TSS-mediating staphylococcal toxins: A novel quantitative real-time immuno-PCR approach for ultra-sensitive detection of SEB and TSST-1

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Staphylococcus aureus is a major human pathogen characterized by a strain-dependent spectrum of virulence factors. One of these is the family of the bacterial pyrogenic toxin superantigens (PTSAg), comprising the toxic shock syndrome (TSS) mediating toxins TSST 1 and staphylococcal enterotoxin B (SEB). As morbidity and mortality from TSS are substantial, early and reliable recognition of TSS is critical. In addition to their nature as superantigens, enterotoxins also function as potent gastrointestinal toxins with a major public health impact.

Immunological methods used until now are known to be limited in sensitivity and specificity, revealing an obvious need for new highly sensitive and specific methods for the detection of staphylococcal toxins. For this reason, two quantitative real-time immuno-PCR (qRT-iPCR) approaches for the detection of SEB and TSST-1 have been developed.

The detection of TSST-1 and SEB, respectively, was achieved by coating toxin-specific polyclonal sheep antibodies (ABs) to microtiter plates in order to capture the target superantigens followed by specific detection of the antigen-AB complex. The resulting immunocomplex was subsequently detected using a covalent antibody-DNA complex, which was synthesized using amino-modified and maleimide-activated reporter DNA and N-succinimidyl-S-actyl-thioacetate-modified secondary detection antibodies. Quantitative real-time amplification of the reporter-DNA was performed for final detection of the toxins.

By usage of this qRT-iPCR technique, superantigen toxin was highly reproducible detected at approximately 10 to 100 pg/ml (0.4 to 4 amol/µl), thereby lowering the limit of detection (LOD) of these toxins by a factor of up to 100 compared to commercially available EIAs. Furthermore, qRT-iPCR offers a high versatility, giving the opportunity of adapting the protocol to detect a broad range of antigens, provided that antibodies for the desired antigen are available. Covalent attachment of different reporter-DNAs to specific antibodies could be used to extend the qRT-iPCR to a multiplex detection platform.

Principle of Real-time Quantitative Immuno-PCR

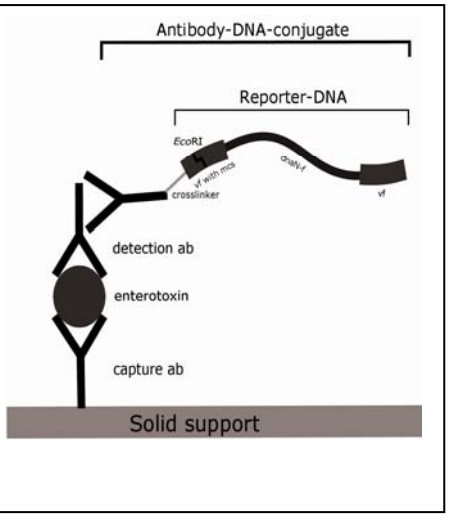

Fig. 1: Principle of an qRT-iPCR- assay
The antigen is bound to a microtiter plate coated with polyclonal sheep- anti-enterotoxin antibodies and is subsequently detected by polyclonal rabbit- anti-toxin antibodies.
A covalent antibody-DNA-conjugate is used for detection of the immuno-complex. The reporter-DNA molecule consists of a fragment of *dnaN* from *E. coli* flanked by short residues of a pUC19 vector (vf), including the MCS with an EcoRI restriction site.
In this study, the DNA reporter molecule is detected by real-time PCR in a Bio-Rad iCycler iQ real-time PCR system using SYBR® Green.

Materials and Methods

Antibodies. Polyclonal sheep and rabbit antibodies against TSST-1 and SEB were purchased from Toxin Technology Inc., Sarasota, Fl. Sheep capture 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, 0.01 M Tris/HCl, pH 7.3) and were subsequently blocked with 4.5% (w/v) 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 *E. coli* DNA polymerase III (*dnaN*) gene was amplified and cloned into a pUC19 vector plasmid. Afterwards, fragments of the resulting plasmid pAF1 were amplified using 5'-amino-modified primers (MWG Biotech AG), resulting in a PCR product containing the *E. coli* fragment flanked by short sequences of the vector, including the restriction sites of the multiple cloning site.

Covalent coupling of antibody and DNA. The amplified NH_2-DNA-fragment was purified by phenol/chloroform extraction and adjusted to a concentration of 1.5 µg/ml (approx. 5 µM) and modified with sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, Sigma). Secondary goat-anti-rabbit antibodies were modified using SATA (N-Succinimidyl-S-acetylthioacetate, Pierce) and finally coupled to the modified reporter-DNA molecules.

Immuno-PCR. The immuno-PCR assays were carried out comparable to sandwich ELISA. 60 µl of sample were incubated in microtiter plates coated with specific capture antibodies and blocked with skim milk powder. After one hour of incubation at 37°C, plates were washed four times with buffer D (buffer B + 5 mM EDTA + 0.05 % Tween 20). For detection, specific rabbit anti-toxin antibodies were diluted 1:1000 (1 µg/ml) in LowCross® buffer (Candor Bioscience, Münster, Germany) and incubated for 1 h at 37°C. After washing, secondary goat-anti-rabbit antibodies (Dako, Denmark) were added and incubated for 1 h at 37°C. To remove unbound antibodies, the plates were washed with buffer D 12 times.

Reporter-DNA-molecules were digested with EcoRI-restriction endonuclease (2 U/cavity; Fermentas) for two hours for detachment of the DNA from the antibodies. The detector DNA molecule was cut at the restriction site and can be transferred into the PCR plate.

Real-time PCR was performed on a Bio-Rad iCycler iQ thermocycler with iQ SYBR®-Green Supermix (Bio-Rad). Data analysis was done using the iCycler iQ software.

Conclusions

- **Currently, qRT-iPCR is up to 100 times more sensitive than ELISA. Additional optimization procedures may lead to a further enhancement of the sensitivity.**
- **The qRT-iPCR may serve as an ultra-sensitive high-throughput method for screening clinical, food-derived, and environmental samples.**
- **The qRT-iPCR for the detection of TSS-mediating toxins TSST-1 and SEB may serve as an easily adaptable platform for the detection of other members of the pyrogenic toxin superantigen family and other exotoxins.**
- **The use of a covalent DNA-antibody conjugate enhances the iPCR to a universal tool for the detection of all kinds of antigens. This approach can be adapted for multiplex applications.**

Results and Discussion

Immuno-PCR is an ultra-sensitive method for detecting smallest amounts of proteins, thus, this method is excessively susceptible to false-positive results due to unspecific binding of the reporter DNA-molecule to the polycarbonate surfaces of the microtiter plate cavities, whereas unspecific binding of antibodies is negligible. Even with excessive blocking with Herring sperm DNA, BSA and/or skim milk, and reducing the number of PCR cycles, false positive results occurred. It was observed that the use of LowCross®-Buffer (Candor 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 covalent DNA-antibody conjugate was 1:200.

Comparison of qRT-iPCR and ELISA

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

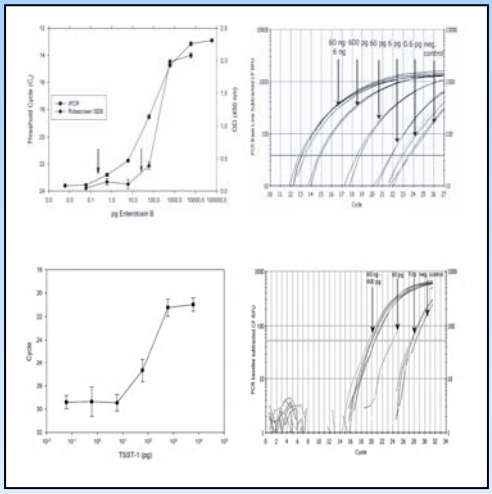

Fig. 2
Right: Amplification graphs (log-scale) of real-time immuno-PCR detection of SEB (top) and TSST-1 (below) using BioRad iCycler iQ and SYBR® Green. Detection limit was 0.6 pg (50 amol, equivalent to 0.4 amol/µl) of total SEB and 60 pg (5 fmol, 400 amol/µl) of TSST-1. PCR of samples was conducted in duplicate, negative controls in triplicate.
Left: Comparison between standard immunological methods and qRT-iPCR: detection limit of SEB was lowered by a factor of ~100 compared to ELISA. qRT-iPCR for TSST-1 was compared to a latex agglutination assay (RPLA) and showed a 10 – 100fold improvement and delivered more reproducible results.

Specificity of the qRT-iPCR

The specificity of the assay was determined using culture supernatants of toxin producing *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 iPCR 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-iPCR 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-iPCR remained superior to these methods. Fig. 3 shows the detection of SEB in milk, providing a LOD of 100 pg/ml.

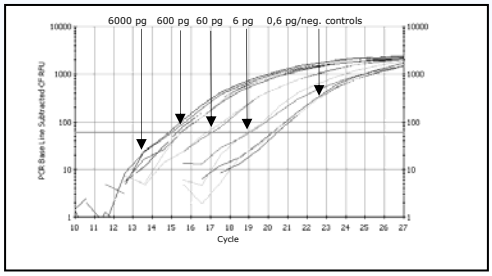

Fig. 3
Amplification graph (log scale) of the detection of SEB milk. Milk was spiked with SEB, centrifuged to remove cream, and subsequently diluted 1:2 in Buffer D.
6 pg (100 pg/ml) of SEB could be detected.

References and related literature

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