**Results and Discussion**

Immu-PCR is an ultra-sensitive method for detecting smallest amounts of proteins, thus, this method is excessively sensitive to false-positive results due to unspecific binding of the reporter DNA-molecules to the polycrystalline surfaces of the microtiter plate cavities, whereas unspecific binding of antibodies is negligible. Even with excessive blocking with dextran sparm DNA, BSA, and/or skim milk, and reducing the number of PCR cycles, false positive results occurred. It was observed that the use of latexSpher™ (Sender Bioscience) could strongly reduce these unspecific reactions without substantial reducing the detection limit (data not shown).

In our hands, the optimal capture antibody concentration was found to be 20 µg/ml. Detection antibodies were used at a concentration of 1 µg/ml. The optimal dilution of the covariant DNA-antibody conjugate was 1:200.

**Comparison of qRT-PCR and ELISA**

In a first approach, samples with purified toxins diluted in buffer were tested. The detection limit for purified TSST-1 containing toxin was as low as 0.6 pg of total SEB, equivalent to approx. 0.4 amol/µl. In comparison to conventionally available conventional ELISA-assays, the detection limit was lowered by a factor of 100. Furthermore, the qRT-PCR is providing a broader linear range.

**Specificity of the qRT-PCR**

The specificity of the assay was determined using culture supernatants of toxigenic S. aureus strains as samples. Positive signals were only observed in SEB or TSST-1 positive samples, respectively. Other PTSAg did not significantly influence the assay. Culture supernatants also served as a proof that the PCR-protocols can be used for the detection of staphylococcal toxins in complex matrices.

**Enterotoxin detection in clinical relevant specimens.**

To test the performance of the qRT-PCR protocol in more complex applications, clinical specimens such as urine and blood were spiked with SEB and TSST-1, respectively. To test the application in food derived samples, milk was spiked with the toxins.

Due to the excess of interfering proteins, the samples had to be diluted 1:2, and the LOD was limited to 100 pg/ml – 1 ng/ml of total toxin, depending on the sample and/or toxin being tested. This effect was also observed with other immunological methods, such as ELISA and RPLA, so that the LOD of qRT-PCR remained superior to these methods. Fig. 3 shows the detection of SEB in milk, providing a LOD of 100 pg/ml.

**Comparison of qRT-PCR and ELISA**

**Principle of Real-time Quantitative Immuno-PCR**

![Principle of Real-time Quantitative Immuno-PCR](image)

**Materials and Methods**

**Antibodies.** Polyclonal sheep and rabbit antibodies against TSST-1 and SEB were purchased from Dako, Denmark. Polyclonal sheep antibodies were coated to the surface of polycarbonate microtiter plates (Nunc MediSorp plates) over night at 4°C at a concentration of 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B (0.15 M NaCl, 0.02 M Tris, pH 7.5) and blocked with 4.5% skim milk powder. Polyclonal rabbit antibodies for detection were used at a concentration of 1 µg/ml.

**Synthesis of a DNA-reporter molecule.** A fragment of the 3’-DNA polymerase III (3’-DNA) gene was amplified and cloned into a pGEM vector plasmid. Afterward, both fragments of the polymerase III gene were ligated to a 32P labeled restriction enzyme site. The resulting product containing the 3’-DNA fragment flanked by short sequences of the vector including the restriction sites of the multiple cloning site.

**Covariant coupling of antibody and DNA.** The amplified 32P-DNA-reporter fragment was purified by phosphoamino acid extraction and ligated to a 3’-DNA fragment (6µg). The ligated fragment was modified with sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, Sigma). Secondary anti-rabbit antibodies were modified using SATA (N-Succinimidyl-S-acrylamide, Pierce) and finally coupled to the modified DNA-reporter molecules.

**Immuno-PCR.** The immuno-PCR assays were carried out comparable to sandwich ELISA. 60 µl of positive and negative controls in 384 well microtiter plates (Nunc MediSorp plates) were incubated with specific capture antibodies and blocked with 1% milk powder. After one hour of incubation at 37°C, plates were washed four times with buffer B (buffer B + 5 mM EDTA + 0.05 % Tween 20). For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 mM carbonate coating buffer (pH 9.6). Plates were washed four times with buffer B + 5 mM EDTA + 0.05 % Tween 20. For detection, specific rabbit anti-toxin antibodies were added to the wells at 20 µg/ml using 15 M

**Conclusion**

Currently, qRT-PCR is up to 100 times more sensitive than ELISA. Additional optimization procedures may lead to a further enhancement of the sensitivity.

The qRT-PCR may serve as an ultra-sensitive high-throughput method for screening and early-stage detection.

The qRT-PCR for the detection of TSST-mediating toxins TSST-1 and SEB may serve as an easily adaptable platform for the detection of other members of the pyrogenic toxin superfamily and other exotoxins.

The use of a covariant DNA-antibody conjugate enhances the PCR to a universal tool for the detection of all kinds of antigens. This approach can be adapted for multiplex applications.

**References and related literature**

5. Niemeyer, C.M., Adler, M., Blohm, D. Fluorometric polymerase chain reaction (PCR) of samples was conducted in duplicate, and the results were calculated. The results were compared to a standard curve. The LOD for SEB was 0.6 pg/µl, equivalent to 0.4 amol/µl.
6. Niemeyer, C.M., Adler, M., Blohm, D. Fluorometric polymerase chain reaction (PCR) of samples was conducted in duplicate, and the results were calculated. The results were compared to a standard curve. The LOD for SEB was 0.6 pg/µl, equivalent to 0.4 amol/µl.