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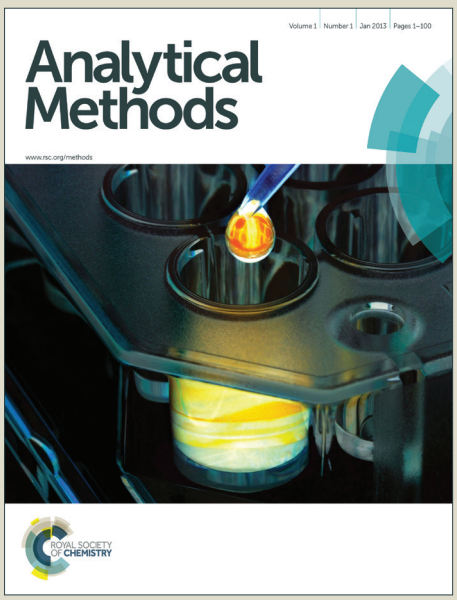

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Gold nanoparticle based immunodetection of *Staphylococcus aureus* LeukotoxinM/F´-PV in subclinical samples of bovine mastitis

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Abstract

In the present study, an immunosensor was designed to assess bovine mastitis at the earliest by quantifying leukotoxin M/F´-PV (LukM/F´-PV), a potent neutrophil targeting and beta barrel pore forming toxin secreted by bovine strains of *S. aureus*. Polyclonal antibodies to recombinant LukF (rLukF) component of LukM/F´-PV was raised and purified by affinity chromatography. Further, anti-rLukF antibody was used to design a classical ELISA detection system in which we obtained 1000 ng/mL LOD. Considering drawbacks of classical detection system, antibodies were functionalized to gold nanoparticles of large surface plasmon band providing opportunity to design immunoassays based on Nanosurface energy transfer (NSET) from dye to gold nanoparticles (GNPs). In the experimental set up, rLukF was incubated with functionalized GNPs. Fluorescein isothiocyanate (FITC) labeled secondary antibodies were added and fluorescence quenching was monitored as function of toxin concentration. With this method, leukotoxin was detected in the range 100 ng-0.1 ng/mL, with LOD of 0.1ng/mL and R^2=0.9908. In addition, above proposed assay was applied to detect toxin in spiked and field samples with 89-95% recovery. Thus, proposed method outcomes major drawbacks of ELISA systems and can provide a window for sensitive detection of toxin before onset of mastitis.

Keywords: Mastitis, LukM/F´-PV, NSET, Immunoassay

1. Introduction

The pathogenesis of *S. aureus* initiating subclinical mastitis creates an epidemiological link of disease severity with persistent shifts in bovine health deterioration have recently become a subject of clinical importance.[5,21,29,36,38] This accounts for an estimated economic loss of $2 billion annually or $140 to $300/cow per year.[20] Among the secreted homologically similar bicomponent leukotoxin counterparts,[3] bovine strains of *S. aureus* secreting LukM/F´-PV is considered as an extremely virulent trait for provoking mastitis in bovine, ewe and goat population under native and experimental conditions.[12,34-36] It is roughly estimated that 10-50% of *S. aureus* isolates comprise *luk*M/F´-PV gene[15] and are known to produce LukM/F´-PV toxin in culture supernatants as analyzed by leukocytotoxicity assay.[30] Subsequently, LukM/F´-PV at its lowest concentration of 3.6 ng/ml binds to immune target cells (monocytes, macrophages and polymorphoneutrophil), oligomerize as octamers and disrupt the membrane integrity by inserting beta barrel pores leading to osmotic imbalance and cell death.[25,34,39] The inflammatory mediators released through formed pores ultimately necrotize mammary gland tissues causing chronic illness accompanied with blood stained milk and clogged udder ducts.[26,43] Therefore, resulting leukotoxicity along with the pathogen eventually decreases milk production and may later lead to the death of animal.[2,24]

As it is crucial to prevent such spread of mastitis, analysis of toxic subunits in raw milk samples may facilitate an early and accurate hidden infection status or the intensity of disease progression. Accordingly to the available literature, the antigen is usually detected through sandwich ELISA formats, where 30 ng/ml and 0.4 ng/ml of individual toxic subunits in milk samples were detected by Loeffler et al. (1988)[22] and Rainard (2007)[35], respectively. The conventional LukM/F´-PV ELISA involves multiple steps of incubation, requires large sample volume for the assay to be carried out and in fact considering the toxicity involved, it becomes very important to detect this toxin near/below infection causing levels (3.6 ng/mL). To address this issue, the

present work aims to exploit the Nano Surface Energy Transfer (NSET) principle between gold nanoparticles and fluorescein isothiocyanate (FITC) labeled secondary antibody for rapid detection of leukotoxin as early as possible and even below the point for causing intramammary infection.

Nano-structured platforms for ultrasensitive detection have become an important tool for biosensing of toxins and other key analytes.[1,7,8,18,19,41] In particular, gold nanoparticles (GNP) based diagnostic systems have attained major focus due to its unique surface plasmon band (SPB), broad absorbance, around 520 nm[23,27,28,32,33,37] and moreover, uniqueness arises due to the fact of having high surface area of metallic nanoparticles. Additionally, GNPs are highly efficient quenchers over long distance[6,42,44] and due to GNPs large SPB it can be efficiently used as quencher for fluorescent dyes/nanoparticles. Considering no defined dipole movement, NSET can take place on nano-surface in any possible orientation of donor.[23] In comparison with NSET, it is critical for Fluorescence Resonance Energy Transfer (FRET) based immunoassays to have donor-acceptor pair to be close enough for resonance energy transfer to be turned on, thus making it a limitation.[13] Accordingly, Yun et al. (2005)[42] have proved that FRET detection limits are low and it does not offer wide range of detection by comparing FRET efficiency over NSET. Considering all these facts, it is a better choice to use NSET phenomenon for immunoassay. Although the distance between GNP-primary antibody and FITC labeled secondary antibody is relatively large, surface energy transfer from FITC to GNPs over large distance can be used as a sensitive tool for monitoring toxins. There are many reports where in GNP based fluorescence quenching is used to design ultrasensitive immunoassays, where Ambrosi et al. (2010)[1] designed immunoassay for sensitive detection of CA15-3 antigen using gold nanoparticle as enhancer. Similarly, long range quenching principle of GNP for detection of cardiac troponin (0.7 ng/ml) was used[23] and europium nanoparticles was used for ultrasensitive detection of anthrax protective antigen at 0.01 ng/ml detection limit.[40]

Despite the progress of NSET phenomenon between GNPs and dyes, practical application involving these for leukotoxin detection is not explored. Of the recent reports, Tao et al.[17] has applied NSET assay for detection of human immunoglobulin G with a detection range of $4.0x10^{-9}$ to $2.2x10^{-7}$ g mL^{-1}. Tao et al., used FITC labeled secondary IgG antibody as probe for detection of IgG antibody which was aided by GNP mediated quenching and used naked GNPs as quencher and it may not serve biorecognition purpose efficiently. To overcome this problem, In our studies we immobilized primary antibody to GNPs so that biorecognition of antigen followed by addition of FITC labeled secondary antibody will bring donor acceptor pair close enough for NSET. Present work was aimed at "turn-off" fluorescence method wherein our strategy was to detect LukF toxin causing bovine mastitis rather than detecting antibody. In the proposed method, anti-rLukF polyclonal antibodies were immobilized on gold surface to develop nanoprobe specific to LukF component (Scheme 1). Upon addition of FITC labeled secondary antibody, fluorescence quenching was monitored with respect to toxin concentration as depicted in scheme 1. The effectiveness of nanogold with antibody in the conjugate system was studied and applied for ultrasensitive detection of LukF. To the best of our knowledge, this is the first report to apply NSET phenomenon for detecting LukF component in spiked and subclinical mastitis samples with ultra sensitivity.

2. Experimental

2.1. Fine chemicals and Reagents

Protein A Sepharose (GE healthcare), nitrocellulose membrane (Sigma Aldrich, USA), biotin conjugated goat anti-rabbit IgG and avidin tagged alkaline phosphatase (Biorad), nitro blue tetrazolium/5-Bromo-4-Chloro-3-Indoylphosphate (NBT/BCIP), p-nitrophenyl phosphate (PNPP) and goat anti-rabbit IgG-FITC (Merck, Bangalore), Polystyrene plates (Tarsons, Bangalore), prestained marker (#SM0671-Fermentas, Bangalore) were purchased for experimental studies. For synthesis of gold nanoparticles, Gold (III) chloridetrihydrate ($HAuCl_4$. $3H_2O$), Tri sodium citrate, Silver nitrate ($AgNO_3$) were procured from Sigma Aldrich chemicals, USA and used as received. Ultra purified water for the experiments was collected using Milli-Q three stage water purification system (Millipore India Pvt Ltd).

2.1.1. Instrumentation

ELISA reader (Thermo scientific USA), spectrophotometer UV-1601 (Shimadzu, Japan), Transmission Electron Microscopy (Jeol 2100, USA) and Spectrofluorimeter (RF-5301 PC, Shimadzu, Japan) were used for analytical studies.

2.2. Experiments

2.2.1. Production and purification of anti-LukF-PV polyclonal antibodies

Anti-rLukF antiserum was obtained previously by immunizing New Zealand rabbits with rLukF´-PV antigen which was expressed in *E. coli* BL21pLysS under the control of T7 promoter of pRSETA vector.[3] The polyclonal antibodies from the immunized serum were purified using Protein A Sepharose column according to manufacturer's protocol with slight modification. Briefly, the column was washed with 10 column volume of 1x PBS (pH 7.4) to which 2 ml of crude serum was applied and the flow through was collected. With a final wash of 20 column volume of 1x PBS, antibody bound to column was eluted as fractions with the addition of 100 µl of 50 mM Glycine (pH 2.7) and in parallel, the elutes were neutralized

with 10 µl neutralization buffer (1 M Tris-HCl, pH 8.0; 1 mM EDTA and 0.05% sodium azide). Protein assay was performed on all collected fractions spectrophotometrically at A_{280}. The fraction with highest concentration of protein was pooled, specific amount of antibody (2 mg/ml) diluted in 100 mM phosphate buffer (pH 7.2) was further analyzed through western blot and indirect enzyme linked immunosorbent assay.

2.2.2. Western blotting

The rLukF along with prestained marker resolved in 12% SDS-PAGE was electrophoretically transferred to nitrocellulose membrane at 100 mA for 1 h. The membrane was blocked overnight in blocking buffer (2% gelatin in 1x PBS-0.05% Tween 20) at room temperature. The blocked membrane was washed three times at 10 min interval with 1x PBS-T and incubated for 2 h with 80 µl of 2 mg/ml purified anti-rLukF antibody (diluted in 10 ml blocking buffer). With intermittent washes, the membrane was subsequently probed with biotin conjugated goat anti-rabbit IgG and avidin tagged alkaline phosphatase (both diluted at 1:3000 in blocking buffer) for 2 h at room temperature. Finally, the blot was developed with the addition of NBT/BCIP substrate (1 mg/ml dissolved in alkaline phosphatase buffer) and when the purple band was visualized, the reaction was terminated by soaking the membrane in distilled water.

2.2.3. IgG-ELISA

Polystyrene plates with 96 wells (Tarsons, India) were coated with 100 µl of rLukF from 1000 ng-50 ng/well in 0.05M sodium carbonate buffer (pH 9.6) and incubated overnight at 4° C. The plate was washed once with 1x PBS-Tween20 (0.05% v/v) and then the unbound sites were blocked with PBS-Gelatin (1.5%) for 2 h at 37° C. After washing with 1x PBST; 20, 40, 60, 80, 100 µl (made up to 100 µl with blocking buffer) from 2 mg/ml of purified antibody was dispensed into respective labeled wells and the plate was incubated for 2 h at 37° C. Sequentially, 100 µl of biotin tagged Goat anti-rabbit IgG and avidin tagged alkaline phosphatase was added with consequent washing steps followed by incubation for 2 h at 37° C.

Given a last wash, the plate was observed for alkaline phosphatase activity with pNPP substrate (1 mg/ml, 100 µl/well) was dissolved in 1% Diethanolamine buffer (pH 9.8) and the reaction was terminated with the addition of 3M NaOH (50 µl/well) after an incubation step of 30 min at 37 °C. The absorbance was read at 405 nm using Multiskan ELISA microplate reader (Thermo Scientific, USA) and the graphical plots for ELISA reading was constructed with averaged OD_{405} values expressed in mean deviation. A two fold increase in cut off mean absorbance value of antigen coated well than blank reading was considered positive for the executed immunosorbent assay.

2.2.4. Gold nanoparticle synthesis and characterization

Spherical gold nanoparticles were synthesized according to the method described by[16] and modified to our requirements. In brief, 5 ml of $HauCl_4$ aqueous solution (0.1%wt) and 45 µl of $AgNO_3$ solution (0.1%wt) added to a given amount of citrate aqueous solution (1% wt).The volume was made up to 10 ml and allowed to incubate for 10 minutes. The reaction mixture was then added to 40 ml of boiling water under reflux with constant stirring. The reaction was allowed to proceed for 30 min to form uniform spherical nanoparticles. The cooled GNPs were filtered through 1 µm filter to remove any aggregates and stored at 4 °C till further use. Synthesized nanoparticles were characterized by spectrophotometry and transmission electron microscope (TEM).

2.2.5. Preparation of anti-rLukF antibody functionalized GNPs and optimization of concentration

GNP having 0.9 absorption units at 520 nm was functionalized with anti-rLukF antibody. Various concentrations (0.04, 0.08, 0.12, 0.16 and 0.2 mg) of anti-rLukF antibodies were prepared in PB and functionalized as described earlier[41] with slight modifications; briefly, antibody solution in PB (50 mM, pH

7.4) was added drop wise to GNP with gentle stirring and incubated for 60 min at 32 °C followed by overnight incubation at 4 °C. The reaction mixture was centrifuged at 10,000 RPM for 15-20 min at 4 °C and the obtained pellet was re-dissolved in 1 ml of PB. Functionalization of GNP with anti-rLukF antibody was confirmed by absorption spectral analysis in comparison with pure GNP. To observe the effect of antibody concentration on proposed assay format, fluorescence quenching experiments were performed by keeping constant (100 ng/ml) antigen concentration. To this, 50 µl of (1:100) FITC labeled secondary antibody was added to primary antibody-antigen mixture after incubating for 20 min. Fluorescence quenching experiments were performed by exciting FITC component at 490 nm and monitoring emission at 516 nm.

2.2.6. Standardization of NSET assay format for leukotoxin detection

Various concentrations of rLukF (0.1-100 ng/ml) were prepared using PB. Optimized concentration of primary antibody functionalized GNP was then added separately to 100 µl of toxin of varying concentrations and incubated at RT for 20 min. To this solution, 50 µl of FITC labeled secondary antibody was added and incubated for 20 min at RT. Fluorescence spectra were recorded and fluorescence quenching was monitored by exciting FITC at 490 nm.

2.2.7. Validation of NSET assay

NSET based assay was developed and used for the detection of LukF component in water and milk samples by monitoring fluorescence quenching of FITC. To study the recovery and detection efficiency by NSET methodology, rLukF of 20 ng/ml and 100 ng/ml was spiked in water and raw milk respectively to study the detection efficiency in complex samples. Optimized concentrations of functionalized primary antibody and FITC labeled secondary antibody were added as indicated in the above said protocol and fluorescence quenching was monitored. To apply the developed method for real time analysis, raw milk of bovine affected with sub-clinical mastitis was obtained from local area and was analyzed for the presence of

native LukF by ELISA and NSET method. To check the sensitivity in real samples, milk was further diluted to obtain ~10 ng/ml toxin and assay was carried out by NSET methodology for recovery and sensitivity determination.

3. Results and discussion

3.1. Western blot and Indirect ELISA

The immunogenicity as evaluated by purified anti-rLukF antibodies through western blot analysis revealed a significant and specific response towards rLukF (39 kDa) protein (Fig. 1a). Similarly, indirect IgG-ELISA was had maximum detection limit of rlukF antigen at 50 ng coated/well (Fig. 1b). The wells with no antigen were considered as negative control (blank) where the calculated OD_{405} of non specific binding of antibodies was used as correction factor. When background signal of first dilution (0.18) was compared to subsequent dilution of antibody, a slight increase in OD values was observed with a ratio of 0.04. Accordingly the cut off was considered for the Ag-Ab interaction, where 80 µl (0.16 mg of antibody) was optimized for further assays. As suggested by[4] that interaction of immobilized antibody on gold nanoparticle to the respective antigen can be confirmed with prior optimization of ELISA for increasing the sensitivity of reaction format. By this method we were able to detect rlukF with Limit of Detection (LOD) of 500 ng/ml.

3.2. GNP synthesis and preparation of anti-rLukF antibody functionalized GNPs

Generally aqueous phase synthesis is preferred over chemical synthesis in biological studies, because of its biocompatibility and ease of use. The absorbance spectrum of GNPs solution displayed a characteristic surface Plasmon resonance (SPR) peak at 520 nm (Fig. 2a). Size and concentration of GNPs are determined from the UV-Vis spectra of GNPs. By substituting the values of absorbance at surface plasmon resonance peak and 450 nm in A_{SPR}/A_{450} gave a dimensionless number of 1.63, which corresponded to the size of 15 nm and correlates well with the TEM characterization (inset of Fig. 2a). Similarly the concentration of GNPs solution was calculated according to Haiss et al. (2007)[16] and was found to be 2.275 x 10^{-9} $M^{-1}cm^{-1}$. Biofunctionalization of GNP with anti-rLukF antibody was confirmed by absorption spectral analysis (Fig 2b). In general, efficient bioconjugation is dependent on optimized concentration, pH and appropriate functional group of any nanoparticles and biomolecules. Citrate capped GNPs possess net negative charge that can be targeted for bioconjugation with immunoglobulins based on charge-coupled electrostatic interactions.[40] The absorption spectrum of GNP plasmon resonance band at 520 nm was shifted towards longer wavelength with increase in absorbance (530 nm, 10 nm shift) (Fig. 2c). This could be due to the change in local dielectric constant upon conjugation with antibody which confirms the successful attachment of IgG to GNP.

3.3. Optimization of Primary Antibody/GNP concentration and NSET assay

An immunoassay format was developed for sensitive detection of leukotoxin by exploiting the optical properties of GNP upon interacting with fluorophores. It was observed that binding of FITC labeled secondary antibody to toxin bound GNP-primary antibody probe, resulted in fluorescence quenching of FITC by GNP. This can be attributed to the fact that donor-acceptor pair was close enough for efficient spectral cross talk. It is well documented that GNPs dipole movement is not defined in comparison with dye molecules. This creates an opportunity for energy transfer to GNPs at any possible orientation of donor.[23]

Optimization of Ag-Ab concentration was performed so that efficient Ag-Ab interaction persists and no free antigen is left in the solution. Whereas, dye labeled secondary antibody concentration was kept low to maximize the optical signals thereby avoiding over saturation which can lead to increase in fluorescence rather than quenching. Also, high concentration of dye labeled antibody may interact with Ag-Ab binding site and create obstructions in assay format. This kind of interaction wherein, Ab acts as a spacer between dye and nanoparticle for nano surface energy transfer is often referred as NSET.[11]

The fluorescence quenching may be attributed due to radiative rate modification and energy transfer. As reported by[23] high energy transfer can be achieved by using antibody-dye of high quantum yield. Moreover, FITC emission overlapping with GNP absorption provides a window for immunoassay. Since GNP used was 15 nm, energy transfer process dominated, where the surface plasmon of GNP efficiently acted as an energy acceptor from dye-labeled antibody in close vicinity within the immunocomplex.

Antibody-GNP concentration plays a crucial role in determining assay sensitivity. GNP of 0.9 absorbance units was functionalized with various concentrations of antibody (0.04, 0.08, 0.12, 0.16 and 0.2 mg). Earlier, ELISA results provided an insight that antibody concentration of 0.16 mg is required for detecting toxin at 500 ng/ml. Further, efficient fluorescence quenching with varying concentrations of antibody was also studied to check the possibility of using standardized (0.16 mg) antibody concentration for NSET based immunoassay. NSET based immunoassay was applied to detect leukotoxin of various concentration ranging from 0.1ng-100 ng/ml. We found that quenching of fluorescence was proportional to the toxin concentration with R^2 of 0.9908 (Fig. 3a and 3b). Ultrasensitive detection of leukotoxin was achieved mainly due to GNPs as optical labels which attributed to long range quenching arised from surface Plasmon band. In fact many studies are reported wherein use of GNP as fluorescence quencher increased sensitivity levels dramatically.[9,10,14]

3.4. NSET assay to detect leukotoxin in spiked and field samples

NSET assay format was used to detect toxin in raw milk samples and the results were compared with ELISA (Table 1). To check the sensitivity and recovery of spiked samples by using proposed assay, rLukF of 20 ng/ml was spiked in water (Table 2). Percent recovery was calculated comparing fluorescence quenching profile with the respective standard graph. In recombinant toxin spiked samples of 20 ng/ml we obtained 94% recovery. Further, to check the applicability and sensitivity in milk samples, raw milk was spiked with 100 ng/ml of recombinant toxin. Assay was performed by proposed method and we obtained 92.5% recovery in comparison with standard toxin with least matrix effect (as observed in ELISA also). For field applicability of NSET method, milk sample (n=10) was obtained from bovine with subclinical phase of mastitis and assay was performed by both ELISA and NSET method. In both the assays we were able to detect 100 ng of toxin. To prove the sensitivity of the developed assay, same sample was further diluted to 10 ng/ml, which was undetectable in ELISA and assay was performed by new NSET method. We were able to obtain 89.1% recovery in real samples without spiking. These analyses proved that developed NSET phenomenon based biosensor can be ultrasensitive tool for detection of the analyte. [42] had also observed that surface energy transfer can be more efficient and could detect analyte at very sensitive levels in comparison with FRET. [23] also attained high sensitivity due to strong fluorescence quenching caused by energy transfer from the excited dye molecules to the GNPs, which occurs with efficiencies as high as 95%. Non interference of milk matrix in detecting toxin component in the present assay was an added advantage for screening samples at field level.

4 Conclusion

To conclude, we successfully raised polyclonal anti LukF antibodies using rabbit models. Further, antibody was affinity purified and ELISA was designed to detect LukF component with LOD of 1000 ng/ml. To address the issue of sensitivity we used primary antibody functionalized GNPs as optical probes for NSET from FITC labeled secondary antibody. By this method we were able to enhance sensitivity dramatically with LOD of 0.1 ng/ml. Newly developed NSET assay was applied for the detection of spiked water and milk samples. In addition, bovine milk with sub-clinical mastitis was also used to detect toxin with good percent recovery and which can be applicable in veterinary diagnostics for controlling mastitis at entry level of subclinical mastitis.

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References

1. A. Ambrosi, F. Airo, A. Merkoçi, *Anal. Chem.*, 2010, 82, 1151–1156.
2. H.A. Bachaya, M.A. Raza, S. Murtaza, I.U.R. Akbar, *J. Anim. Plant Sci.*, 2011, 21, 16–19.
3. M.B. Barrio, P. Rainard, G. Prévost, *Microbes Infect.*, 2006, 8, 2068–2074.
4. H. Berney, P. Roseingrave, J. Alderman, W. Lane, J.K. Collins, *Sensor Actuat.* B, 1997, 44, 341-349.
5. P. Boutet, D. Boulanger, L. Gillet, A. Vanderplasschen, R. Closset, F. Bureau, P. Lekeux, *J. Dairy Sci.*, 2004, 874, 104-114.
6. X. Cao, Y. Ye, S. Liu, *Anal. Biochem.*, 2011, 417, 1–16.
7. P. Čapek, T.J. Dickerson, *Toxins (Basel)*, 2010, 2,24–53.
8. S. Carregal-Romero, E. Caballero-Díaz, L. Beqa, A.M. Abdelmonem, M. Ochs, D. Hühn, B.S. Suau M. Valcarcel, W.J. Parak, *Annu. Rev. Anal. Chem.*, 2013, 6, 53–81.
9. J. Chen, A. Zheng, A. Chen, Y. Gao, C. He, X. Kai, G. Wu, Y. Chen, *Anal. Chim. Acta.*, 2007, 599, 134–142.
10. W. Chen, X. Tu, X. Guo, *Chem. Commun.*, 2009, 1736–1748.
11. Y. Chen, M.B. O'Donoghue, Y.F. Huang, H. Kang, J.A. Phillips, X. Chen, M.C. Estevez, C.J. Yang, W. Tan, *J. Am. Chem. Soc.*, 2010, 132, 16559–16570.
12. W. Choorit, J. Kaneko, K. Muramoto, Y. Kamio, *FEBS Lett.*, 1995, 357, 260–264.
13. A.R. Clapp, I.L. Medintz, J.M. Mauro, B.R. Fisher, M.G. Bawendi, H. Mattoussi, *J. Am. Chem. Soc.*, 2004, 126, 301–310.
14. E. Dulkeith, M. Ringler, T.A. Klar, J. Feldmann, A. Muñoz Javier, W.J. Parak, *Nano Lett.*, 2005, 5, 585–589.
15. J.M. Fueyo, M.C. Mendoza, M.R. Rodicio, J. Mun, M.A. Alvarez, *J. Clin. Microbiol.*, 2005, 43, 1278–1284.

16. W. Haiss, N.T.K. Thanh, J. Aveyard, D.G. Fernig, *Anal. Chem.*, 2007, 79, 4215–4221.
17. H. Tao, X. Liao, M. Xu, X. Xie, F. Zhong, Z. Yi, *Anal. Methods*,2014, 6, 2560-2565.
18. M.R. Kierny, T.D. Cunningham, B.K. Kay, *Nano Rev.*, 2012, 1, 1–24.
19. A. Kumar, B. Mazinder Boruah, X.J. Liang, *J. Nanomater.*, 2011, 2011, 1–17.
20. T. Lam, R. OldeRiekerink, O. Sampimon, H. Smith, *Irish Vet. J.*, 2009, 62, 34–39.
21. C. Le Maréchal, N. Seyffert, J. Jardin, D. Hernandez, G. Jan, L. Rault, V. Azevedo, P. François, J Schrenzel, M. van de Guchte, S. Even, N. Berkova, R. Thiéry, J.R. Fitzgerald, E. Vautor, Y. Le Loir, *PloS one*, 2011, 6, e27354.
22. D. Loeffler, M.T. Creasy, N.L. Norcross, M.J. Paape, *J Clin. Microbiol.*, 1988, 26, 1331–1334.
23. S. Mayilo, M.A. Kloster, M. Wunderlich, A. Lutich, T. A. Klar, A. Nichtl, K. Kürzinger, F.D. Stefani, J. Feldmann, *T. Nano Lett.*, 2009, 9, 4558–4563.
24. J. Memon, J. Kashif, M. Yaqoob, W. Liping, Y. Yang, F. Hongjie, *Pakistan Vet. J.*, 2012, 33, 170-174.
25. G. Menestrina, M. Dalla Serra, M. Comai, M. Coraiola, G. Viero, S. Werner, D. Colin, H. Monteil, G. Prévost, *FEBS Lett.*, 2003, 552, 54–60.
26. G. Miles, L. Movileanu, H. Bayley, *Protein Sci.*, 2002, 11, 894–902.
27. N. Nagatani, R. Tanaka, T. Yuhi, T. Endo, K. Kerman, Y. Takamura, E. Tamiya, Sci. Technol. *Adv. Mater.*, 2006, 7, 270–275.
28. S. Oaew, R. Charlermroj, T. Pattarakankul, N. Karoonuthaisiri, *Biosens. Bioelectron.*, 2012, 34 238–243.
29. M.J. Paape, D.D. Bannerman, X. Zhao, J.W. Lee, *Vet. Res.*, 2003, 34, 597–627.
30. R.J. Padmaja, P.M. Halami, *Ind. J. Microbiol.*, 2013, 53, 276-282.

31. R.J. Padmaja, P.M. Halami, *Vet. Microbiol.*, 2014, 170(3-4), 358-367.
32. C. Parolo, A. de la Escosura-Muñiz, A., Merkoçi, *Biosens. Bioelectron.*, 2013, 40, 412–416.
33. V.H. Pérez-Luna, S. Yang, E.M. Rabinovich, T. Buranda, L.A. Sklar, P.D. Hampton, G.P. López *Biosens. Bioelectron.*, 2002, 17, 71–78.
34. P. Rainard, J.C. Corrales, M.B. Barrio, T. Cochard, B. Poutrel, *Clin. Diagn. Lab Immunol.*, 2003 10, 272–277.
35. P. Rainard, *Vet. Res.*, 2007, 38, 685–696.
36. K. Schlotter, R. Ehricht, H. Hotzel, S. Monecke, M. Pfeffer, K. Donat, *Vet. Res.*, 2012, 43, 42–50.
37. M. Seydack, *Biosens. Bioelectron.*, 2005, 20, 2454–2469.
38. J. Sommerhäuser, B. Kloppert, W. Wolter, M. Zschöck, A. Sobiraj, K. Failing, *Vet. Microbiol.*, 2003 96, 91–102.
39. Staali, L. Monteil, H, Colin, D.A, *J. Membr. Biol.*, 1998, 162, 209–216.
40. S. Tang, M. Moayeri, Z. Chen, H. Harma, J. Zhao, H. Hu, R.H. Purcell, S.H. Leppla, I.K. Hewlett, *Clin. Vaccine Immunol.*, 2009, 16, 408–413.
41. A.C. Vinayaka, P.S. Muthukumar, M.S. Thakur, *BioNano Sci.*, 2013, 3, 232-240.
42. C.S. Yun, A. Javier, T. Jennings, M. Fisher, S. Hira, S. Peterson, B. Hopkins, N.O. Reich, G.F Strouse, *J. Am. Chem. Soc.*, 2005, 127, 3115–3119.
43. X. Zhao, P. Lacasse, *J Anim. Sci.*, 2008, 86, 57–65.
44. Y. Zhou, X.L. Tian, Y.S. Li, F.G. Pan, Y.Y. Zhang, J.H. Zhang, L. Yang, X.R. Wang, H.L. Ren, S.Y. Lu, Z.H. Li, Q.J. Chen, Z.S. Liu, J.Q. Liu, *Biosens. Bioelectron.*, 2011, 26, 3700–3704.

Legends to Figures and Tables.

Fig. 1 Immunoreactive ability of purified anti-rLukF antibodies (a) Western blot analysis; Lane 1: Marker (SM0671), Lane 2: Ni-NTA purified r LukF, (b) Titration plot of r-LukF to purified anti r-LukF antibodies by Indirect IgG-ELISA.

Fig. 2 (a) Synthesized Gold nanoparticles with 0.9 absorption units at 520 nm, (b) Gold nanoparticle functionalized with anti-rLukF antibody (c) Absorption spectral shift observed in functionalized anti-rLukF antibody (0.16mg) with gold nanoparticle

Fig. 3 (a) Fluorescence quenching of FITC due to the interaction of GNP bound primary antibody. Toxin concentration ranging from 0.1 ng-100 ng/ml, (b) Standard graph for rLukF component with relative fluorescence unit (RFU).

Scheme 1: Representing proposed NSET assay for detection of LukF in sub-clinical mastitis bovine milk samples.

Table 1. Comparison of proposed NSET method with state-of-art techniques.

Table 2. Detection of LukF component in spiked and real samples by NSET method (n=10).

Table 1.

State of art techniques	**Leukotoxin detected (**ng/mL)	**Reference**
Sandwich ELISA	30	Loeffler et al., 1988
Sandwich ELISA	0.4	Rainard 2007
Classical indirect ELISA	1000	Present study
NSET assay	**0.1**	**Present study**

Table 2.

Sl. No	**Sample**	**Quantity (**ng/mL)	**Recovery (%)***
1.	rLukF spiked to water	20	94.0 ± 0.3
2.	Raw milk spiked with rLukF	100	92.5 ± 0.1
Analysis of real time samples			
3.	Raw Milk of bovine with sub-clinical mastitis (100 ng detected by ELISA was diluted to 10 ng/mL for NSET assay)	10	89.1± 0.3

***Average±SD of ten determinations.**

Fig. 1

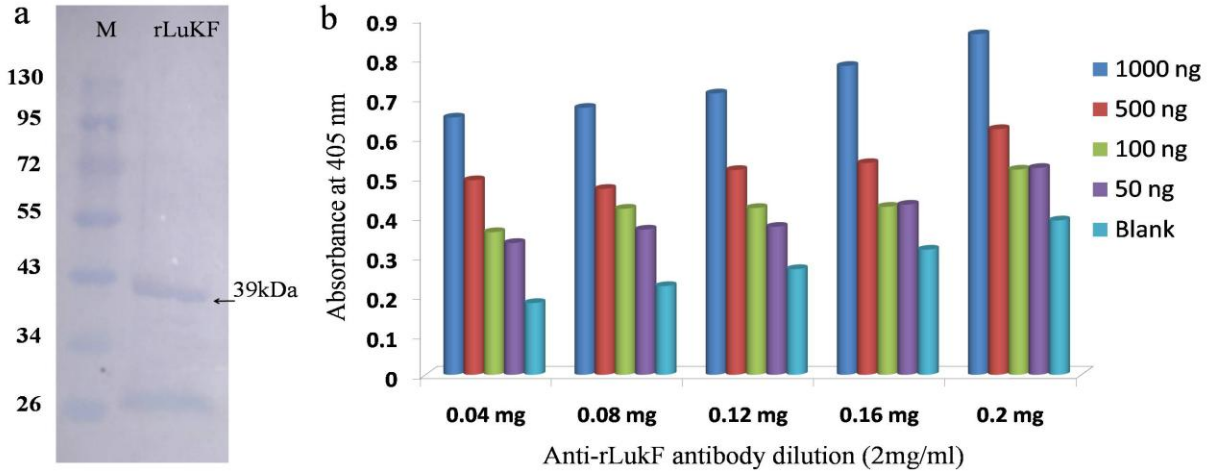

Fig. 2

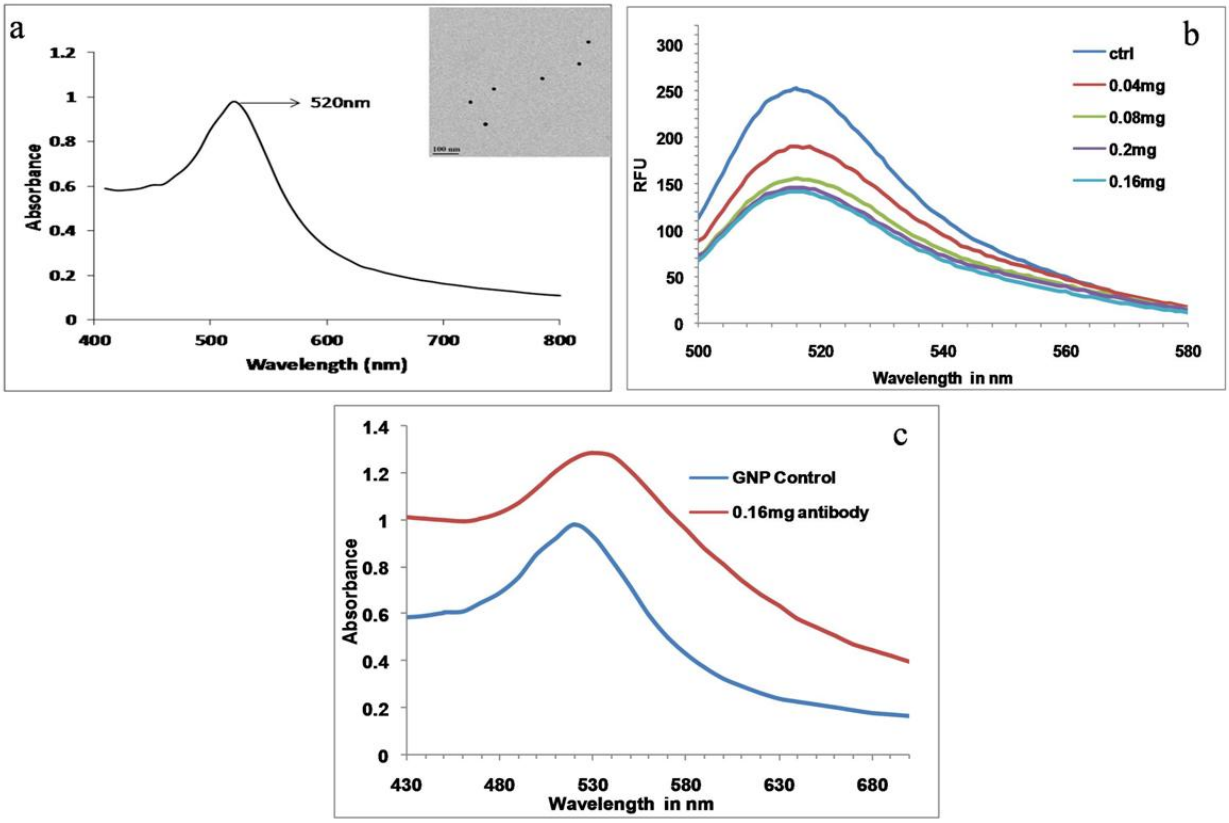

Fig. 3a

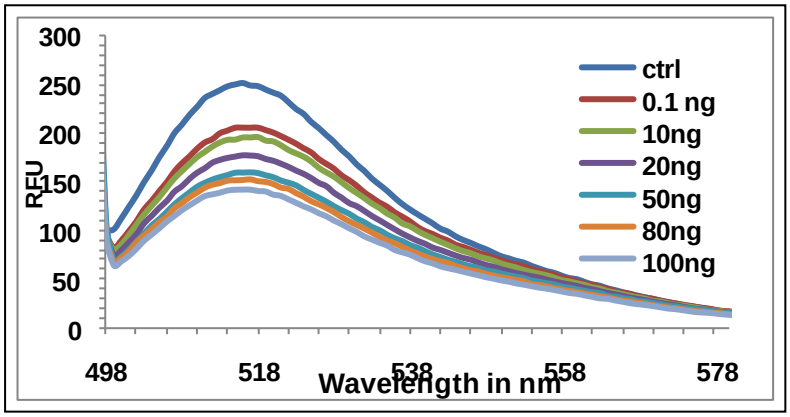

Fig. 3b