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## DETECTION OF ENTEROTOXIGENIC *STAPHYLOCOCCUS AUREUS* BY IMMUNOCHEMICAL METHODS AND BY THE POLYMERASE CHAIN REACTION

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*Staphylococcus aureus*, staphylococcal enterotoxin, immunoprecipitation, Western blot, RIA, PCR, sea, seb

### Summary

*Staphylococcus aureus* has been one of the most important microorganisms responsible for food borne disease. Food-associated intoxications are commonly mediated by heat-stable staphylococcal enterotoxins (SEs). The production of staphylococcal enterotoxin A (SEA) and B (SEB) in reference strains of *S. aureus* and in 40 *S. aureus* isolates was examined by immunochemical methods such as Ouchterlony immunoprecipitation, Western blot and RIA method. The presence of sea and seb genes have been tested by polymerase chain reaction. Minimal detection limit was established as 50 pg/50 µl of reaction mixture to detect sea and seb genes by PCR. In reference *S. aureus* strains, PCR results were identical with SEs production. In *S. aureus* isolates, sea gene was detected in 8 (20%) out of 40 ones. In this 8 *S. aureus* isolates SEA production was proved either alone or with SEB. Gene seb was confirmed in 17 (42,5%) *S. aureus* isolates producing SEB or combination of SEB with SEA

### Introduction

Staphylococcal enterotoxins (SEs) (MW 27 900-29 600) are exotoxins of *Staphylococcus aureus* and of some other types of staphylococci. Along with toxic shock syndrome toxin (TSST-1), exfoliative toxins (ETs), hemolysins and other extracellular proteins, SEs contribute to pathogenicity and virulence of the above microorganisms. SEs cause foodborne diseases (food poisoning) - i.e. afebrile alimentary enterotoxicoses with short incubation time (2-6h) and concomitant symptoms which include nausea, emesis and diarrhoea. It has been proved that SEs may participate in the toxic shock syndrome (TSS) cases (Munson et al., 1998) and play an important role in the pathogenesis of a number of infectious, inflammatory and autoimmune diseases in humans (nonfoodborne diseases) and animals. In animals mastitis in cattle and sheep are of the great importance.

The aim of the present study was to examine the production of staphylococcal enterotoxin A and B in 40 *S. aureus* field isolates by three different immunochemical methods and to detect sea and seb genes by PCR.

### **Material and methods**

The reference (control) strains used were *S. aureus* FRI 722 (Food Research Institute, Madison, University of Wisconsin, U.S.A.) and *S. aureus* CCM 5757 (Czechoslovak Collection of Microorganisms, Brno, Czech Republic), that produced SEA and SEB, respectively. As negative controls, non-enterotoxigenic strains of *S. aureus* CCM 2351(α-hemolysin) and *S. aureus* CCM 6188 (β-hemolysin) were used (Czechoslovak Collection of Microorganisms, Brno, Czech Republic). *S. aureus* field isolates were obtained from smears of technological equipment from food industry, swabs of throat and respiratory passages of food handlers, samples of pasta and sheep cheese. Bacteriological examination of samples has been done according to standards STN ISO 6888 (1997). Bacterial strains were cultured in 5 ml of Brain heart infusion broth for 18-24 h at 37°C with shaking. Supernatants of culture media were examined for the presence of SEA and SEB by modification of Ouchterlony method (Fotta, 1997) and by radioimmunoassay (RIA) (Gondol' et al., 1997). In reference strains the enterotoxigenicity was determined by Western blot method as well (Harlow et Lane, 1988). The presence of genes for staphylococcal enterotoxins in *S. aureus* strains has been tested by PCR technique. Genomic DNA was isolated by phenol-chlorophorm method according to Sambrook et al. (1986). DNA concentration was determined spectrophotometrically (Spectronic Genesys™ 5). Oligonucleotide primers (SEA1, SEA2-20-mer, SEB1, SEB-20-mer) were selected on the basis of the study of Johnson et al. (1991). The total volume of PCR reaction mixture was 50 µl with 3,0 mM MgCl<sub>2</sub>, 2,0U AmpliTaq polymerase (Perkin Elmer) and 0,3 µM specific primers. PCR was performed in Genius thermocycler (Techne, U.S.A) and comprised of: 94°C for 2 min.; 35 cycles of 94°C for 1 min., 55°C for 30 sec., 72°C for 30 sec; followed by a final extension at 72°C for 2 min.30 sec. Minimal detection limit for sea and seb *S. aureus* genes was determined by ten-fold dilutions of DNA template with initial DNA concentration of 500 µg/ml. PCR products were identified on 2 % agarose gel after staining with ethidium bromide (0,5 µg/ml) and visualization by UV transilluminator.

### **Results**

#### *Immunoprecipitation, RIA, Western blot*

The immunoprecipitation revealed the synthesis of relevant staphylococcal enterotoxins (SEA, SEB) in all enterotoxigenic reference strains. In reference strains synthetising α-hemolysin or β-hemolysin the production of SEs has not been proved. The production of SEA and SEB was retested in control *S. aureus* strains by RIA method. The presence of SEA in culture medium supernatant was proved in *S. aureus* strain FRI 722, that of SEB in *S. aureus* strain CCM 5757. In remaining reference strains, RIA results were negative. The bands obtained on nitrocellulose membrane by Western blot were at the same position as purified staphylococcal enterotoxins A and B (MW 27 800 and 28 336, respectively) on polyacrylamide gel. In 40 field isolates of *S. aureus* the presence of one of SEA or SEB enterotoxins or their combinations was indicated by immunoprecipitation and RIA in 20 (50%) samples. In case of 14 isolates from sheep cheese the production of SEB was the most often indicated (57% of isolates). One field isolate from smears from technological equipment produced SEB. Six isolates of *S. aureus* obtained from pasta produced SEA alone, SEB alone or SEA plus SEB. Production of these toxins in pasta was not confirmed in 12 isolates (67%).

#### *Polymerase chain reaction (PCR)*

By amplification of the target DNA sequence of reference *S. aureus* strain FRI 722 the 120 bp PCR product was obtained so the presence of sea gene in this strain was confirmed. In non-enterotoxigenic control *S. aureus* strains the result of amplification was negative. Using the reference strain *S. aureus* CCM 5757 the sequence with a length of 476 bp was determined by PCR. The lowest DNA dilution in which specific PCR products were still detected was 50 ng/ml. Based on this, minimal PCR detection limit was determined for 50 pg/50µl of reaction mixture to detect sea and seb genes. sea gene was detected by PCR in 8 (20%) *S. aureus* isolates in which the production of staphylococcal enterotoxin A was confirmed by immunoprecipitation and RIA. seb gene was detected in 17 (42,5%) out of total 40 examined DNA samples.

### **Discussion**

Staphylococcal food poisoning (SFP) belongs to food borne diseases that are subjected to regular control in countries with developed food industries. SEA and SEB are the toxins most often detected in foods (Rasooly et Rasooly, 1998). SEA in particular causes staphylococcal food poisoning even at very low concentrations (0,6 ng/ml). McLauchlin (2000) reported that after 1990, the share of SEA in food borne disease was 23%. In comparison with this result 20% of our field isolates of *S. aureus* produced SEA and 42,5% produced SEB. Many methods have been developed for the detection of staphylococcal enterotoxins and enterotoxin-producing staphylococci. Three groups of detection methods are currently available, including biological assays, immunochemical assays and polymerase chain reaction-based assays (Su et Wong, 1997). In our work three immunochemical methods which differ by their detection limit (0,5-2,5 µg/ml for immunoprecipitation, 2,5-25 ng/ml for Western blot and 0,5-1 ng/ml for RIA) were used for detection of SEA and SEB. Immunoprecipitation can detect only strains with relatively high production of SEs in contrast with RIA which can detect also low-producing strains. In our study it was found out that in one field isolate of *S. aureus* the production of SEB was indicated only by RIA but not by

immunoprecipitation. The presence of gene for expression of SEB was confirmed by PCR in this field isolate. The advantage of Western blot is detection of enterotoxins on the basis of their molecular weights. PCR offers the possibility of specific amplification of fragments of the genes responsible for enterotoxin production. It can also be used for the detection of staphylococcal enterotoxin genes the expression of which has not been proved for different reasons. The results of PCR obtained in our study were consistent with results of immunoprecipitation, Western blot and RIA in 97,5% of examined isolates of *S.aureus*.

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Fig.1.

Detection of staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) in culture supernatant fluids of *S. aureus* FRI 722-SEA and *S. aureus* CCM 5757-SEB reference strains by means of SDS-polyacrylamide electrophoresis (SDS-PAGE) and Western blot. Monoclonal antibodies against SEA and against SEB were used as the first antibodies.

- A. SDS-PAGE
1. molecular weight markers (14, 20, 30, 43, 67, 94 kDa)
  2. purified antigen SEA
  3. crude antigen SEA (culture supernatant fluid)
  4. purified antigen SEB
  5. crude antigen SEB
- B. Western blot
6. purified SEA
  7. crude SEA
  8. purified SEB
  9. crude SEB

Fig.2.

Detection of sea gene encoding staphylococcal enterotoxin A (SEA) of *S.aureus* by means of PCR on 2% agarose gel.

1. standard: λDNA digested by EcoRI/HindIII
2. 120 bp product of amplification-DNA *S.aureus* FRI 722-SEA
3. 120 bp amplicon-*S.aureus* FRI 722-SEA
4. negative control-*S.aureus* CCM 5757-SEB
5. negative control -*S.aureus* FRI 361- SEC
6. negative control- *S.aureus* 1151m-SED
7. negative control-*E.coli* M1

Fig.3.

Detection of seb gene encoding staphylococcal enterotoxin B (SEB) of *S.aureus* by means of PCR on 2% agarose gel.

1. standard:  $\lambda$  DNA digested by EcoRI/HindIII
2. 478 bp product of amplification. 3,0 mM MgCl<sub>2</sub>
3. 2,5 mM MgCl<sub>2</sub>
4. 2,0 mM MgCl<sub>2</sub>
5. 1,5 mM MgCl<sub>2</sub>
6. 1,0 mM MgCl<sub>2</sub>
7. 0,5 mM MgCl<sub>2</sub>
8. standard:  $\lambda$  DNA digested by HindIII