Development and evaluation of an indirect ELISA for detection of exfoliative toxin ExhA, ExhB or ExhC produced by *Staphylococcus hyicus*

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Abstract

Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA) confirmed previous reports that the *Staphylococcus hyicus* exfoliative toxins ExhA and ExhB are metalloproteins, and further indicated that ExhC is also a metalloprotein. An indirect ELISA was developed for the detection of toxigenic strains as an alternative method to the use of phage typing for selection of *S. hyicus* isolates to be used in autogenous vaccine against exudative epidermitis in pigs. The indirect ELISA was evaluated by investigating the presence of toxin among a total of 655 *S. hyicus* isolates from 69 pig skin samples, one from each of the 69 pig herds with outbreak of exudative epidermitis. Toxigenic *S. hyicus* were detected in 74% of the cases by ELISA. From each of the five cases, in which initially no toxigenic *S. hyicus* were found, a further 40 *S. hyicus*-like colonies were tested in ELISA. Testing of this number of colonies has a >99% probability of disclosing toxigenic *S. hyicus*. Toxin-producing isolates were found in only two of the five cases investigated. This may indicate the existence of one or more variants of the exfoliative toxin of *S. hyicus* that are not detected in the indirect ELISA or that *S. hyicus* may be displaced from lesions of exudative epidermitis. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Exudative epidermitis (EE) in pigs is caused by infection with virulent strains of *Staphylococcus hyicus* (Amtsberg, 1979; Wegener et al., 1993; Tanabe et al., 1996). Sale

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of autogenous vaccine for prevention of EE produced at the Danish Veterinary Laboratory (DVL) has, from 1986 to 1997, increased from 13,260 to 77,880 doses per year (Anno., Annual Report, 1986–1997, Danish Veterinary Laboratory and Danish Veterinary Institute for Virus Research) indicating that EE in pigs has become of increasing significance during the past decade in the Danish pig industry. Previously, phage and antibiogram typing and plasmid profiling in combination have been recommended for selection of *S. hyicus* isolates for production of autogenous vaccine against EE (Wegener, 1993). At DVL phage typing alone is used in selection of isolates for autogenous vaccines. Representative phage types found among up to 10 isolates of *S. hyicus* are selected and used for preparation of a mixed autogenous vaccine. *S. hyicus* is part of the normal skin flora of pigs (Devriese et al., 1985) and can be characterised as either virulent or avirulent on the basis of their ability to induce EE (Amtsberg, 1979; Wegener et al., 1993; Tanabe et al., 1996). Virulent *S. hyicus* produce exfoliative toxin of \( \approx 27 \text{kDa} \) (Tanabe et al., 1993) or 30 kDa (Andresen et al., 1997) and purified toxins caused characteristic alterations in the skin of piglets similar to the lesions of EE. Recently, three antigenic variants of the toxin have been identified and designated ExhA, ExhB and ExhC, respectively (Andresen, 1998). Although the production of toxin variants was predominantly associated with certain phage groups, toxin producing (toxigenic) isolates could be assigned to each of the known phage groups. Thus, phage typing could not presumably be used to identify toxigenic isolates. Studies have indicated that the *S. hyicus* exfoliative toxins ExhA and ExhB are metalloproteins, and that the interactions between the toxins and the monoclonal antibodies MabEXH7.7 and MabEXH5.1, directed against ExhA and ExhB, respectively, are stabilised by the presence of certain divalent metal ions (Andresen, 1999).

The aim of this work was to develop an indirect enzyme-linked immunosorbent assay (ELISA) for discrimination between isolates of *S. hyicus* that produce ExhA, ExhB or ExhC and isolates that do not produce any of these toxins. The ELISA was developed in order to select *S. hyicus* isolates for the preparation of autogenous vaccines against EE. The ELISA makes use of the metalloprotein nature of the exfoliative toxins and was evaluated by investigating the presence of toxin producing *S. hyicus* from pig herds with EE.

2. Materials and methods

2.1. Strains

*S. hyicus* strains NCTC 10350 (Devriese et al., 1978), 1289D-88 and 842A-88 (Wegener et al., 1993) were used as strains of reference with regard to production of the exfoliative toxins ExhA, ExhB and ExhC, respectively (Andresen, 1998). *S. hyicus* strains A3793/76, A4596/76, A72/75, 842G-88, 842J-88, 1403B-88, 1403H-88, 1289E-88 and SK170, previously shown to be avirulent (Wegener et al., 1993) and not producing ExhA, ExhB or ExhC (Andresen, 1998), were used to define the optical density (OD) values for non-toxigenic *S. hyicus* in the indirect ELISA. Additionally, three sets of 30 toxin producing *S. hyicus* each (Andresen, 1998), representing different phage groups, and the
three variants of the toxin were used to establish the OD values for toxigenic *S. hyicus* in the indirect ELISA.

2.2. **Growth media**

Bacteria from the skin of pigs were cultivated on selective/indicative medium (Devriese, 1977) (primary plates). *S. hyicus* was subcultivated on Columbia agar-base (Oxoid, Unipath, Basingstoke, UK) plates containing 5% bovine blood (C-blood agar). Liquid growth medium was 30 g l\(^{-1}\) Trypticase soy broth (4311768, Becton Dickinson, Cockeysville, MD) supplemented with 10 g l\(^{-1}\) yeast extract (Oxoid, L21); pH was adjusted to 7.2 before autoclaving the medium. The liquid growth medium was used either with both 0.5 mM CoCl\(_2\) and 0.5 mM ZnSO\(_4\) added after sterilization or without these supplementary metal salts. Cultures were grown for toxin assay in tightly closed 10-ml tubes containing 5 ml liquid growth medium inoculated with a single colony from an overnight culture on C-blood agar. Cultures were incubated for 18–24 h at 37°C with shaking at 130 rpm.

2.3. **SDS-PAGE and immunoblotting**

Ten-microlitre samples were analyzed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared from liquid culture supernatant mixed with sample buffer 1 : 1. SDS-PAGE (Laemmli, 1970) was performed using 4% polyacrylamide (50 V, constant voltage) in the stacking gel and 16.6% polyacrylamide (200 V, constant voltage) in the separation gel prepared from a 40% acrylamide : N,N’-methylenebisacrylamide mixture (29 : 1, 3.3% C) (Bio-Rad, Hercules, CA). Transfer of proteins separated in SDS-PAGE gels to nitrocellulose membranes was performed using the semi-dry blotting technique (Kyhse-Andersen, 1984). The monoclonal antibodies MabEXH7.7 (Andresen, 1998) and MabEXH5.1 (Andresen et al., 1997), raised against ExhA and ExhB, respectively, and polyclonal rabbit antibodies raised against ExhC (Andresen, 1998) were used as primary antibodies. MabEXH7.7 reacted with all three types of exfoliative toxin (Fig. 1). MabEXH5.1 was specific for ExhB. Visualisation of bands in immunoblots was performed using alkaline phosphatase conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulins (D0486 or D0487, Dako, Glostrup, Denmark) and the 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazonium staining method (Harlow and Lane, 1988).

2.4. **Enzyme linked-immunosorbent assay (ELISA).**

Indirect ELISA was performed by coating ELISA plates (MaxiSorp 442404, Nalge Nunc International, Roskilde, Denmark) with heat-treated culture supernatant. After cultivation of the isolates in a liquid growth medium the supernatant was isolated by centrifugation. In order to facilitate the adsorption of the exfoliative toxin to the polystyrene surface of the ELISA plate, samples of supernatant were heated for 10 min in a boiling water bath. The heat-treated supernatant was subsequently coated on an ELISA plate in a 1 : 2 dilution in 200 mM carbonate buffer (60 mM Na\(_2\)CO\(_3\), 140 mM NaHCO\(_3\),
pH 9.6) in duplicate (100 µl/well) and left at 4°C overnight. On each plate supernatants from Staphylococcus hyicus strains NCTC 10350 and 1289D-88 were included as positive controls and 10 wells containing only carbonate buffer were used for determining the background OD value. Plates were washed four times in a washing buffer between each step in the ELISA procedure, using an ELISA plate washer (Microwash II, Skatron Instruments, Lier, Norway). Washing buffer was phosphate buffered saline (PBS) (2.3 mM KH₂PO₄, 7.7 mM Na₂HPO₄, 140 mM NaCl, pH 7.2) with 0.05% Tween-20.

The plates were pre-incubated with 0.05% Tween-20, 1% bovine serum albumin in PBS (PBS-T-BSA) (200 µl/well) for 1 h. All antibodies were diluted in PBS-T-BSA (100 µl/well) and the incubation time was 1 h in each step. Exfoliative toxins were detected using the monoclonal antibodies MabEXH7.7 and MabEXH5.1 in a mixture as primary antibodies. Secondary antibodies were anti-mouse Ig horseradish peroxidase linked F(ab’)₂ fragment polyclonal sheep antibodies (NA9310, Amersham, UK). A solution containing 670 µg ml⁻¹ 2,3,5-triphenyltetrazolium chloride and 0.0125% H₂O₂ dissolved in 35 mM citric acid, 67 mM Na₂HPO₄ buffer, pH 5.0, was used as substrate (100 µl/well). Colour was allowed to develop for 20 min and the reaction was stopped by the addition of 0.5 M sulphuric acid (150 µl/well). Optical density (OD) was measured at 490–650 nm.

Fig. 1. Immunoblot analysis of culture supernatant from Staphylococcus hyicus strains grown with, and without, addition of Co²⁺ and Zn²⁺ to the liquid growth medium using MabEXH7.7 as primary antibody. Lane M: molecular weight markers; lanes 1 and 2: strain NCTC 10350 producing ExhA; lanes 3 and 4: strain 1289D-88 producing ExhB; lanes 5 and 6: strain 842A-88 producing ExhC; lanes 1, 3 and 5: strains grown without additional metal ions; lanes 2, 4 and 6: strains grown with 0.5 mM Co²⁺ and 0.5 mM Zn²⁺ added. In lane 2, MabEXH7.7 reacted as previously observed (Andresen, 1998) with a few minor bands, which could be due to degradation of the toxin or to heterogeneity of the toxin.
by dual wavelength endpoint, read in an ELISA plate reader (V max, Molecular Devices, Menlo Park, CA).

2.5. Sampling and identification of S. hyicus field isolates

From each of 69 pig herds, a skin fragment or carcass exhibiting EE was submitted to the Danish Veterinary Laboratory during the period August 1997 to May 1998. Isolates were randomly picked from primary plates in sets of 10 S. hyicus per specimen, for routine testing. In cases where colonies were sparse, the sample size was reduced to 4–9 isolates. S. hyicus was identified as described previously (Andresen, 1998). A total of 655 field isolates of S. hyicus were obtained from the skin of 69 pigs with EE. Among specimens from which all the S. hyicus isolates initially tested were found non-toxigenic in the indirect ELISA, five specimens were selected for a further investigation. In these five cases, the initially tested sets contained 9 (2 sets) or 10 (3 sets) S. hyicus isolates. This prompted a test of a larger number (n = 40) of S. hyicus-like colonies from each of the five cases in order to confirm or disprove the initially obtained result. The number of colonies chosen was 40, because it would give a probability of 99% for the coming test result to be correct. This assumption was based on previous results (Andresen, 1998), indicating that toxigenic S. hyicus could be detected in 72% of 60 sets of S. hyicus, each set comprising 8–10 isolates. From primary plates of each of the five specimens non-pigmented, lipase-positive colonies that were non-haemolytic when subcultivated on C-blood agar were isolated. Subsequently, the 5 × 40 S. hyicus-like colonies were tested for production of exfoliative toxin in ELISA.

3. Results

3.1. Development of indirect ELISA

S. hyicus strains NCTC 10350, 1289D-88 and 842A-88 were grown in a liquid medium with, and without, addition of both, 0.5 mM CoCl₂ and 0.5 mM ZnSO₄ and supernatants from these cultures were analysed in immunoblotting using MabEXH7.7 as primary antibody. Fig. 1 shows that all three types of exfoliative toxin were detected with higher intensities of their bands when grown with metal salts added to the growth medium. This result was confirmed in indirect ELISA. Table 1 shows the results of the indirect ELISA performed on supernatant from isolates of S. hyicus producing ExhA, ExhB or ExhC and from non-toxigenic S. hyicus strains grown in a liquid medium with, and without, 0.5 mM CoCl₂ and 0.5 mM ZnSO₄ added using a mixture of MabEXH5.1 and MabEXH7.7 as primary antibodies. The results show that the OD values of the toxigenic isolates were increased when the metal salts were included in the growth medium. The mean background OD value ± standard deviation (SD) of wells incubated with carbonate buffer only during the antigen coating step of the ELISA-procedure was 0.05 ± 0.03 which was not significantly different from the mean OD value ± SD of the non-toxigenic strains (Table 1). Using a metal salt-supplemented liquid growth medium, the mean OD values ± SD of the ELISA-measurements on the supernatants from the nine non-toxigenic strains and from the 30 ExhC producing isolates were 0.06 ± 0.04 and
The cut-off value of the ELISA was set to 0.200 which was a value just above the mean OD value of the non-toxigenic strains + 3SD and lower than the mean OD value of the ExhC-producing isolates – 1SD. For an isolate to be considered positive for production of ExhA, ExhB or ExhC, both OD values in a double determination should be ≥0.200.

Ten out of the 30 ExhC-producing S. hyicus isolates were selected for investigating if one or both the metal ions Co^{2+} and Zn^{2+} had an enhancing effect on the detection of ExhC in the ELISA. Mean OD values ± SD were 0.5 ± 0.1, 0.07 ± 0.01 and 0.7 ± 0.2, when the 10 ExhC producing isolates were cultivated in the presence of 0.5 mM ZnSO_{4}, 0.5 mM CoCl_{2} and 0.5 mM of both ZnSO_{4} and CoCl_{2}, respectively. The mean OD value ± SD for cultivation of the 10 ExhC producing isolates grown without additional metal salt was 0.07 ± 0.01, i.e. the same as the OD value when the strains were cultivates in the presence of 0.5 mM Co^{2+}. These results show that only Zn^{2+} had an enhancing effect on the OD values for ExhC as measured in the indirect ELISA.

### Table 1

<table>
<thead>
<tr>
<th>Type of toxin produced</th>
<th>No. of S. hyicus (isolates/strains)</th>
<th>Grown without metal salts added&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Grown with metal salts added&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExhA</td>
<td>30</td>
<td>1.3 ± 0.8</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td>ExhB</td>
<td>30</td>
<td>1.1 ± 0.4</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>ExhC</td>
<td>30</td>
<td>0.1 ± 0.06</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Non-toxigenic</td>
<td>9</td>
<td>0.08 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean OD values ± SD. All toxigenic isolates were tested in duplicate.

<sup>b</sup> The nine non-toxigenic strains were tested three times in duplicate. The data presented are mean OD values ± SD of all the determinations.

### 3.2. Investigation of field isolates

The indirect ELISA was used for detecting the presence of S. hyicus field isolates producing the exfoliative toxins ExhA, ExhB or ExhC. A total of 655 S. hyicus isolates were initially tested in sets of 4–10 isolates from one pig from each of the 69 herds with EE. Toxigenic isolates were detected in 51 (74%) of the specimens investigated. In 18 (26%) of the specimens no toxigenic isolates were found. Two out of the five selected specimens, which initially yielded only toxin negative S. hyicus isolates, demonstrated toxin-producing colonies when the additional 40 colonies were tested. In the remaining three specimens no toxin producing colonies were found.

In only one of the 69 specimens investigated, the presence of more than one type of toxin was indicated. Nine of the 10 S. hyicus isolates from this specimen had OD values of 0.2–0.4, indicating the production of ExhC, and one isolate had OD values of 4.0 indicating production of ExhA or ExhB (Table 1). Further investigation by immunoblot analysis using MabEXH7.7, MabEXH5.1 and polyclonal rabbit antibodies specific for ExhC, respectively, showed that the latter isolate produced ExhB and the other nine isolates produced ExhC.
4. Discussion

A previous study (Andresen, 1999) on the effect of a number of different divalent metal ions on the exfoliative toxins ExhA and ExhB from *S. hyicus* showed that addition of 0.5 mM Zn$^{2+}$ and 0.5 mM Co$^{2+}$ to the growth medium had an enhancing effect on the detection of ExhA and ExhB, respectively, in ELISA. Cobalt(II) and zinc(II) ions were, therefore, added to the liquid growth medium for enhancing the detection of the exfoliative toxins in this study. The immunoblotting (Fig. 1) of culture supernatant from the strains NCTC 10350, 1289D-88 and 842A-88 which produce ExhA, ExhB and ExhC, respectively, (Andresen, 1998) confirmed that addition of Co$^{2+}$ and Zn$^{2+}$ improved the detection of ExhA and ExhB. The immunoblot in Fig. 1 and the results of ELISA on the 30 ExhC-producing *S. hyicus* in Table 1 indicate that ExhC also is a metalloprotein. ExhC was more easily detected by the monoclonal antibody MabEXH7.7, when the ExhC-producing *S. hyicus* were grown in liquid medium containing the divalent metal ions Co$^{2+}$ and Zn$^{2+}$. Separate investigations on 10 of the 30 ExhC-producing isolates indicated that the metal ion that had an enhancing effect on the detection of ExhC in the indirect ELISA was Zn$^{2+}$.

In the present study, toxin producing isolates were found in 74% and no toxin producing isolates were found in 26% of the 69 specimens investigated. This is in agreement with a previous study on the prevalence of ExhA, ExhB and ExhC producing *S. hyicus* from pigs with EE (Andresen, 1998). In that study, toxin producing isolates were found in specimens from 72% of 60 pig herds with EE, whereas in specimens from 28% of the herds no toxin producing isolates were found. So far, investigations have shown that the production of exfoliative toxin by *S. hyicus* is necessary in order to be able to induce EE in pigs (Wegener et al., 1993; Tanabe et al., 1996; Andresen, 1998).

Investigation of a total of 49 or 50 *S. hyicus* or *S. hyicus*-like colonies from each of the five specimens showed that in only two of the five specimens toxigenic colonies could be found. The number of colonies tested should give a probability of >99% for finding a toxigenic isolate on the assumption that any type of toxin could be detected by the assay described. The criteria used for inclusion of colonies in this part of the investigation should ensure that they were *S. hyicus*. However, colonies were only regarded as *S. hyicus*-like. In particular, *S. hyicus*-like colonies were distinguished from *S. chromogenes* by being lipase positive (able to hydrolyse Tween-80 (polyoxyethylensorbitanmonoo- leat)) (Devriese et al., 1978) and from *S. aureus* by being non-haemolytic on bovine blood agar. The absence of toxin-producing *S. hyicus* in three of the five specimens may suggest that there are one or more variants of the exfoliative toxin from *S. hyicus* not detected in the ELISA or that the toxigenic *S. hyicus* had been displaced by other bacteria in the specimens.

In one case, immunoblot analysis showed that different *S. hyicus* isolates from the same specimen produced different types of toxin. In a previous study, where the three types of toxin were tested separately (Andresen, 1998), none of the 60 sets of 8–10 *S. hyicus* investigated showed production of more than one type of toxin. Although all the sets of isolates in this study were not analysed for the production of the three toxins separately, the occurrence of different isolates of *S. hyicus* from the same specimen producing different types of toxin is regarded as rare.
The present study has provided a simple indirect ELISA for the detection of isolates of *S. hyicus* producing the exfoliative toxin ExhA, ExhB or ExhC. Selection of isolates for preparation of autogenous vaccine against EE should be done by identification of the disease causing agent, i.e. toxigenic *S. hyicus*. However, in 26% of the specimens investigated, the ELISA could not detect any toxigenic *S. hyicus*. Improvement of the detection rate of toxigenic *S. hyicus* using indirect ELISA may be achieved if other variants of the *S. hyicus* exfoliative toxin are identified or if studies show that the timing of, and conditions for, the sampling of material from pigs with EE are of significance for the detection of toxigenic isolates.

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**References**


