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Taming C-terminal peptides of *Staphylococcus aureus* leukotoxin M for B-cell response: Implication in improved subclinical bovine mastitis diagnosis and protective efficacy *in vitro*

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ABSTRACT

Leukotoxin M/F′-PV (LukM/F′-PV) produced by bovine mastitis causing *Staphylococcus aureus* structurally comprises three domains, the β-sandwich, rim and stem domain. The rim and stem domains interacting with target cell membrane lipid rafts contributes to the virulent trait of the toxin. In the present study, two facts were hypothesized that neutralization of these domains will ebb LukM/F′-PV leukotoxicity. Secondly, the neutralizing antibodies can improve the leukotoxin detection sensitivity in bovine mastitis milk samples. The *in silico* mapping of *S. aureus* LukM C-termini comprising these domains predicted seven linear B-cell antigenic epitopes. The immune response of C-terminal truncated recombinant peptides $rCtM_{19}$ (19 kDa; near carboxy-terminal) having four epitopes and $rCtM_{15}$ (15 kDa; C-terminal) with three epitopes were evaluated for their diagnostic and neutralization potential. Anti-$rCtM_{19}$ and anti-$rCtM_{15}$ antibodies with enhanced immunogenicity had the most striking outcome in IgG-ELISA for detecting native determinants of leukotoxin. For the obtained ELISA values, ROC curve inferred a cut-off score of >0.102 OD_{405}. The assay sensitivity in the range of 90–96% along with 100% specificity and AUC of 0.93–0.98 categorized subclinical and clinical from healthy bovine milk samples. As observed through *in vitro* neutralization and LDH assays, C-terminus specific antibodies (1:42 titer) deactivating leukotoxicity abolished LukM from interacting with lipid bilayer and LukF for forming pores on bovine neutrophil membrane. As a proof of concept, it was proved that peptide antibodies can be a more specific serodiagnostic and passive therapeutic molecules.

1. Introduction

Leukotoxin M/F′-PV, the virulent bicomponent β-pore forming toxin secreted by *Staphylococcus aureus* is widely implicated in bovine mastitis (Fluit, 2012; Rainard et al., 2003). Structurally the toxin monomers are comprised of three domains; the β-sandwich, rim and stem domain (Guillet et al., 2004). These proteins exert their leukotoxicity through receptor-dependent mechanism (Nishiyama et al., 2006). Binding of LukM to their respective membrane receptors, which are preferentially associated with lipid rafts favor toxin oligomerization required for pore formation, a key step critical for leukotoxicity (Fromageau et al., 2010). Staphylococcal leukotoxins thus interacting with these lipids rafts by specific domains induce Ca^{2+} influx into the cytosol and subsequent pore formation (Barrio et al., 2006). The reports on experimental binding of *S. aureus* leukotoxin have also provided evidence that LukM/F′-PV mainly target the cells of myeloid lineage, migratory or residing inflammatory phagocytes, making them the most susceptible victims (Rainard, 2007). Therefore, leukotoxin domains may be considered as an interesting target for mastitis detection and prevention strategy at the earliest.

In our previous studies, we have exploited the immune response of individual recombinant components of LukM/F′-PV for developing a diagnostic agent to detect subclinical phase of mastitis (Padmaja and Halami, 2014). However, without knowing the particular detailed epitope-mapping of leukotoxin, the specific molecular basis of the humoral immune protective response to LukM in bovine mastitis remain unclear. For understanding the mechanism of antigen antibody interactions, knowledge of epitopes is prerequisite and such epitopes can be identified through rapid computational methods with ease (Sharma et al., 2013). Although, a detailed study on C-terminus of LukM is not elucidated

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and thus far nothing is known about the nature and presence of B-cell epitopes responsible for the induction of immune response in infected animals. C-termini have become important for two reasons: (i) The studies on truncation mutagenesis yielded that C termini of LukS and LukF should be intact for a proficient oligomerization and (ii) Luk subunits are vulnerable to short deletions at the C-terminus (Nariya et al., 1993).

Apparently, C-terminus is represented by conserved sequences of rim and stem domain that may serve as B-cell epitopes can be helpful in the development of species-specific diagnostic assays. To gain insight, linear B-cell epitopes mapped immune-dominant peptides of LukM were identified. The diagnostic efficacy of immune response elicited for two truncated recombinant peptides comprising C-terminal epitopes of LukM was evaluated for enhanced leukotoxin detection sensitivity. Additionally, neutralization of LukM by the immune dominant-epitope specific antiserum was also determined.

2. Materials and methods

2.1. Reagents and instrumentation

Luria Bertani and Brain Heart Infusion (HiMedia, Mumbai) broth was obtained for growing *E. coli* and *S. aureus* respectively. Molecular biology grade components such as *Taq* DNA polymerase, dNTPs, $MgCl_2$, primers, T_4DNA ligase and gel extraction kit were procured from Sigma-Aldrich, USA. *Xho*I and *Hind*III restriction enzymes were purchased from Fermentas, USA. *Escherichia coli* DH5α (Invitrogen, Carlsbad) and the expression host BL-21(DE3)(pLysS) (Novagen, Madison), cloning vector pGEMT-Easy (Promega), expression vector pRSETA (Invitrogen, Carlsbad), Biotin labeled secondary antibody and streptavidin alkaline phosphatase conjugate (Biorad, India), MTT (HiMedia), 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (BCIP/NBT), Ni-nitrilotriacetic acid (NTA) beads and p-Nitrophenyl Phosphate (pNPP) from (Merck Biosciences, Bangalore) were purchased for the experimental studies. All chemicals and reagents used were of analytical grade until otherwise mentioned. Ninety-six-well flat-bottom polystyrene ELISA plates were purchased from Tarsons, Bangalore. ELISA absorbance was observed at 450 nm using Multiskan ELISA microplate reader (Thermoscientific, Waltham, MA, USA). Buffers and solutions for IgG-ELISA: (a) Coating buffer- 50 mM sodium carbonate-bicarbonate buffer (pH 9.6), (b) Wash buffer- 1xPBS-T (0.05% v/v), (c) Blocking buffer- 1xPBS-gelatin (1.5% w/v), (d) Diethanolamine buffer (pH 9.8).

2.2. Study population

As per the diagnosis of veterinary professionals and disease characteristics, mastitis milk samples (n = 101) were collected under aseptic condition from different localities of Mysore district, India. The obtained test samples were grouped as milk samples of healthy cows with no disease characteristics (n = 13), presumptive subclinical samples (n = 24) and samples obtained from clinical cases with blood stains, curdled or watery appearance (n = 64). Apparently, the sample characteristics were further confirmed by calculating the somatic cells in healthy and clinical samples via direct microscopic count method.

2.3. Epitope mapping of LukM C-terminal region

For creating $rCtM_{15}$ and $rCtM_{19}$, potential B-cell linear epitopes of LukM COOH-terminal were mapped *in silico*, using the free downloadable software Linear Epitope Prediction System (LEPS, http://leps.cs.ntou.edu.tw/) developed based on support vector machine (SVM) algorithm (Wang et al., 2011). The two C-terminal peptides were spanned using the selected antigenic epitopes predicted by Hopp and Woods hydropathy plot (Hopp and Woods, 1983) at window size = 7. Vaxijen scoring (Doytchinova and Flower, 2013) for the peptides was also analyzed to determine the antigenicity.

2.4. Cloning, expression and purification of recombinant $rCtM_{15}$ and $rCtM_{19}$

The genomic DNA of *S. aureus* 17a (native isolate of bovine mastitis, Accession number: MCC2984) (Padmaja and Halami, 2014) was used as the template for the PCR amplification of CtM_{15} and CtM_{19} (GenBank ID: D83951.1). The *Xho*I and *Hind*III tagged primers for CtM_{19} (Forward: 5′-GTG*CTCGAG*TTCCAGTCAG-TACCATCTATA-3′ and Reverse: 5′-CTC*AAGCT T*CTCGTATCGCCT GAATCTTT-3′) and CtM_{15} (Forward: 5′-TTA*CTCGAG*GATTCAGGCG ATACGAGC-3′ and Reverse: 5′-CTC*AAGCTT*TAGTTGTGCCCCTT-TACTTT-3′) were designed to amplify the epitomic encoding peptides. The amplification reaction was programmed as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 45 s, extension at 72 °C for 105 s and final extension at 72 °C for 10 min. Amplicons of CtM_{15} and CtM_{19} were purified using gel extraction kit according to manufacturer's specifications and the extracted products were sequenced (Vimta labs, Hyderabad, India) and cloned individually to pGEM-T easy vector. The coding regions excised from pGEM- CtM_{19} and pGEM- CtM_{15} was subcloned to *Xho*I and *Hind*III sites of pRSETA expression vector, respectively. The resultant pRSETA-CtM_{19} and pRSETA-CtM_{15} cassettes were verified for coding sequences to be in frame before transforming into the cells of *E. coli* BL21(DE3)pLysS strain for expression studies. The expression and purification of $rCtM_{19}$ and $rCtM_{15}$ were performed as described previously (Padmaja and Halami, 2014).

2.5. Immunization of rabbits, dot blot and western blot assay

Immunization of New Zealand rabbit was carried out as per the standardized protocol described (Padmaja and Halami, 2014). A 250 μg of 6× His-LukM and/or 6× His-LukF in Freund's complete adjuvant was injected to experimental rabbits as the prime dose respectively. The same antigens in Freund's incomplete adjuvant at a concentration of 125 μg was injected into rabbits on 4th, 6th and 8th week after the first injection day as booster dose. Sera from immunized rabbits were collected 10 days after the final immunization. In Dot blot assay, anti-$rCtM_{15}$/$rCtM_{19}$ antibodies were validated for detecting rLukM (test control, Padmaja and Halami, 2014) blotted at varying concentration of 1 μg, 0.5 μg and 0.1 μg on nitrocellulose membrane at marked locations. Western blot analysis was also performed to evaluate the immunogenicity of recombinant peptides with slight modification. Nitrocellulose membranes were assayed with $rCtM_{15}$ and $rCtM_{19}$ positive sera diluted at 1:10,000 for detecting their respective recombinant antigens.

2.6. IgG-enzyme linked immunosorbent assay

The amount rCtM immunogens for reacting with immunoglobulin G (IgG) of antiserum was optimized through checkerboard ELISA format. The purified $rCtM_{19}$/$rCtM_{15}$ with varying concentrations from 0.1 to 1000 ng per well in coating buffer were coated in 96-well polystyrene microtiter flat bottom plates. With overnight incubation at 4 °C, the plates were washed once with wash buffer and blocked with blocking buffer for 2 h at 37 °C. The incubated plates were washed three times with wash buffer and 100 μl of