Arch. Biol. Sci., Belgrade, 65 (3), 1113-1121, 2013 DOI:10.2298/ABS1303113D

IMMUNOCHEMICAL AND PCR ANALYSIS OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXIN B (SEB) IN MILK AND FRUIT JUICES COLLECTED IN LAHORE, PAKISTAN

NADIA DAR[1], MEHWISH JAVED[2] and ZAHOOR QADIR SAMRA[2]

[1] *Department of Biology, Jinnah Degree College for Women, Mozang Road,* Lahore, Pakistan
[2] *Institute of Biochemistry and Biotechnology, Quaid-i-Azam Campus, University of the Punjab,* Lahore 54590, Pakistan

Abstract - Enterotoxins secreted by *S. aureus* are known as a food-poisoning agent that is associated with various gastrointestinal pathological conditions. In this study, a one-step immunodetection method was devised for routine checking of SEB in milk and fruit juices available locally. Antibodies against recombinant SEB were raised, purified, and cross reactivity was checked against clinically important bacteria (*Shigella flexneri, Streptococci, Salmonella typhi, Klebsiella and Bacillus subtilis*). Purified anti-SEB antibodies were conjugated with gold nanoparticles (Ab-GNPs) for direct detection of SEB in samples. SEB (33%, 4.76% and 15%) was found in non-sterilized milk (118), sterilized milk (42) and juices (60), respectively. Coagulase, MSA tests and PCR amplification of 725 bp of the SEB gene confirmed the presence of *S. aureus* in the collected samples positive for SEB. Immunoassay is easy, reliable and less time consuming and will be helpful to detect the SEB in food samples at local level.

Key words: SEB, gold nanoparticles, antibodies, food, Pakistan

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is considered as a pathogen and food-poisoning illness-causing agent. The food poisoning is associated with the ingestion of food contaminated with the *S. aureus* enterotoxins. Enterotoxins are stable and retain their biological activity during heat treatment and normal cooking. Generally, staphylococcal enterotoxins (SEs) are resistant to proteases and retain toxic properties in dairy products. Indeed, enterotoxins in food have caused outbreaks where the incriminated food had already undergone heat treatment (Asao et al., 2003).

Gastrointestinal disturbance is the major symptom of the food poisoning caused by SE. Even a low dose of non-degraded toxin is sufficient to cause food poisoning and disturb normal physiological conditions (Asao et al., 2003). Among SE, SEB is a low molecular weight protein of nearly 28 KDa and is important due to its rapid production in dairy products. SEB can be associated with atopic eczema (Breuer et al., 2000; Bunikowski et al., 1999; Mempel et al., 2003), rheumatoid arthritis (Howell et al., 1999; Uematsu et al., 1991), and toxic shock syndrome (Herz et al., 1999). SEB has also been reported as potential bioweapon (Henghold, 2004; Ler et al., 2006; Rosenbloom et al., 2002; Wiener, 1996).

SE are divided into two groups: classical SE and new SE. The classical SE are well-recognized and includes SEA, SEB, SEC, SED and SEE. In recent years, SEG, SEH, SEI, SElJ, SElK, SElL, SElM, SElN, SElO, SElP, SElQ, SElR, SElU and SElV have been identified as new SE and SE-like toxins (Lee et al., 1993;

Omoe et al., 2003; Lina et al., 2004; Thomas et al., 2006). As superantigens, SEB can initiate the activation and proliferation of T-cells through interaction with receptors on T-cells, and can cause toxic shock syndrome (Marrack and Kappler, 1990). This condition can cause pyrogenicity, enhance lethal endotoxin shock, and induce the release of inflammatory cytokines such as tumor necrosis factor and interleukin 1 (IL-1) (Bohach et al., 1990).

The polymerase chain reaction (PCR) has been developed to detect SEB producing *S. aureus* (Hohnson et al., 1991; Sharma et al., 2000). However, this assay is labor-intensive, time-consuming and requires post-PCR electrophoresis to detect the amplified products. The real-time PCR assay is used for the quantitative detection of enterotoxigenic *S. aureus* but this method needs expensive equipment (Sharma et al., 2000). Loop-mediated isothermal amplification (LAMP) of DNA is time consuming for the routine diagnostic analysis of *S. aureus* (Klotz et al., 2003; Notomi et al., 2000). The sensitivity of the LAMP assay is generally higher than the conventional PCR assay and can detect enterotoxigenic *S. aureus* strains within 60 min. Immuno-PCR is used to detect *S. aureus* and is also a cumbersome procedure (Goto et al., 2007). The magnetic bead immunoassay for SEB detection is difficult for routine analysis by technicians (Alefantis et al., 2004).

PCR and RT-PCR assay are used to detect the SE gene, however, they require skill and equipment that are not easily available in under-developed countries. Another approach is using gold nanoparticles in the detection system, which is easy and no specific skill is required. The gold nanoparticles can be synthesized easily and used for antibody or protein immobilization due to their physical adsorption mechanism.

In the Lahore metropolitan area, the available milk, milk products and juices are prepared in unhygienic conditions. It has been observed that a large number of humans, especially children, suffer from gastrointestinal problems from April to September. The objective of this study was to devise an easy and cheap method for routine detection of SEB in food. Prior detection of SEB in food will be helpful to monitor food poisoning as well as to reduce gastrointestinal pathological conditions.

MATERIALS AND METHODS

Sample collection and processing

Raw milk samples (n = 118, cow and buffalo) were collected under sterile conditions from different distribution shops in the Lahore metropolitan area, Pakistan. Boiled and then cooled milk samples (n = 42) were also collected from shops near bus stops and railway station. Sealed juices (n = 60) of different fruits, sold at bus stops and the railway station, were also collected. 100 ml of each milk sample was kept in an ice bath and transported to the laboratory for storage at 4°C. The samples were centrifuged at 7000 rpm for 15 min at 4°C. The cream layer was removed and the remaining part was stored at -20°C until further processing. The collected juice samples were also stored at -20°C until further process. The juice and milk samples were diluted (1:5 dilution) with sterilized normal saline (0.85 % NaCl) for immunochemical assays. 0.5 ml aliquots of samples was used to inoculate a 5.0 ml mannitol salt medium and kept for 16 h at 37°C. The next day, the growing culture was centrifuged at 7000 rpm at 4°C. Bacterial pellets were separated and used for chromosomal DNA extraction and PCR analysis of SEB gene.

Maintenance and culturing of bacterial strains

S. aureus (IBB-2011, SEB producer, locally isolated and characterized) was obtained from IBB, University of the Punjab, Lahore and maintained on minimal salt agar medium (mannitol 1.0%, NaCl 7.5%, beef extract 0.1%, peptone 1.0%, phenol red 0.0025%, pH 7.4). This strain was used as reference control in all assays. Other clinically important bacterial strains (*Shigella flexneri, Streptococci, Salmonella typhi, Klebsiella and Bacillus subtilis*) were also obtained (IBB, University of the Punjab, Lahore, Pakistan) as control and maintained on LB-agar medium. The bacterial strains mentioned above were cultured in 5.0 ml

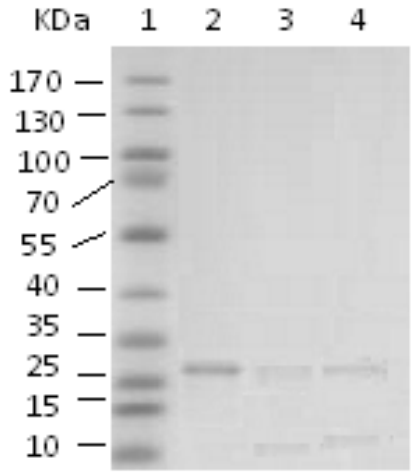

Fig. 1. Western blot analysis for specificity of anti- SEB antibodies.
Purified SEB, *S. aureus* (IBB-2011) producing SEB and culture supernatant of contaminated milk with *S. aureus* were separated on a 10% SDS-polyacrylamide gel, followed by Western blot analysis. Lane 1 - molecular weight protein marker (Fermentas, SM0671); lane 2 - recombinant SEB; lane 3 - supernatant of growing culture of *S. aureus* (IBB2011); lane 4 - culture supernatant of contaminated milk with *S. aureus* (IBB 2011). A 28 KDa protein band indicated the specificity of antibody in pure and contaminated samples. A light band of the blue formazan reaction product was observed below the 28 KDa protein in lanes 3 and 4. This band may be the degraded product of SEB protein.

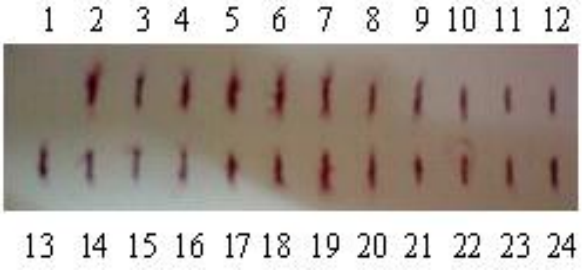

Fig.2. Immunoslot blot analysis of SEB.
Detection of SEB in milk and fruit samples by gold conjugated anti-SEB antibodies. Slot 1 - negative control (milk sample contaminated with non-pathogenic *E.coli* (DH5α); slot 2 - purified SEB; slot 3, growing culture of *S. aureus* (IBB-2011); slots 4 to 9 - non-sterilized milk; slots 10 to 12 - boiled then cooled milk; slots 13-24 - juice samples. The reactivity indicated the direct detection of SEB in the contaminated samples. Ab-GNPs did not show reactivity with culture supernatant of *Shigella flexneri, Streptococci, Salmonella typhi, Klebsiella and Bacillus subtilis* (data not shown).

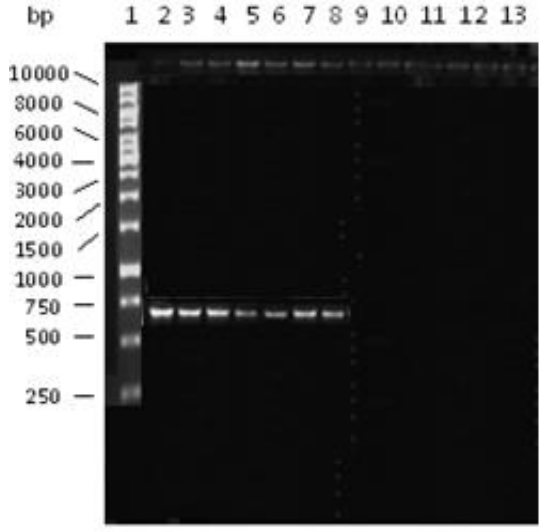

Fig. 3. Agarose gel electrophoresis analysis of PCR amplification of the SEB gene. 5 µl of each amplified sample was loaded. Lane 1 - DNA marker (Fermentas); lane 2 - *S. aureus* IBB 2011; lanes 3-5 - *S. aureus* isolated from milk samples; lanes 6–8 - *S. aureus* isolated from fruit juice samples; lane 9 - *Shigella flexneri*; lane 10 - *Streptococci*; lane 11 - *Salmonella typhi*; lane 12 - *Klebsiella*; lane 13 - *Bacillus subtilis* A single amplified PCR product 725 bp of the SEB gene.

sterilized milk for 16 h at 37°C separately. The cell-free supernatants were separated by centrifugation of the culture at 8000 rpm for 20 min at 4°C and used in immunoassays to test the specificity of antibodies.

Preparation of SEB

An *E. coli* strain (BL21-codon plus) carrying a pET28a-SEB hybrid vector expressing recombinant SEB was obtained from Dr. Zahoor Q. Samra (Molecular biotechnology lab, IBB, University of the Punjab). The recombinant SEB was purified on nickel-agarose affinity resin as described (Sambrook and Russel, 2001). The fractions collected from the nickel resin were loaded on a Sephadex G-75 column (1.5 x 30 cm) equilibrated with 20 mM Tris-Cl, pH 7.4 buffer, and 1.0 ml fractions were collected. The collected fractions were checked on 10% SDS-PAGE (Laemmli, 1970). The 28 KDa protein bands were pooled and the protein concentration was de-

Table 1. Tests of SEB in milk and juice samples.

No.	Samples size positive (n =)	Immunoslot blot analysis & %	MSA test positive & %	Coagulase test positive & %	PCR Assays & %
1	Milk* (118)	39 (33.05%)	63 (53.38%)	39 (33.05%)	39 (33.05%)
2	Milk** (42)	3 (7.14%)	11 (26.19%)	3 (7.1466%)	3 (7.14%)
3	Juices (60)	9 (15%)	17 (28.33)	9 (15%)	9 (15%)

Non-sterilized milk *, Boiled-cooled milk **, MSA (Minimal salt agar).

termined by the Bradford reagent assay using bovine serum albumin as standard (Bradford, 1976). The SEB protein concentration was adjusted to 1.0 mg/ml and stored at -20°C until further use.

Production and affinity purification of antibodies

Polyclonal antibodies against the SEB antigen were developed in mice (Balb/C, 5 to 6 weeks old) by adopting the guidelines for laboratory animals. 5.0 ml of purified recombinant SEB was mixed with 2.0% buffered-formalin (v/v, in 10 mM Tris-Cl, pH 7.4) to diminish the toxicity to animals. Formalin-treated SEB protein was precipitated with 80% ammonium sulphate saturation. The precipitated SEB protein was collected at 13000 rpm for 20 min at 4°C. The pellet was resuspended in 10 mM Tris-Cl, pH 7.4, and dialyzed against the same buffer. The formalin-treated SEB protein was mixed with Freund's complete adjuvant in 1:1 ratio and used as an immunogen. Each mouse received 50 to 60 µg protein/per administration, and a total six injections were subcutaneously applied at two-week intervals. Blood (0.1 ml) was drawn from the mouse tail and checked for the anti-SEB antibody titer by enzyme-linked immunosorbent assay (ELISA). After checking the antibody titer, blood was isolated from the heart and serum was separated (Harlow and Lane, 1988). The purified recombinant SEB antigen was conjugated with CNBr-activated sepharose 4B according to the manufacturer's instruction (Pharmacia) and used to affinity purify the anti-SEB antibodies (Harlow and Lane, 1988). The concentration of purified antibodies was checked by Bradford reagent assay and stored at -20°C until further process.

Characterization of antibodies
Enzyme-linked immunosorbent assay

Ten µl of purified recombinant SEB (0.1 mg/ml) and culture supernatant of bacterial strains (*S. aureus* IBB 2011, *Shigella flexneri, Streptococci, Salmonella typhi, Klebsiella and Bacillus subtilis*) was mixed separately with 0.09 ml of 0.05 M carbonate buffer, pH 9.0, and was absorbed onto microtiter ELISA plates for one hour at 37°C. The wells were treated with 3% bovine serum albumin in Tris-buffered saline-Tween 20, (TBST) for 45 min at 37°C. The plates were washed with TBST and then affinity-purified mouse anti-SEB antibody (1:2000 dilutions) was added and kept at 37°C for 50 min. After washing with TBST, rabbit anti-mouse IgG antibody – alkaline phosphatase conjugated (1:5000 dilution), was added and incubated at 37°C for 40 min. After the washings with TBST, a substrate of alkaline phosphatase (para-nitrophenyl phosphate in 0.01 M ethanolamine buffer pH 9.00, 1.0 mM $MgCl_2$) was added to each well (Harlow and Lane, 1988). The pre-immune serum collected before the immunization was used as control.

Western blot analysis

The monospecificity of anti-SEB antibodies was also analyzed by Western blot analysis. Purified recombinant SEB and the culture supernatants of growing a native strain of *S. aureus* (IBB-2011) in minimal salt medium and LB medium, were run on 10% SDS-poly acrylamide gel (Laemmli, 1970) and then transferred onto a nitrocellulose (NC) membrane (Towbin et al., 1979). Non-specific sites on the NC-membrane were blocked with 3% BSA in TBST for 45 min at 37°C. The NC membrane was further incubated with puri-

fied anti-SEB antibodies (1:2000 dilution) and then with rabbit anti-mouse IgG antibody – alkaline phosphatase conjugated (1:5000 dilution). After extensive washing, the blots were exposed to nitroblue tetrazolium and 5-bromo 4-choloro 3-indolyl phosphate as substrates and the results were noted.

Preparation of colloidal gold nanoparticles and conjugation

Colloidal gold nanoparticles (20-25 nm) were prepared by reducing chloroauric acid ($HAuCl_4$) (Walker, 1994). Chloroauric acid (0.1 g) was dissolved in 1 l of double-distilled deionized water and boiled for 30 min; 12.5 ml of 1% trisodium citrate was added under vigorous stirring. The gold solution turned from yellow to dark blue and finally to red. The gold nanoparticle solution was cooled to room temperature and pH 7.5 was adjusted with 0.1 M K_2CO_3 solution. The final color of the prepared gold-particle solution was deep red and stored at 4°C in an airtight container. Affinity-purified anti-SEB antibody was conjugated with gold nanoparticles. The amount of antibody necessary to stabilize the gold solution was determined by flocculation test. Briefly, two-fold dilutions of anti-SEB antibody were made. Gold solution (2.5 ml) was added to each 0.5 ml of diluted antibodies and the color of the solution was noted. The lowest concentration of antibody that stabilizes the red-colored gold solution was noted.

For conjugation, 0.1 ml of affinity-purified antibody (20 μg) was mixed with 1.0 ml of gold-nanoparticle solution and agitated for 30 min at 25°C. The solution was centrifuged at 13000 rpm for 35 min at 4°C. Supernatant was discarded and the red pellet of antibody-gold complex (Ab-GNPs) was resuspended in 0.5 ml of stabilizing buffer (10 mM Tris-Cl, pH 7.5, 0.0125 gm polyethylene glycol, 0.15 M sodium chloride, and 2.0 mg NaN_3 dissolved in 25 ml deionized H_2O) and stored at 4°C.

Immunodot blot analysis for Ab-GNPs complex

Nitrocellulose membrane was cut (3.0 cm x 5.0 cm) and 5.0 μ of purified recombinant SEB antigen was applied to the membrane and allowed to dry. The membrane was stained with acidic Ponceau-S stain (0.1% in 1.0% acetic acid solution) for 5 min in order to check the presence of antigen. The membrane was destained with several changes of Tris-buffered saline. The membrane was dipped in blocking buffer (3% BSA in TBS or 2% gelatin in TBS) for 30 min at room temperature to block the nonspecific binding sites. After washing with 1 x TBS, 0.01 ml of Ab-GNPs solution was diluted in 0.5 ml Tris-buffered-saline solution and added to the membrane for 10-15 min. The blots were rinsed with TBS and the color was noted.

Detection limit of SEB

A serial dilution of purified recombinant SEB antigen (1, 5, 10, 20, 30, 40, 50 ng/ml in 0.85 % normal saline) was made. Ten μl of each dilution was applied onto the nitrocellulose membrane and used for immunodot blot reaction as described above. The deposition of Ab-GNPs complex on a minimum amount of SEB in immunodot blot was noted.

Immunoslot blot analysis for SEB antigen in samples

Diluted samples (0.2 ml) of milk and juices were absorbed in a slot blot apparatus (Bethesda Research Laboratories, USA) containing the NC membrane according to the manufacturer's instructions. The NC membranes were rinsed with TBST and stained with Ponceau S to check for the presence of proteins. The blots were rinsed again with TBST and incubated with blocking buffer (3% BSA in TBST) for 40 min at room temperature. The blots were further incubated with Ab-GNPs for 15 min at room temperature and results were noted. The same quantity of diluted samples was also checked for the presence of SEB by commercially available SEB test strips (Standard diagnostic Inc, Korea).

Confirmation of S. aureus in food samples

The presence of *S. aureus* in the milk and juice samples, which were positive for SEB in immunoslot blot analysis, was further checked by biochemical and

PCR tests to confirm our immunodetection results. All tests were conducted in triplicate.

MSA test

Food samples (milk and juices; 0.2 ml) were taken in a tube containing 5 ml mannitol salt medium (1% mannitol, 7.5% NaCl, 0.1% beef extract, 1% peptone, 0.0025% phenol red, pH 7.4), and incubated at 37°C. The growing culture was further spread on minimal salt-agar medium and incubated for 24-30 h at 37°C. The growing culture was further processed for Gram staining (Benson, 1998).

Blood agar and coagulase test

A single colony from the MSA medium was picked and spread on the blood agar plate. The plate was kept for 24 h at 37°C. The same colony was used to inoculate 1 ml of human serum and kept at 37°C.

PCR analysis

The bacterial pellets isolated from the milk and juice samples were also processed for SEB gene detection by PCR. The chromosomal DNA of bacterial strains (*S. aureus* IBB 2011, bacterial culture isolated from milk and juice samples and the above-mentioned clinically important bacterial strains) was isolated as described (Qiagen, USA) and used for amplification of the SEB gene. The primers for the SEB gene were designed using the available nucleotide sequence of SEB gene at the NCBI (accession number AY852244). F-primer, (5′-ATGGAGAGTCAACCAGATC-3′) and R-primer, (5′-TCACTTTTTCTTTGTCGTAAC-3′) and were custom synthesized from Fermentas. The SEB gene was amplified in iCycler (Biorad) by using 1 µl of template DNA (0.1 mg/ml), 2.5 units of Taq DNA polymerase, PCR buffer 1 x, 0.25 mM dNTPs,, 2.5 mM $MgCl_2$, 0.5 µM of each forward and the reverse primer. The conditions for amplification are as follows: initial denaturation at 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 10 min. After amplification, an aliquot of PCR was mixed with commercially available DNA loading dye (Fermentas) and analyzed on a 1% agarose, 0.5 x Tris-Borate-EDTA buffer.

RESULTS AND DISCUSSION

Characterization and immunodetection of SEB

The recombinant SEB was used to develop the polyclonal antibodies and its monospecificity was also characterized by Western blot analysis (Fig. 1). Affinity-purified anti-SEB antibodies were conjugated with gold nanoparticles for direct detection of toxins in the milk and juice samples. The development of a pink color on the samples in immunodot blot indicated the specific binding of the antibody with SEB. The minimum appropriate detection limit in the positive control was 1-5 ng/ml. A light color of the Ab-GNPs complex was observed on the blot with 1.0 ng/ml of pure SEB. An appropriate detection was observed in 5 ng/ml. Milk and juice samples were collected from different public areas to examine the presence of SEB by Ab-GNPs (Fig 2).

Immunoslot blot analysis indicated that out of 118 raw milk samples, 39 (33.05%) were contaminated with SEB, 3 (7.14%) out of the 42 boiled-cooled milk samples, and 9 (15%) out of 60 juice packets were also contaminated with SEB. The overall percentage contamination of SEB in the milk and juices were 33% and 15%, respectively (Table 1).The culture supernatants of other microbes (described above) were also tested to cross-check reactivity by Ab-GNPs. It was observed that no other microbes tested for immunochemical reaction showed any reaction except *S. aureus* IBB 2011. Commercially available immunotest strips also detected the SEB in the samples and similar results were observed.

Biochemical characterization of S. aureus in test samples

Milk and fruit juice samples were further processed to confirm the presence of *S. aureus*. An aliquot of milk and fruit juice samples was cultured on MSA medium and processed for the coagulase test. The appearance of yellow color in MSA medim and co-

agulation of plasma confirmed the presence of *S. aureus* in the samples. Sixty three (53.38%) non-sterilized milk samples were positive for the MSA test, and 11 (26.19%) of the boiled-cooled milk samples were positive. A total 17 (28.33%) juice samples were also positive for the MSA test. The coagulase test was positive in 39 (33.05%), 3 (7.14%) and 9 (15%) non-sterilized milk samples, boiled-cooled milk samples and juice samples, respectively. The samples positive for the coagulase test were also tested for the SEB gene of *S. aureus*. The appearance of a 725 bp PCR product in the tested samples (milk and juices) further indicated the contamination of *S. aureus* (Fig. 3). PCR analysis revealed that 39 (33.05%) of the non-sterilized milk samples, 3 (7.14%) boiled-cooled milk samples and 9 (15%) juice samples were contaminated with *S. aureus*. The contamination rate was found to be high in the milk samples. The other clinically important bacteria did not show a positive PCR test.

The presence of enterotoxin SEB of *S. aureus* in milk and fruit juices is a major problem of public health and the local food industry. The food poisoning due to *S. aureus* toxin occurs in children and adults depending on the sources that help in the survival of *S. aureus*. At the moment, there is no effective test available to early check the SEB contamination of food. Generally, biochemical tests are used to check *S. aureus* contamination/presence of SEB. The expertise for other methods for routine checking of SEB or *S. aureus* (PCR, immune-PCR) is not available locally. There is a need to devise a simple and reliable method to check the SEB in food and clinically important samples.

In this study, gold conjugated anti-SEB antibodies were prepared to examine the presence of SEB in milk and juice samples. Our studies indicated a 33% and 15% SEB contamination in the milk and juice samples, respectively. Clinically important microbes did not show any cross reactivity with Ab-GNPs reagent, which indicates the specificity and reliability of the devised detection system. The biochemical and PCR tests further provided supporting evidences for the immunodetection of SEB in the samples. The presence of *S. aureus* was found to be higher in milk compared to juices. The SEB contamination in milk and juices may be due to the handling of the raw materials under unhygienic conditions and the use of non-sterilized contaminated water. The quantity of enterotoxin SEB and *S. aureus* in the samples depends on many factors: (i) a contaminated carrier, (ii) unhygienic conditions in factories as well as in household production, (iii) ignorance about hygiene, (v) uses of contaminated water and (vi) sterilization procedure.

The presence and proliferation of *S. aureus* in food for human consumption is a major health problem for children here. The contamination level of SEB should confirm whether a disease is due to S. *aureus* infection or to other microbial contamination. Other staphylococcus strains, such as *S. intermedius* and *S. hyicus*, are also enterotoxigenic. *S. intermedius* is generally considered a veterinary pathogen and has been isolated and characterized from butter and margarines. Food poisoning due to *S. aureus* generally occurs in meat and meat products, cream-filled milk products as well as salads. All these items become contaminated at home or by food supplier companies at local level if handled in unhygienic conditions. It is also reported that if the fermentation of milk products is not completed (e.g. failure of lactic acid consumption), the proliferation of *S. aureus* is rapid.

In an underdeveloped country like Pakistan, it is necessary to observe sanitary and hygienic conditions and the proper disposal of wastes according to international standards. Strict hygienic conditions will be helpful in reducing *S. aureus* contamination. The existence of enterotoxin is associated with the origin and identity of *S. aureus* and the environmental conditions of the host. It is observed that bacteria isolated from cow milk secrete SEA and SEB enterotoxins and bacteria isolated from human skin and wounds secrete SEB. However, only biochemical tests are not be reliable because some other microbes also produce the same biochemical results. PCR assay is a cumbersome process and expensive chemicals, instruments and skills are required. The immuno-

chemical assays are more sensitive, specific and easy to handle. They can detect a very small amount of antigen in samples.

The direct detection of SEB in food samples by this Ab-GNPs complex is not the replacement of other available detection methods for SEB. Detection of a low concentration of SEB in flow immunochromatography assay (1.0 ng/ml) has been reported by utilizing the combination of two antibodies (Hwa et al., 2010; Schutt et al., 2002). In this immunoslot blot assay or immunodot blot assay, the SEB was detected by utilizing only the labeled antibody specific for SEB. Ab-GNPs reagent is reliable, easy to handle and less time is required to detect the SEB in the samples.

CONCLUSION

Anti-SEB antibodies were conjugated successfully with gold nanoparticles. The Ab-GNPs were used to detect SEB in milk and fruit juice samples. The percentage contamination of SEB in food was observed to be high, which reflects unhygienic conditions. The devised method may be used for early detection of SEB in food samples.

Acknowledgments - The authors are thankful to the Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore, for providing the chemicals and instruments for this study. We are grateful to our colleagues for the constructive discussions during this study.

REFERENCES

Alefantis, T., Grewal, P., Ashton, J., Khan, A.S., Valdes, J.J., and *V.G Del Vecchio* (2004), A rapid and sensitive magnetic bead-based immunoassay for the detection of staphylococcal enterotoxin B for high-through put screening. *Mol Cell Probes*; 18, 379-382.

Asao, T., Kumeda Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., Nakazawa, H., and *S Kozaki* (2003). An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiol. Infect*; **130**, 33-40.

Benson, H.J (1998). Microbiological applications, 7th edn, McGraw Hill companies, Inc, USA.

Bohach, G.A., Fast, D.J., Nelson, R.D., and *P.M Schlievert* (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Crit. Rev. Microbiol;* **17**, 251-272.

Bradford, M.M (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. *Anal. Biochem*; **72**, 248-251.

Breuer, K., Wittmann, M., Bosche, B., Kapp, A., and *T. Werfel* (2000). Severe atopic dermatitis is associated with sensitization to staphylococcal enterotoxin B (SEB). *Allergy*; **55**, 551–555.

Bunikowski, R., Mielke, M., Skarabis, H., Herz, U., Bergmann, R. L., Wahn, U., and *H. Renz* (1999). Prevalence and role of serum IgE antibodies to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *J. Allergy Clin. Immunol*; **103**, 119–124.

Goto, M., Hayashidani, H., Takatori, K., and *Y. Hara-Kudo* (2007). Rapid detection of enterotoxigenic *Staphylococcus aureus* harbouring genes for four classical enterotoxins, SEA, SEB, SEC and SED, by loop-mediated isothermal amplification assay. *Letters in Appl. Micro*; 266-8254.

Harlow, D and D. Lane (1988). Antibodies: A Lab Manual of Gold Spring Harbor Laboratory. Gold Spring Harbor, NY,

Henghold, W.B. (2004). Other biologic toxin bioweapons: ricin, staphylococcal enterotoxin B, and trichothecene mycotoxins. *Dermatol. Clin;* **22**, 257-262.

Herz, U, Bunikowski, R, Mielke, M., and *H. Renz* (1999). Contribution of bacterial superantigens to atopic dermatitis. Int. Arch. *Allergy Immunol*; **118**, 240-241.

Howell, M.D, Diveley, J.P, Lundeen, K.A, Esty, A, Winters, S.T, Carlo, D.J, and *S.W. Brostoff* (1991). Limited T-cell receptor beta-chain heterogeneity among interleukin 2 receptor-positive synovial T cells suggests a role for superantigen in rheumatoid arthritis. *Proc. Natl. Acad. Sci;* **88**, 921-10,925.

Hwa, S.R., Shek, T.S., Jiang, C.D., and *Wen, H.Y* (2010). Gold nanoparticle-based lateral flow assay for detection of staphylococcal enterotoxin B. *Anal Meth;* **118** (2), 462-466.

Johnson, W.M., Tyler, S.D., Ewan, E.P., Ashton, F.E., Pollard, D.R., and *K.R, Rozee* (1991). Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *J Clin Microbiol*; **29**: 426-430.

Klotz, M., Opper, S., Heeg, K., and *S. Zimmermann* (2003). Detection of *Staphylococcus aureus* enterotoxins A to D by real-time fluorescence PCR assay. *J Clin Microbiol*; **41**:4683-4687.

Laemmli, U.K (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature;* **227**,680-685.

Lee, J.C, Takeda, S., Livolsi, P.J., and *L. C. Paoletti* (1993). Effects of in vitro and in vivo growth conditions on expression of type-8 capsular polysaccharide by Staphylococcus aureus. *Infect. Immun;* **61**, 1853-1858.

Ler, S.G, Lee, F.K, and *P. Gopalakrishnakone* (2006). Trends in detection of warfare agents. Detection methods for ricin, staphylococcal enterotoxin B and T-2 toxin. *J. Chromatogr. A*; **1133**, 1-12.

Lina, G., Bohach, G.A., Nair, S.P., Hiramatsu, K., Jouvin-Marche, E. and *R. Mariuzza* (2004). International nomenclature committee for Staphylococcal superantigens. Standard nomenclature for the superantigens expressed by Staphylococcus. *J Infect Dis*; **189,** 2334-2336.

Marrack, P., and *K. Kappler* (1990). The staphylococcal enterotoxins and their relatives. *Science*, **248**, 705-711.

Mempel, M., Lina, G., Hojka, M., Schnopp, C., Seid, H.P., Schafer, T., Ring, J., Vandenesch, F., and *D. Abeck* (2003). High prevalence of superantigens associated with the egc locus in Staphylococcus aureus isolates from patients with atopic eczema. *Eur. J. Clin. Microbiol. Infect. Dis*; **22**, 306-309.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and *T. Hase* (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*; **28**, e63.

Omoe K, Hu DL, Takahashi-Omoe H, Nakane A and *K. Shinagawa* (2003). Identification and characterization of a new staphylococcal enterotoxin-related putative toxin encoded by two kinds of plasmids. *Infect Immun*, **71**, 6088-6094.

Rosenbloom, M., Leikin, J.B., Vogel, S.N., and *Z.A. Chaudry* (2002). Biological and chemical agents: a brief synopsis. *Am. J. Ther;* **9**, 5-14.

Sambrook, J and *D. Russel* (2001). Molecular cloning. Cold spring harbor laboratory press, N.Y. A lab manual.

Schutt, U., Langfeldt, N., Peruski, A.H., and *H. Meyer* (2002). Detection of Staphylococcal enterotoxin B (SEB) by enzyme linked immunosorbent assay and by a rapid hand-held assay. *Clin Lab*; **48** (7-8), 395-400.

Sharma, N.K., Rees, C.E., and *C.E. Dodd* (2000). Development of a single-reaction multiplex PCR toxin typing assay for *Staphylococcus aureus* strains. *Appl Environ Microbiol*; **66**, 1347-1353.

Thomas, D.Y., Jarraud, S., Lemercier, B., Cozon, G., Echasserieau, K., Etienne, J., Gougeon, M.L, and *G. Lina,* (2006). Staphylococcal enterotoxin-like toxins U2 and V, two new staphylococcal superantigens arising from recombination within the enterotoxin gene cluster. *Infect Immun*, **74**, 4724-4734.

Towbin, H.K,. Staehelin, T., and *Gordon, J* (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membrane. *Proc Natl. Acad Sci;* **76**, 4350-4359.

Uematsu, Y, Wege, H, Straus, A, Ott, M., Bannwarth, W., Lanchbury, J., Panayi, G, and *M. Steinmetz* (1991). The T-cell receptor repertoire in the synovial fluid of a patient with rheumatoid arthritis is polyclonal. *Proc. Natl. Acad. Sci*; **88**, 8534-8538.

Walker, J.M (1994). Basic protein and peptide protocols; Methods in molecular biology. Humana press Inc. Totowa, NJ.

Wiener, S.L (1996). Strategies for the prevention of a successful biological warfare aerosol attack. *Mil. Med*; **161**, 251-256.