Detection of immunoglobulin-G-binding proteins in *Streptococcus suis*

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This study was undertaken to search for the presence of immunoglobulin G (IgG)-binding proteins in *Streptococcus suis*, an important swine pathogen. Whole bacterial cells were incubated with human or pig IgG conjugated to gold particles and examined by transmission electron microscopy. Cells of some *S. suis* strains were labelled as were cells of the positive control strain, *Staphylococcus aureus* Cowan I. Binding of pig and human IgG to five different bacterial species of group D streptococci, to reference strains representing the 29 capsular types of *S. suis*, and to 12 *S. suis* capsular type 2 strains was then examined using Western blotting. All strains interacted with pig and human IgG, although the binding profiles were slightly different. A 52 kDa protein was observed in all capsular types of *S. suis*. This protein, absent in other group D streptococcal species, was observed in all capsular type 2 isolates originating from diseased or clinically healthy pigs, and was shown to bind human IgG-Fc fragments. The IgG-binding activity was also observed in the culture supernatant and was sensitive to proteolysis.

Introduction

Several bacterial species express surface proteins that interact with immunoglobulin (Ig) G molecules in non-immune reactions. These binding proteins have been reported on staphylococci (Forsgren & Sjöquist, 1966), streptococci of serological groups A, B, C, G, L and U (Kronvall, 1973; Chhatwal *et al.*, 1985), and other micro-organisms such as *Haemophilus somnus*, *Clostridium perfringens* and *Mycoplasma salivarium* (Lindahl & Kronvall, 1988; Yarnall *et al.*, 1988; Sawa *et al.*, 1992).

Based on the non-immune reactivity of intact bacteria with various IgG species and subclasses, a functional classification was established and six different types of IgG-binding proteins were defined (Myhre & Kronvall, 1981; Reis *et al.*, 1988). The most extensively studied is type I, expressed by the majority of *Staphylococcus aureus* strains and designated protein A (Boyle, 1990), followed by type III, found on the surface of most human groups C and G streptococcal strains and commonly designated protein G (Björck & Akerström, 1990). The type II, associated with group A streptococci, represents the most diverse and heterogeneous group of IgG-binding proteins (Reader *et al.*, 1991). Type IV is found on certain bovine group G streptococci, and types

V and VI are found on different *Streptococcus zooepidemicus* strains (Myhre & Kronvall, 1981).

The presence of immunoglobulin-binding proteins on the surface of different pathogens led to the suggestion that these molecules provide pathogens with the potential to evade or elude the host defences by interfering with opsonization, phagocytosis or complement consumption (Widders, 1990).

The presence of an IgG-binding activity has been reported on some human group D streptococci (Christensen & Kronvall, 1974). *Streptococcus suis*, which also belongs to the Lancefield group D, is a common cause of septicaemia, meningitis and other serious infections in pigs (Higgins & Gottschalk, 1990). It has also been isolated from other animal species (Hommez *et al.*, 1988; Higgins *et al.*, 1990), as well as from humans (Arends & Zanen, 1988; Trottier *et al.*, 1991). Twenty-nine capsular types of *S. suis* have been described (Gottschalk *et al.*, 1991). In many countries, capsular type 2 is considered as the most virulent and prevalent capsular type in diseased pigs (Higgins *et al.*, 1992), but isolates belonging to this capsular type have also been found in nasal cavities of clinically healthy pigs (Brisebois *et al.*, 1990). Little is known about the virulence factors involved in the pathogenesis of *S. suis* infections. It has been shown that surface components such as the capsular polysaccharides and some cell wall proteins as well as an extracellular protein could be associated with the virulence of *S. suis*

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capsular type 2 (Vecht *et al.*, 1991; Gottschalk *et al.*, 1992). The purpose of the present study was to search for the presence of IgG-binding proteins in *S. suis*.

Methods

Bacterial strains and growth conditions. Reference strains of *S. suis* capsular types 1 through 8 and 1/2 were kindly supplied by Jorgen Henriksen, Statens Serum Institute, Copenhagen, Denmark. Reference strains of *S. suis* capsular types 9 through 28, *Enterococcus faecalis* (ATCC 19433), *Enterococcus faecium* (ATCC 2440), *Streptococcus bovis* (ATCC 9809), *Streptococcus equinus* (77-1020), *Staphylococcus aureus* Cowan I (ATCC 12598) and *Staphylococcus epidermidis* (ATCC 14990) were from our laboratory. Twelve *S. suis* capsular type 2 field strains originating from clinically healthy or diseased pigs and from different geographical areas were selected according to data obtained from studies based on restriction endonuclease fingerprinting and ribotyping (Beaudoin *et al.*, 1992). A non-capsulated *S. suis* strain (92-2872), obtained from our laboratory, was also included in the study. All *S. suis* strains were identified with biochemical tests (Sanford & Higgins, 1992), and capsular typing was performed using the coagglutination and the capsular reaction tests (Higgins & Gottschalk, 1990). The Lancefield group D antigen was detected using a coagglutination reagent (Phadebact; Pharmacia Diagnostic). Bacteria were grown on blood agar plates (trypticase soy agar containing 5%, v/v, bovine blood; BBL) and in Todd-Hewitt broth (Difco) for 18–24 h at 37 °C in a 5% CO₂ atmosphere.

Electron microscopy. (i) *Whole cell labelling.* Colloidal gold particles (15 nm) were prepared by the sodium citrate method as described by Frens (1973). The optimal concentration of human or pig IgG (Sigma) needed to stabilize the colloidal gold solution was estimated by a salt flocculation test (Bendayan, 1984). IgGs were then mixed with the colloidal gold, and these complexes were sedimented by ultracentrifugation at 60000 g for 40 min. The pellet was resuspended in 0.01 M-phosphate-buffered saline (PBS; 0.01 M-potassium phosphate, 0.85% NaCl, pH 7.2); pH 7.2 and stored at 4 °C. A drop of a bacterial suspension was placed on Formvar-coated grids for 1 min. After blocking for 5 min with 1% (w/v) bovine albumin, grids were placed on drops of colloidal gold particles conjugated to human or pig IgG for 30 min. Grids were then rinsed with distilled water, and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV.

(ii) *Post-embedding labelling.* Bacterial cells were fixed for 2 h at room temperature in 0.1 M-cacodylate buffer pH 7.2 containing 1% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde. Bacteria were then immobilized in 4% agar (w/v), washed five times in cacodylate buffer and placed in 30%, then in 40% (v/v) ethanol, both for 30 min at 4 °C. The bacterial cells were then processed through the following ethanol dehydration series with 30 min incubation at –20 °C: 50%, 60%, 70%, 80%, 90% and 100% (v/v) ethanol. The following embedding procedure also was done at –20 °C. Bacteria were transferred to Lowicryl K4M resin/ethanol (1:1, v/v) for 60 min. Further transfers included resin/ethanol (2:1) and pure resin, both for 60 min. Infiltration was continued in pure resin overnight. Cells were transferred to pure resin in Beem capsules. Polymerization was done in a UVC1 Cryo chamber (Ted Pella Inc.) by illumination with a 360 nm UV light at –35 °C for 48 h, and then at room temperature for a further 4 d. Blocks, stored with desiccant at 4 °C until use, were sectioned. Thin sections were labelled with IgG–gold as described above, and were stained with uranyl acetate and lead citrate.

SDS-PAGE and Western blot analysis. SDS-PAGE was done by using the discontinuous buffer system according to Laemmli (1970), with a 4.5% (w/v) polyacrylamide stacking gel and a 12.5% (w/v)

polyacrylamide running gel. Samples were mixed with solubilization buffer (2%, w/v, SDS, 10%, w/v, glycerol, 5%, v/v, β -mercaptoethanol, 0.025% bromophenol blue in 62.5 mM-Tris/HCl, pH 6.8) to a final concentration of 0.1 g ml^{–1}, and boiled for 10 min. Low-molecular-mass markers and prestained low-molecular-mass markers obtained from Bio-Rad were used. Gels were run on a Mini-Protean II vertical slab electrophoresis cell (Bio-Rad). Western blot analysis was done as described by Towbin *et al.* (1979) with some modifications. The separated proteins were transferred to a nitrocellulose membrane (Bio-Rad) which was then blocked with 2% (w/v) skim milk in Tris-buffered saline (TBS; 10 mM-Tris/HCl, 150 mM-NaCl, pH 7.4) for 60 min at room temperature, and incubated with human or pig IgG at a concentration of 10 μ g ml^{–1} in the blocking solution overnight at 4 °C; control membranes were incubated overnight without IgG. The membrane was washed four times in TBS containing 0.02% (v/v) Tween 20, then reacted with horseradish-peroxidase-conjugated goat anti-human or anti-pig IgG (Jackson ImmunoResearch Laboratories) also in blocking solution. After a subsequent washing, bands were developed by adding a solution of 4-chloro-1-naphthol. For the detection of human IgG (Fc)-binding activity, the membrane was incubated with 10 μ g ml^{–1} of peroxidase-labelled human Fc fragments (Jackson ImmunoResearch Laboratories) for 2 h at room temperature, and was developed as described above.

Proteolytic treatment. Agar-grown bacteria were treated with proteinase K (1 mg ml^{–1}; Sigma) in 50 mM-Tris/HCl, pH 8.0, containing 1 mM-CaCl₂; the mixture was incubated at 60 °C for 60 min. Treated bacterial cells were examined for their IgG-binding activity by electron microscopy.

Detection of IgG-binding proteins in culture supernatants. Bacteria, grown in liquid medium overnight at 37 °C with agitation, were removed by centrifugation at 10000 g for 10 min and filtration through a 0.2 μ m membrane (Nalge Co.). The cell-free culture supernatant was concentrated 20-fold by ultrafiltration using a YM10 membrane (Amicon). The concentrated supernatants were subjected to SDS-PAGE and Western blot analysis as described above.

Results

Pig IgG-binding activity

Colloidal gold particles (15 nm) were conjugated to pig IgG, and these complexes were allowed to react with whole bacterial cells. The IgG-binding activity was examined by electron microscopy, and it was observed that the surface of the non-capsulated strain 92-2872 was labelled (Fig. 1a).

Approximately 70 gold particles (on average) were distributed uniformly on the cell surface. This labelling was similar to the one observed with the positive control, *Staphylococcus aureus* Cowan I (Fig. 1b). Labelling was absent or weak (less than 8 gold particles by cell) on the surface of capsulated strains of *S. suis* (Fig. 1c), similar to that observed with the negative control, *Staphylococcus epidermidis* (data not shown). The IgG-binding activity was also examined on thin sections of *S. suis* capsular type 2, and labelling of the cytoplasm and the cell wall region was observed (Fig. 1d).

Binding of pig IgG to five different species of group D streptococci, to the reference strains representing the 29 capsular types of *S. suis*, and to 12 *S. suis* capsular type

Fig. 1. Transmission electron micrographs of *S. suis* 92-2872 (a), *Staphylococcus aureus* Cowan I (b) and *S. suis* capsular type 2 (c) whole cells, as well as thin sections of *S. suis* S735 (d) labelled with pig IgG conjugated to gold particles (15 nm). Bars, 200 nm.

2 strains was then examined using Western blotting. Negative (*S. epidermidis*) and positive (*Staphylococcus aureus*) bacterial controls were included in each test. All strains interacted with pig IgG. Analysis of five group D streptococcal species revealed the presence of distinct IgG-binding profiles (Fig. 2a). *S. suis* was represented by

the reference strain of capsular type 2 (lane 3). Molecular masses of the major binding proteins varied between 52 and 80 kDa, and no common protein was observed between *S. suis* capsular type 2 and the other group D species, which were *E. faecalis* (lane 1), *E. faecium* (lane 2), *S. equinus* (lane 4) and *S. bovis* (lane 5).

Fig. 2. Western blot of whole bacterial cells probed with pig IgG. (a) Lanes: 1, *E. faecalis* 19433; 2, *E. faecium* 2440; 3, *S. suis* capsular type 2; 4, *S. equinus* 77-1020; 5, *S. bovis* 9809. (b) Lanes 1 to 4, reference strains of *S. suis* capsular types 7, 2, 3 and 16 respectively. (c) Lanes 1 to 4, different field strains of *S. suis* capsular type 2. The arrowhead indicates the 52 kDa binding protein. Molecular mass markers (in kilodaltons) are indicated on the left.

Fig. 3

Fig. 3. Western blot of whole bacterial cells of *S. suis* probed with human IgG. Lanes 1 to 4, reference strains of capsular types 7, 3, 16 and 2 respectively; lanes 5 and 6, reference strains of *S. suis* capsular type 2 and *S. suis* 92-2872, respectively, probed with human IgG-Fc fragments conjugated to peroxidase. The arrowhead indicates the 52 kDa binding protein. Molecular mass markers (in kilodaltons) are indicated on the left.

Fig. 4

Fig. 4. Western blot of whole bacterial cells of *S. suis* capsular type 2 grown on blood agar plates (lane 1) or in Todd-Hewitt broth (lane 2), of *S. suis* capsular type 2 Todd-Hewitt broth culture supernatant (lane 4), and of a control, non-inoculated Todd-Hewitt broth concentrated 20-fold (lane 3) probed with pig IgG. The arrowhead indicates the 52 kDa binding protein. Molecular mass markers (in kilodaltons) are indicated on the left.

The 29 reference strains of *S. suis* capsular types were examined to determine whether these strains displayed IgG-binding activity, and had similar binding profiles. Molecular masses of the major IgG-binding proteins varied between 52 and 70 kDa; minor bands of higher molecular mass (> 80 kDa) were also present (Fig. 2*b*). No characteristic profile was associated with strains from diseased pigs (e.g. capsular types 2, 1/2, 3, 8), or from

clinically healthy pigs (e.g. capsular types 17, 18, 21). All 29 reference strains of *S. suis* presented a common protein with an approximate molecular mass of 52 kDa.

Twelve capsular type 2 isolates from clinically healthy pigs, or animals with septicaemia, pneumonia or meningitis, were analysed by Western blot. These capsular type 2 strains expressed a more homogeneous IgG-binding profile (Fig. 2*c*). No particular binding profile

could be associated to strains from diseased (meningitis, lane 1; pneumonia, lane 2; septicaemia, lane 3) or healthy (lane 4) pigs. The common 52 kDa protein, observed in all *S. suis* capsular types, was also present in all capsular type 2 strains tested.

Human IgG-binding activity

Using electron microscopy, the surface of the non-capsulated *S. suis* strain was labelled with human IgG-gold particles, and the density of the labelling was similar to the one observed previously with pig IgG-gold. Thin sections of *S. suis* capsular type 2 were also labelled with human IgG-gold, and labelling was observed on the cell wall and in the cytoplasm (data not shown). By Western blotting analysis, proteins reacting with pig IgG also bound human IgG. In addition, proteins in the 33 to 40 kDa region were observed (Fig. 3, lanes 1 to 4). The non-capsulated *S. suis* strain 92-2872 as well as the different capsular types reacted with human IgG (Fc) fragments using Western blotting; the proteins in the 33 to 40 kDa region did not bind the Fc fragment (Fig. 3, lanes 5 and 6), but the 52 kDa protein did. After treatment of *S. suis* cells with proteinase K, no binding activity was observed by electron microscopy analysis; both human and pig IgG-binding activities were completely abolished.

IgG-binding proteins in the culture supernatant

IgG-binding activity was observed in *S. suis* culture supernatants (Fig. 4, lane 4). The binding profile was similar to that of whole bacterial cells grown on blood agar plates (lane 1) or in Todd-Hewitt broth (lane 2).

Discussion

The ability to bind IgG by a non-immune mechanism is a property shared by certain staphylococcal strains and streptococcal groups (Forsgren & Sjöquist, 1966; Kronvall, 1973). This property has been reported once for some human group D streptococci (Christensen & Kronvall, 1974). The results of this study demonstrate the presence of human and pig IgG-binding activities in five species of group D streptococci, particularly in the important swine pathogen *S. suis*. The binding was mediated by a proteinaceous molecule since activity was abolished following digestion with proteinase K.

The five group D streptococcal species as well as all *S. suis* strains tested bound both pig and human IgG. A variation in the molecular mass of the different IgG-binding proteins was observed between the group D species. This heterogeneity was less pronounced among the 29 capsular types of *S. suis*, and even less within

strains of the same capsular type. This is in agreement with previous studies which demonstrated variations in the molecular mass of Ig-binding proteins of groups B and C streptococci (Yarnall & Widders, 1990). Studies, in group B streptococci, suggest that the β antigen which binds the IgA Fc fragment is expressed as a low-molecular-mass molecule, but can be processed to a higher molecular mass (Brady & Boyle, 1990). These findings could explain the presence of multiple bands of molecular mass > 52 kDa in *S. suis*. Field strains of *S. suis* capsular type 2 of different origins (pneumonia, meningitis, septicaemia or healthy animals) were genotypically characterized by fingerprinting and ribotyping (Beaudoin *et al.*, 1992). Our results did not demonstrate any correlation between the IgG-binding profile of *S. suis* strains and their origin. Interestingly, a 52 kDa protein was observed in all *S. suis* capsular types examined, and was characteristic of this species. Variation in the expression level of this protein was, however, noted among the different capsular types. This protein was also able to bind human IgG (Fc) fragments, which suggests that the 52 kDa IgG-binding protein of *S. suis* is a Fc-receptor.

Like the majority of bacterial IgG-binding proteins (Yarnall *et al.*, 1988), the *S. suis* proteins were associated with the cell surface, and also released in a soluble form during bacterial growth. Their presence, observed by electron microscopy, in the central region of the cell could be explained because they are synthesized in the cytoplasm, then carried to the cell surface. These proteins have an important role in virulence, perhaps by favouring bacterial persistence *in vivo* (Widders, 1990).

It is quite possible that the capsule layer of *S. suis* (Jacques *et al.*, 1990) masked the binding proteins because, using electron microscopy, they were best observed on the surface of the non-capsulated *S. suis* strain but could be detected on sections of capsulated strains. This might explain why Lämmler *et al.* (1986) did not observe any IgG-binding activity on the surface of cells from six different group D streptococcal cultures.

This study has shown that group D streptococci exhibit pig and human IgG-binding activities, and that a 52 kDa IgG-binding protein is present in all *S. suis* strains examined. This protein may therefore represent a common molecular marker for the rapid identification of *S. suis*. Further studies will be needed to determine the role of these IgG-binding proteins in *S. suis* pathogenesis.

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