β-mercaptoethanol (0,1%)	10 min, 20°C	789
H_2O_2 (0,1%)	10 min, 20°C	605
TLCK (0,1%)	10 min, 20°C	730
Cholesterol (0,1%)	10 min, 20°C	533

According to Feder *et al.* (1994), hemolytic activity of *S. suis* was not detectable after 24 h at 24°C and after treatments of supernatants with amylase or trypsin. This study showed that hemolytic activity of *S. suis* in the culture supernatant appeared to be heat labile and susceptible to proteinase K treatment. These findings indicated the presence of a soluble proteinaceous hemolysin in the culture supernatant. Heat labile hemolysins are also reported for *Actynomyces pyogenes* (Ding, 1994), *Clostridium botulinum* (Haque *et al.*, 1992). The *S. suis* hemolysin, like many other bacterial hemolysins are probably proteins (Feder *et al.*,

1994; Haque *et al.*, 1992). Jacobs *et al.* (1994) described the hemolysin produced by *S. suis* type 2 was thiol-activated hemolysin and designated as suilysin. Partial restoration of hemolytic activity which are resulted after addition of excess ß-mercaptoethanol to the TLCK-treated preparation explained the presence of oxidized thiol groups (Jacobs *et al.*, 1994). In this study, TLCK reduced hemolytic activity of *S. suis* strain D282 (serotype 2). Reducing agent of hemolytic activity in this study might be free cholesterol which is a potent inhibitor of cytolytic activity (Jacobs *et al.*, 1994).

Analysis of the hemolysin of *S. suis* strain D282 used in this study by SDS-PAGE showed that the hemolysin had molecular weight of about 31,000-55,000. Jacobs *et al.* (1994), reported that suilysin had apparent molecular mass of 54,000, for other thiol-activated toxin found the molecular weight was within the range of 52,000 to 60,000. *S. suis* cultures used in this study caused a-hemolysis (8 cultures) and ß-hemolysis (2 cultures) on sheep blood agar plates. All 10 cultures were screened for their hemolytic activities, and six cultures showed hemolytic activity. The hemolytic units varied among the cultures (Table 1).

Most of the different serotype strains tested appeared to secrete hemolysin. In this study suggested that hemolysin activity was detected in 6 (serotype 1, 2, 4, 11 and NT) of 9 S. suis cultures. Feder *et al.* (1994) screened 23 serotypes (1 to 22 and 1/2) of *S. suis* for their hemolytic activities, 10 serotypes (1/2, 1, 2, 4, 5, 14, 15, 17, 19 and 20) showed hemolytic activity. The role of hemolysin produced by *S. suis* is probably related to listeriolysin (Chanter *et al.*, 1993). Lack of listeriolysin (streptolysin O produced by *Listeria monocytogenes*) caused an inability of mutant listeriae to leave the phagolysosome and reside in the cytoplasm where they would escape from the bactericidal mechanisms of the macrophage. The possibility of macrophage modulating activity for the hemolysin of *S. suis* and its role in the intracellular survival, need further investigation.

Conclusions

The purified hemolysin of *S. suis* strain D282 had an apparent molecular mass of 31,000-55,000 and exhibited a specific activity of 890 U/ml. Hemolytic activity was reduced after incubation at 37°C for 3 hours and after the addition of proteinase K. Most of the different serotype strains appeared to secrete hemolysin.

Acknowledgment

The author gratefully acknowledged Prof. Dr. Christoph Lämmler for advice and Prof. Dr. Soesanto Mangkoewidjojo, M.Sc. for correcting the manuscript. This work was supported by the Univesity Research for Graduate Education (URGE) project, No. 009/PDB/URGE/1996.

References

Arends, J.P. and Zanen, H.C. (1988) Meningitis caused by *Streptococcus suis* in human. *Rev. Infect. Dis.* 10: 131-137.

Chanter, N., Jones, P.W. and Alexander, T.J.L. (1993) Meningitis in pigs caused by *Streptococcus suis*-a speculative review. *Vet. Microbiol.* 36: 39-55

Dharma, D.M.N., Soeharsono, N.L.D., Supartika, K.E. dan Dibia, I.N. (1994) Wabah "Streptococcal meningitis" pada babi dan kera di Bali. Kongres XII dan Konferensi Ilmiah Nasional VI PDHI di Surabaya.

Ding, H. (1994) Untersuchungen zu mutmaßlichen Pathogenitätsfaktoren von *Actinomyces pyogenes*. Vet. Med. Diss. Justus-Liebig-Universität Gießen.

Elliott, S.D., Mc Carty, M. and Lancefield, R.C. (1977) Teichoic acid of group D streptococci with special reference to strains from pig meningitis. *J. Exp. Med.* 145: 490-499.

Estoepangestie, S. and Lämmler, C. (1993) Distribution of capsular types 1 to 28 and further characteristics of *Streptococcus suis* isolates from various European countries. *Zbl. Bacteriol.* 279: 394-403.

Feder, I., Chengappa, M.M., Fenwick, B., Rider, M., and Staats, J. (1994) Partial characterization of *Streptococcus suis* type 2 hemolysin. *J. Clin. Microbiol.* 32: 1256-1260.

Gottschalk, M., Lebrun, A., Jacques, M. and Higgins, R. (1990) Hemagglutination properties of *Streptococcus suis*. *J. Clin. Microbiol.* 28: 2156-2158.

Gottschalk, M., Higgins, R., Jacques, M. and Dubreuil, D. (1992) Production and characterization of two *Streptococcus suis* capsular type 2 mutants. *Vet. Microbiol.* 30: 59-71.

Haque, A., Sugimoto, N., Horiguchi, Y., Okabe, T., Miyata, T., Iwanaga, S. and Matsuda, M. (1992) Production, purification and characterization of botulinolysin, a thiol-activated hemolysin of *Clostridium botulinum*. *Infect. Immun.* 60: 71-78.

Higgins, R. and Gottschalk, M. (1990) Review Article: An update on *Streptococcus suis* identification. *J. Vet. Diagn. Invest.* 2: 249-252.

Jacob, A.A.C., Loeffen, P.L.W., van den Berg, A.J.G., and Storm, P.K. (1994) Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. *Infect. Immun.* 62: 1742-1748.

Salasia, S.I.O. (1994) Untersuchungen zu mutmaßlichen Pathogenitätsfaktoren von *Streptococcus suis*. Vet. Med. Diss. Justus-Liebig-Universität Gießen.

Salasia, S.I.O., Lämmler, C., and Devriese, L.A. (1994) Serotypes and putative virulence markers of *Streptococcus suis* isolates from cats and dogs. *Res. Vet. Sci.* 57: 259-261.

Salasia, S.I.O. and Lämmler, C. (1994) Occurrence of haemagglutinating adhesin among virulent and avirulent isolates of *Streptococcus suis*. *Med. Sci. Res.* 22: 763-764.

Salasia, S.I.O. and Lämmler, C. (1995) Distribution of serotype, virulence markers and further characteristics of *Streptococcus suis* isolates from pigs. *J. Vet. Med.* B42: 78-83.

Salasia, S.I.O., Lämmler, C., and Herrmann, G. (1995) Properties of a *Streptococcus suis* isolate of serotype 2 and two capsular mutants. *J. Vet. Microb.* 45: 151-156.

Vecht, U., van Leengoed, L.A.M.G and Verheijen, E.R.M. (1985) *Streptococcus suis* infections in pigs in the Netherlands (part one). *Vet. Quart.* 7: 315-321.

Vecht, U., Wisselink, H.J., Jellema, M.L., and Smith, H.E. (1991) Identification of two proteins associated with virulence of *Streptococcus suis* type 2. *Infect. Immun.* 59: 3156-3162.

Williams, A.E., and Blackmore, W.F. (1990) Monocyte-mediated entry of pathogens into the central nervous system. Neuropathol. *Appl. Neurobiol.*, 16: 377-392.