



Study on Enzyme-Linked Immunoassay and Polymerase Chain Reaction for the Identification and Quantification of *Staphylococcus aureus* in Pure Culture and Food Samples

Majumdar Tania, Chakraborty Runu and Raychaudhuri Utpal*

Department of Food Technology and Biochemical Engineering, Jadavpur University, Kolkata-700032, INDIA

Available online at: www.isca.in

Received 5th June 2012, revised 11th June 2012, accepted 12th June 2012

Abstract

Search for rapid, accurate and sensitive method of detection for pathogen such as *Staphylococcus aureus* (*S.aureus*) has become a challenge to food and pharmaceutical industries. Our present work is a study on molecular methods of detection of food borne pathogen, *S.aureus* NCIM 2602. Studies showed that protein A (SpA) antigen of *S.aureus* actually contributed to the resistance of the organism to phagocytosis and opsonization. SpA was detected and the interaction between the SpA antigen and mammalian IgG was confirmed by Western blot and Enzyme linked immunosorbent assay (ELISA). Further, the technique was successfully applied to artificially contaminated food samples like milk, meat, and cheese with an inoculation level of 100 CFU/ml of *S.aureus*. Polymerase chain reaction (PCR) was also applied for the identification of the gene coding for SpA (*spa*) for the current strain. These techniques would be useful in analyzing safety of food samples especially meat and dairy based products which are prone to contamination.

Keywords: *Staphylococcus aureus*, protein A, food samples, ELISA, PCR.

Introduction

The genus *Staphylococcus* includes pathogenic microorganisms in which *Staphylococcus aureus* (*S.aureus*) is the most important¹. It has been reported that *S.aureus* was involved in many documented outbreaks of food borne poisoning² and has been estimated to cause much illness, hospitalizations and even deaths per year all from consumption of contaminated foods³. Hospital infection by *Staphylococcus aureus* deserves special attention because of their resistance to various antibiotics and chemotherapeutic agents⁴. However, mostly used industrial methods for detection and quantification of this pathogen such as plate count, most probable number (MPN) determinations etc are not sensitive, rapid and reproducible enough. Therefore the research worker nowadays are undertaking studies of the characteristics of the microorganism that can survive even in pesticide contaminated site⁵ at the molecular level towards generating informations and data desired for successful fabrication of rapid molecular methods of detection. From the previous serologic analyses, it has been shown that the *S.aureus* has a large number of agglutinogens. Treatment with proteolytic enzymes indicates that majority of these agglutinogens are proteins⁶. Protein A (SpA) from *S.aureus* cell wall is one such example, which is having such receptor⁷. SpA which is present on the surface of the cell wall, makes up almost 7% of the cell wall⁸ and is present in around 95% of all pathogenic strains of *S.aureus*⁹. The molecular size ranges from 50-60 kDa¹⁰⁻¹². It has got very high affinity for binding to Fc region of Immunoglobulin G (IgG)¹³, contributing to the resistance of the organism to phagocytosis and opsonization. SpA helps inhibit phagocytic engulfment and acts as an immunological disguise.

Mutants of *S.aureus* lacking SpA are more efficiently phagocytosed in vitro¹⁴. It activates complement¹⁵ and induces delayed and immediate hypersensitivity reactions¹⁶. *S.aureus* expresses the zymogen staphylokinase that cleaves human plasminogen into active plasmin in turn IgG is cleaved. Thus it inhibits the recognition of the pathogen by C1q, initial component of the classical complement activation pathway^{17,18}. In recent times, different strains of *S.aureus* have caused increasingly severe problems of nosocomial infection as well as community acquired diseases¹⁹ and is a leading cause of gastroenteritis resulting from the consumption of contaminated food. Common foods which get easily contaminated are: milk, cheese, meat, chicken, fish, raw vegetables, fruits, etc. Due to staphylococcal food poisoning onset of different symptoms arise such as abdominal cramps, nausea, vomiting along with diarrhea²⁰. The traditional methods of detection need around 1-2 days for the target pathogen to be identified. Ever since these traditional methods evolved into molecular diagnostics rapid and accurate identification has been possible²¹⁻²⁴.

The identification and quantification of cell wall protein, SpA of pathogenic strain like *S.aureus* NCDO 949, NCDO 1022 and NCDO 2044 have been proved to be useful in determining the specific immunogenic property of *S.aureus* from pure culture as well as food samples²⁵. Analyzing those studies, in the present work, we have tried to find the applicability of molecular methods to detect the presence of the cell wall protein, SpA in a specific pathogenic strain *S. aureus* NCIM 2602 with which no reported study available yet. The study was made with food samples like milk, meat and cheese procured from local market in Kolkata, India. The interaction between SpA antigen and

antibody was detected by western blot analysis and colorimetric amplified enzyme linked immunosorbent assay (ELISA). The polymerase chain reaction was successfully applied for the sensitive detection of bacteria by nucleic acid amplification method.

Material and Methods

Bacterial strain and Culture conditions: Pure cultures of *Staphylococcus aureus* (*S.aureus*) NCIM 2602 was procured from National Collection of Industrial Microorganisms (NCIM), National chemical Laboratory (NCL) (Pune, India). The culture was maintained on slopes of nutrient agar (beef extract-10.0g, NaCl-5.0g, peptone-10.0g, Agar-20.0 g/ L D/W) by monthly subculture and storage at 4°C. Broth cultures were obtained by overnight (18 h) growth in sterile nutrient broth at 37°C. Enumeration of test bacteria was done by colony counting method after incubation at 37°C for 18 h.

Food samples: Milk was procured from Mother Dairy, Kolkata, cheese (Amul, Gujarat) and goat meat (local market, Kolkata).

Bacterial Growth and Harvest: A loopful of bacteria was inoculated into a 50 ml liquid broth and incubated for 18 h at 37°C with shaking. Liquid culture was harvested by centrifugation at 2,000g for 10 min (Model-Hanil, supra 22 K). All harvested cells were washed twice with 10 ml of cold 0.9% NaCl for 1 min. Cells were collected by recentrifugation at 7,000 g for 20 min⁶.

Extraction and purification of Cell wall Protein With Lysostaphin: Pellets obtained were suspended in 1.2 ml of digestion buffer (30% raffinose in 1.2 ml of 0.05 M Tris (pH-7.5) with few drops of 0.145 M NaCl to which 200µg of lysostaphin (Sigma, 625.4 units/mg protein) and 20µg of DNase I (Fermentas, 1 u/µl) were added, iodoacetamide and phenylmethylsulfonyl fluoride (PMSF) were added further to yield a final concentration of 1mg/ml and 1 mM/ml respectively). The cell mixture was allowed to react for 1 h at 37°C with gentle shaking followed by centrifugation at 8,000 g for 10 min to remove the protoplasts and the supernatant was recentrifuged⁶. Supernatant was collected and centrifuged with centrifugal filter devices (Amicon ultra, Millipore) for further purification. The cell wall antigen was stored at - 70°C for future work.

Isolation of Protein from Food sample: Food samples (10 ml or 10 gm) were inoculated with 0.1 ml from overnight grown broth culture of *S.aureus* (~10⁸CFU/ml) and incubated overnight. To 10 ml of *S.aureus* inoculated food samples, 90 ml of sterile 0.01M sodium citrate buffer (pH 6) containing 0.05% (vol/vol) tween 20 was added and homogenized for 2 min. The food samples were later diluted with sterile 0.01M sodium citrate buffer (pH 6) to prepare various concentration grades and the test samples were boiled for 15 min to denature endogenous enzymes and to liberate protein A from the cell envelop²⁵.

Assay of isolated Protein sample: Total protein content present in the cell wall antigen was estimated by standard Lowry method²⁶.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE): Protein electrophoresis has been of great value in bacterial classification and identification¹⁰. Molecular weight of cell wall protein A (SpA) antigen was further analysed through sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% gel composition using mini-protean tetra cell (Biorad, USA) by the method of Laemmli²⁷. Sample buffer was boiled for 5 min before being loaded onto the gel. The molecular weight of protein sample was determined by comparing with prestained molecular weight marker (fermentas).

Western Blot or Enzyme-linked Immuno-electrotransfer Blot: Cell wall proteins of *S.aureus* were separated on 7.5% SDS-PAGE and the bands were then transferred to PVDF (polyvinylidenedifluoride) membrane in mini trans blot cell (Biorad). 5% skimmed milk was added to the membrane, which was used as a blocking agent to which primary antibody (anti-protein A antibody developed in rabbit, Sigma) was added further to bind and thereby recognize protein A antigen. Dilution was maintained for antibody and skimmed milk at 1:1000 µl. Membrane was incubated overnight in the cold room with shaking. Again the membrane was washed thrice with PBST followed by PBS. Secondary antibody (anti-rabbit, developed in goat alkaline phosphatase (AP) conjugate, Santa Cruz Biotechnology, 200 µg/0.5 ml) diluted with PBS (phosphate buffer saline) in the ratio of 1:5000 µl and incubated for 90 min. Again the membrane was washed with PBST (phosphate buffer saline tween- 20) and PBS. The bands were detected with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) which acts as the substrate for alkaline phosphatase conjugate²⁸.

Colorimetric amplified enzyme linked Immunosorbent assay (ELISA) Method: Human IgG (200 µl of 5 µg/ml) in PBS (pH 7) was added to the wells of 96 well flat bottom polystyrene microtitre plates (Nunc-immunoplate) and allowed to adsorb by overnight incubation at 4°C. The plates were washed with PBST thrice. 0.5% BSA was used as blocking agent and added to each of the wells, incubated for 1 h and again washed with PBST thrice. Purified protein A was used as the test antigen prepared in 0.01 M sodium citrate buffer (pH- 7.5) containing 0.05% (v/v) tween 20. Test antigen (200 µl) was pipetted into the microtitre plate wells and incubated for 1 h at 20°C with gentle shaking. Control contained 0.5% (w/v) BSA in PBS. Again, the plate was washed with PBST thrice, 200 µl of rabbit anti-protein A antibody (5 µg/ml) in PBST was added to each well and incubated for 1 h at 20°C with gentle shaking. Wells were washed thrice with PBST, 200 µl of anti-rabbit IgG - alkaline phosphatase conjugate (1.2 µg/ml) in PBST was added to each well and incubated with shaking for another 1h. After wells were washed with PBST, 80 µl of substrate, NADP⁺

(nicotinamide adenine dinucleotide phosphate) (SRL) (240 µg/ml in 50 mM diethanolamine hydrochloride buffer, pH- 9.8 was added to each well and the plates were incubated for 15 min. Amplifier solution containing 200 µl of 25 mM PBS (pH-7.2) containing 40 µl of ethanol/ml, 300 µg of *p*-iodonitrotetrazolium violet (INT)/ml (Amresco), 150 µg of diaphorase/ml (Sigma, 7.8 units/mg protein) and 150 µg of alcohol dehydrogenase/ ml (SRL, 382 units/mg protein) was added to each well and further incubated for 30 min at 20°C. Color developed by formation of formazan was measured spectrophotometrically at 492 nm using a microplate reader (Model 680, Biorad) immediately after incubation period was over²⁵. Similar assay procedure was applied for food samples inoculated with *S.aureus* NCIM 2602 as well as food sample naturally contaminated with *S.aureus* and further examined for the presence of test bacteria by plate counts and the MPN method. The minimum concentration of protein A subjected to the assay was 50 µg/ml which corresponded to approximately 100 CFU/ml.

Sample preparation for PCR: Isolation of DNA from *S.aureus*: From pure culture of *S.aureus*, for extraction of DNA cells were harvested from the overnight grown culture by centrifugation at 8000 rpm at 10°C for 15 min and the supernatant was discarded²⁹ (some modifications have been made to the protocol). The pellet was washed twice with normal saline and centrifuged at 8,000 rpm at 10°C for 15 min. 0.5 ml of 10 mM tris-HCl (pH 8) and 2.5 mg/ml of lysozyme was added and incubated at 37°C for 1 h. Then 1 ml of lysis buffer (50 mM tris, 100 mM EDTA, 1% SDS pH 8) and 1 mg/ml of proteinase-K was added. Mixed gently and incubated at 50°C for 1 in a water bath. Digestion with proteinase-K was followed by addition of 1 ml of phenol: chloroform (1:1). Mixed gently for 2-3 min and centrifuged at 10,000 rpm at 4°C for 15 min, transferred the upper layer in a fresh sterile tube and extracted with chloroform: isoamyl alcohol (24:1) by the centrifugation at 10,000 rpm at 4°C for 15 min and the step was repeated again. Supernatant was taken and 50 µl of 5 M NaCl and twice volume of 75% of ethanol was added and left till the precipitation settled down for overnight. Then centrifuged at 10,000 rpm at 4°C for 15 min and decanted the supernatants and air dried the pellet. 50µl of TE buffer (depending upon the size of DNA pellet) was added for re-suspension of DNA and incubated the DNA at 37°C in a water bath for 1 h for the inactivation of DNA degrading enzymes.

For artificially contaminated milk sample, 0.1 ml of fresh broth of *S.aureus* (~10⁸CFU/ml) was added to 10 ml of milk sample and incubated at 37°C for 18 h. DNA was extracted by centrifugation and followed the same procedure as of pure culture and obtained the *S.aureus* artificially contaminated DNA product. For naturally contaminated milk sample, milk was kept at 4°C for overnight. Then 10 µl of one day old milk sample was plated on Mannitol salt agar media (MSA) (Himedia), selective media for *S.aureus* and incubated at 37°C for 18 h. Next day colonies were observed on MSA plate. Loopful of bacteria was

added to nutrient broth and incubated at 37°C with shaking for overnight. From the overnight grown culture, DNA was extracted following the same procedure as mentioned above and all the three samples were subjected to PCR and agarose gel electrophoresis.

Primer Design: The spa gene size was approximately 2.15 Kb³⁰. Table 1 shows the primer sequence designed by using primer 3 for the PCR to be performed³¹.

Table-1
PCR primers used in the study

Gene	Primer	Sequence (5'-3')	Product size
spa	Forward	CGGCACTACTGCTGACAAAA	09 bp
	Reverse	AACGCTGCACCTAAGGCTAA	

PCR: The DNA obtained was amplified by PCR in 50 µl vials. To the 50 µl vials, 2 µl of 10X PCR buffer, 0.8 µl of 2.5mM d NTP, 2 µl of 25 mM MgCl₂, 0.1 µl Taq DNA polymerase (5 unit/ µl), 12.1 µl of double distilled water, forward primer 0.5 µl, reverse primer 0.5 µl and 2 µl DNA sample was added to obtain the final product. Final working volume was 20 µl. The program was run in M J Mini thermal cycler (Biorad) at 94°C for 5 min for an initial denaturation, followed by 35 cycles of DNA denaturation at 94°C for 45 s, annealing at 58°C for 45 s and extension at 72°C for 1 min. After 35 cycles were over, the thermal cycler was set at 72°C for 7 min for final extension.

Agarose gel electrophoresis: PCR products were subjected to electrophoresis on a 1.5 % agarose gel, visualized under UV light and gel image was documented further in gel documentation system (Uvipro, platinum). A 1Kb plus DNA ladder ranges from 20 Kb to 75 bp (Fermentas) was used as marker to indicate the size of amplicon.

Results and Discussion

Cell wall Protein separation by SDS-PAGE: After estimation of the total protein content by Lowry method, the protein antigen sample was loaded to the wells of 7.5% gel. Figure-1 shows the presence of cell wall protein bands through SDS-PAGE analysis and the bands obtained were compared with prestained molecular weight marker and it has been found the presence of protein A was between 86 and 47 kDa.

Identification of Protein A by Immunoblot: For further identification of SpA from *S.aureus* after resolving through 7.5% gel, bands were transferred from gel to the PVDF membrane. Figure- 2 shows lysostaphin extract of cell wall protein, SpA probed with primary and secondary AP conjugate antibody by immunoblot.

ELISA: By applying the enzyme amplification system, sensitivity of the detection method was improved. Nicotinamide adenine dinucleotide phosphate (NADP⁺) was dephosphorylated to nicotinamide adenine dinucleotide (NAD⁺) which catalytically activated a secondary enzyme system consisting of

a redox cycle driven by the enzymes alcohol dehydrogenase and diaphorase. NAD^+ was reduced by ethanol and the NADH so formed reduced a tetrazolium salt to regenerate NAD^+ and to produce an intensely coloured formazan dye³². Figure- 3(a) and 3(b) shows the linear relationship between formazan formation and increasing SpA concentration at $A_{492\text{nm}}$ obtained from pure culture as well as food samples inoculated with *S.aureus*. All measurements were done in triplicate ($n = 3$) and data points were presented as mean \pm S.D, $p < 0.05$.

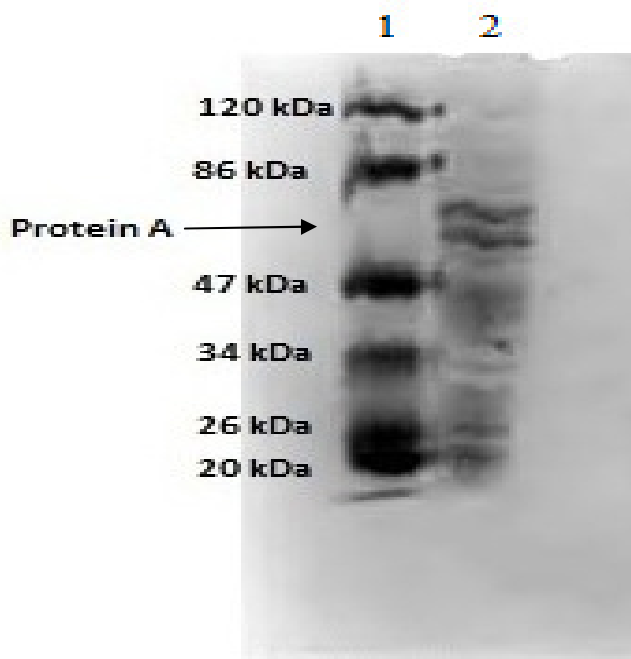


Figure-1
 SDS-PAGE (7.5% Gel) of cell wall lysostaphin extract from *Staphylococcus aureus*. Lane 1 shows marker; lane 2, protein extract from *S.aureus* NCIM 2602

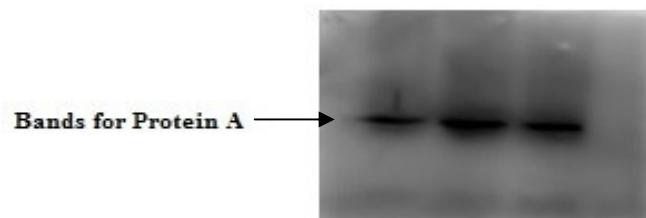


Figure-2
 Western Blot/Immunoblot of cell wall extract of *Staphylococcus aureus* probed with primary and secondary antibody

PCR and Agarose gel electrophoresis: Figure- 4 shows agarose gel electrophoresis of spa gene amplification products. Lane 1 shows ladder, lane 2 with pure culture of *S.aureus*, lane 3 contained *S.aureus* artificially contaminated milk sample and lane 4 had naturally contaminated milk sample. The bands obtained after gel electrophoresis for the amplified products from pure culture (*S.aureus* NCIM 2602), food sample artificially contaminated as well as naturally contaminated with *S.aureus* were nearly at 200bp.

In figure-1, since the band obtained for protein extract from *S.aureus* NCIM 2602 varying between 86-47 kDa, which goes well with the molecular size of standard cowan I SpA, suggests the bands represent SpA only. For figure- 2, the bands on the membrane incubated with primary antibody and skimmed milk at 1:1000 μl , the skimmed milk blocked the sites where the antibody did not bind to the antigen to avoid nonspecific binding between antibody and antigen. Further addition of anti-rabbit IgG AP conjugate secondary antibody allowed the detection to be easier and specific. SpA showed strong binding by color development within 30 min after addition of the substrate (BCIP/NBT) in AP buffer and the bands became prominent. This suggests that the protein of interest i.e SpA from *S.aureus* NCIM 2602 had strong IgG binding properties similar to that of other standard *S.aureus* strain containing SpA³³.

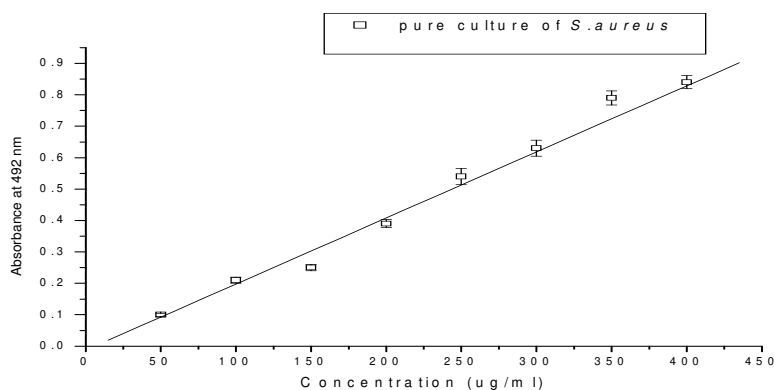


Figure- 3(a)
 Relationship between Formazan formation (measured at 492 nm) and Protein A concentration from pure culture of *S.aureus* NCIM 2602

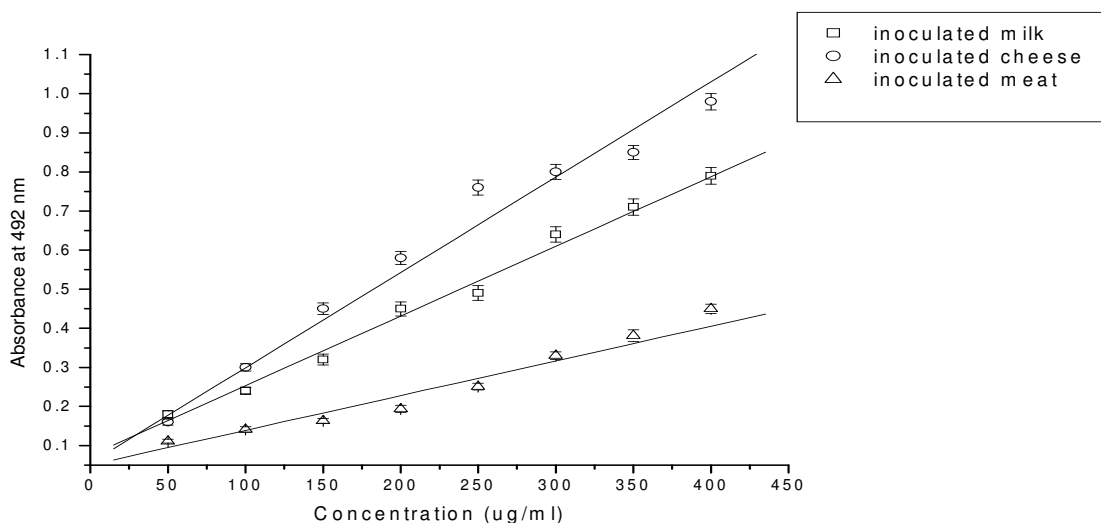


Figure- 3(b)

Relationship between Formazan formation (measured at 492 nm) and Protein A concentration from *S.aureus* NCIM 2602 inoculated food samples milk, Cheese and meat

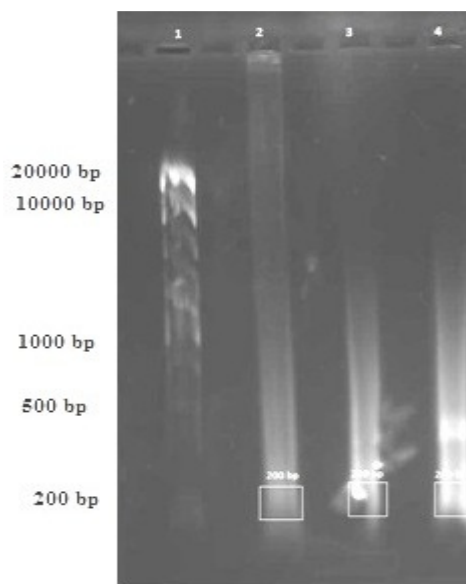


Figure- 4

Electrophoretic analysis of PCR amplified products, Lane 1 Molecular weight marker, lane 2 Pure culture of *S.aureus* NCIM 2602, lane 3 artificially contaminated milk sample by *S.aureus* NCIM 2602, lane 4 naturally contaminated milk sample by *Staphylococcus* strain

When the above mentioned ELISA method for protein A quantification was applied to pure culture as well as contaminated food samples quite similar results were obtained for all the samples. NADP^+ acts as the substrate for alkaline phosphatase conjugated secondary antibody which releases NAD^+ and in presence of amplifier solution gets reduced to NADH. NADH by releasing electron to *p*-iodonitrotetrazolium (INT) gives the red colored product formazan. The linear

relationship between formazan formation (A_{492}) and protein A concentration was good. With application of increasing concentration of protein (SpA), more binding between Ag and antibody occurred. Consequently, there was more of NAD^+ and NADH production leading to increasing response with INT at A_{492} nm. Figure- 3(a) shows R^2 value of 0.98 for protein A from pure culture and figure- 3(b) shows R^2 values of 0.99, 0.98 and 0.95 for *S.aureus* inoculated milk, cheese and meat respectively.

It was obvious from figure- 3(a) and 3(b) that the R^2 value for SpA from pure culture was close to that of other food samples inoculated with *S.aureus* which proved that food constituents did not interfere much with the sensitivity of the assay. In addition, plate counts and MPN determinations for *S.aureus* also supported the above given results. Thus this amplified ELISA method was reproducible for quantification of the particular strain of *S.aureus* containing protein A antigen and would be applicable to all other strains of *Staphylococcus aureus* containing cell wall protein A.

In figure- 4 since the bands obtained after gel electrophoresis from the amplified products for pure culture (*S.aureus* NCIM 2602), food sample artificially contaminated as well as naturally contaminated with *S.aureus* were found nearly at 200bp, it may be assumed that the sample of lane 4 containing the strain isolated from one day old milk sample also expressed the same gene coding for protein A i.e spa gene. Therefore it may be concluded that more studies can be done by designing new primers specific for a wide range of pathogenic microorganisms whose early detection would be beneficial to resist many deadly diseases.

Conclusion

Staphylococcus aureus has been recognized as a frequent pathogen in human and animal infections. The investigation for the presence of such microorganism both qualitatively and quantitatively is very much necessary to maintain the quality and safety of food and pharmaceutical products. In addition this study may lead to fabricate quick, sensitive (for the present study detection limit was 100 CFU/ml for *Staphylococcus aureus* NCIM 2602) and reproducible detection method of pathogen substituting the prevailing conventional, extended, labour intensive and sometimes low precession method of detection of the pathogens. This will ultimately save time and energy resources of food and other allied industries.

Acknowledgement

We would like to express our gratitude to University Grants Commission, New Delhi, India and grant from the Centre for Advanced Studies – I of the department of Food Technology and Biochemical Engineering, Jadavpur University for providing us financial support to carry out our research work.

References

1. Kaleem F., Usman J., Hassan A., Omair M., Khalid A. and Roz Uddin., Sensitivity pattern of methicillin resistant *Staphylococcus aureus* isolated from patients admitted in a tertiary care hospital of Pakistan, *Iran. J. Microbiol.*, **2**, 141-143 (2010)
2. Olsen S.J., Mackinnon L.C., Goulding J.S., Bean N.H. and Slutsker L., Surveillance for foodborne disease outbreaks—United States, 1993–1997, *Morb. Mortal. Wkly. Rep.*, **49 (SS01)**, 1–51 (2000)
3. Mead P.S., Slutsker L., Dietz V., Mc Caig L.F., Bresee J.S., Shapiro C., Griffin P.M. and Tauxe R.V., Food-related illness and death in the United States, *Emerg. Infect. Dis.*, **5**, 605–625 (1999)
4. Nakade Dhanraj B., Antibiotic sensitivity of common Bacterial Pathogens against selected Quinolones, *ISCA J. Biological Sci.*, **1(1)**, 77-79 (2012)
5. Rohilla S.K. and Salar R.K., Isolation and Characterization of Various Fungal Strains from Agricultural Soil Contaminated with Pesticides, *Res. J. Rec Sci.*, **1 (ISC-2011)**, 297-303 (2012)
6. Cheung A.L. and Fischetti V.A., Variation in the Expression of Cell Wall proteins of *Staphylococcus aureus* Grown on Solid and Liquid Media, *Infect. Immun.*, **56**, 1061-1065 (1998)
7. Goding J.W. Use of staphylococcal Protein A as an immunological reagent, *J. Immunol. Method.*, **20**, 241-253 (1978)
8. Forsgren A., Protein A from *Staphylococcus aureus* VIII. Production of protein A by bacterial and L forms of *S.aureus*, *Acta. Pathol. Microbiol. Scand.*, **75**, 481-490 (1969)
9. Forsgren A., Significance of Protein A production by staphylococci, *Infect. Immun.*, **2**, 672-673 (1970)
10. Costas M., Cookson B.D., Talsania H.G. and Hill L.R. Numerical analysis of electrophoretic protein patterns of Methicilin-Resistant Strains of *Staphylococcus aureus*, *J. Clin. Microbiol.*, **27**, 2574-2581 (1989)
11. Manchester L., Pot B., Kersters K. and Goodfellow M., Classification of Streptomyces and Streptoverticillium Species by Numerical Analysis of Electrophoretic protein patterns, *Syst. Appl. Microbiol.*, **13**, 333-337 (1990)
12. Vauterin L., Vantomme R., Pot B., Hoste B., Swings J. and Kersters K., Taxonomic analysis of *Xanthomonas campestris* pv. *begoniae* and *X. campestris* pv. *pelargonii* by means of phytopathological, phenotypic, protein electrophoretic and DNA hybridization methods, *Syst. Appl. Microbiol.*, **13**, 166-176 (1990)
13. Forsgren A. and Sjoquist J., Protein A from *S.aureus*. I. Pseudoimmune reactions with human gamma-globulin, *J. Immunol.*, **97**, 822-827 (1966)

14. Goodyear C.S. and Silverman G.J., Death by a B-cell super antigen: In vivo VH targeted apoptotic supra-clonal B-cell deletion by a staphylococcal toxin, *J. Exp. Med.*, **197**, 1125-1139 (2003)
15. Stalenheim G., Otze O.G., Cooper N.R., Sjoquist J. and Muller-Eberherd H.J., Consumption of human complement components by complexes of IgG with Protein A of *Staphylococcus aureus*, *Immunochem.*, **10**, 501-507 (1973)
16. Forsgren A., Ghetie V., Lindmark R. and Sjoquist J., Protein A and its exploitation, In: Easmon CSF, Adlam C (ed) *Staphylococci and Staphylococcal infections*, pp. 429-480, Academic Press, New York (1983)
17. Salyers A.A. and Whitt D. D., *Salmonella* species. In: Salyers AA and Whitt DD (eds) *Bacterial pathogenesis: a molecular approach*, ASM Press, Washington, DC, 381-397 (2002)
18. Rooijackers S.H., Van Wamel W. J., Ruyken M., Van Kessel K.P. and Van Strijp J., Anti-opsonic properties of staphylokinase, *Microbes. Infect.*, **7**, 476-484 (2005)
19. Berber I., Cokmus C. and Atalan E., Characterization of *Staphylococcus* species by SDS-PAGE of whole-cell and extracellular proteins, *Microbiol.*, **72**, 54-59 (2003)
20. Loir Y.L., Baron F. and Gautier M., *Staphylococcus aureus* and food poisoning, *Gen. Mol. Res.*, **2**, 63-76 (2003)
21. Weinstein M.P., Mirrett S., Reimer L.G., Wilson M.L., Smith-Elekes S., Chuard C.R., Joho K. L. and Reller L.B., Controlled evaluation of BacT/Alert standard aerobic and FAN aerobic blood culture bottles for detection of bacteremia and fungemia, *J. Clin. Microbiol.*, **33**, 978-981 (1995)
22. Carroll K.C., Leonard R.B., Newcomb-Gayman P.L. and Hillyard D.R., Rapid Detection of the Staphylococcal mec A gene from BACTEC blood culture bottles by the polymerase chain reaction, *Am. J. Clin. Pathol.*, **106**, 600-605 (1996)
23. Mata A.I., Gibello A., Casamayor A., Blanco M.M., Dominguez L. and Fernandez J.F., Multiplex PCR assay for detection of bacterial pathogens associated with warm-water Streptococcosis in fish, *Appl. Environ. Microbiol.*, **70**, 3183-3187 (2004)
24. Kamesh R.S. and Roman R.G., Multiplex Detection of Ehrlichia and Anaplasma species pathogens in Peripheral Blood by Real-Time RT PCR, *J. Mol. Diagn.*, **7**, 308-316 (2005)
25. Brooks J.L., Mirhabibollahi B., Kroll R.G., Sensitive Enzyme-Amplified Electrical Immunoassay for Protein A-Bearing *Staphylococcus aureus* in Foods, *Appl. Environ. Microb.*, **56**, 3278-3284 (1990)
26. Wilson K. and Walker J., *Practical Biochemistry: Principles and Techniques*, Cambridge University Press (2000)
27. Laemmli U.K., Cleavage of structural proteins during assembly of the head of bacteriophage T4, *Nature (London)*, **227**, 680-685 (1970)
28. Blake M.S., Johnston K.H., Russell G.J.J. and Gotschlich E.C., A rapid sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots, *Anal. Biochem.*, **136**, 175-179 (1984)
29. Wilson K., Preparation of genomic DNA from bacteria. In: Ausubel FM, Brent R., Kingston R. E., Moore D. D., Seidman J.G., Smith J.A., Struhl K (eds) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York, **1**, 2.4.1-2.4.2 (1987)
30. Frenay H.M., Bunchoten A.E., Schouls L.M., VAN Leeuwen W.J., VAN Denbroucke-Grauls C.M., Verhoef J. and Mooi F.R. Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism, *Eur. J. Clin. Microbiol. Infect. Dis.*, **15**, 60-64 (1996)
31. Uhlen M., Guss B., Nilsson B., Gatenbeck S., Pthilipson L. and Lindberg M., Complete Sequence of the Staphylococcal Gene Encoding Protein A, a gene evolved through multiple duplications, *J. Biol. Chem.*, **259**, 1695-1702 (1984)
32. Lowry O.H., Passonneau J.V., Schulz D.W. and Rock M.K., The measurement of pyridine nucleotides by enzymatic cycling, *J. Biol. Chem.*, **236**, 2746-2755 (1961)
33. Cheung A.L., Bayer A.S., Peters J. and Ward J.I., Analysis by Gel Electrophoresis, Western Blot, and Peptide Mapping of Protein A heterogeneity in *Staphylococcus aureus* strains, *Infect. Immun.*, **55**, 843-847 (1987)