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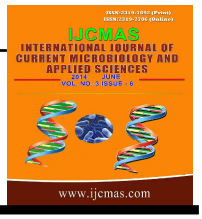


Original Research Article

Detection of Antibodies in Milk for the Pyruvate formate lyase associated with *S.aureus* isolated from mastitis affected milk by serological proteome analysis

T.Anitha[1], J.Thanislass[1*], P.X.Antony [2] and B.Sharanabasav[1]

[1]Department of Veterinary Biochemistry, [2] Department of Veterinary Microbiology, Rajiv Gandhi Institute of Veterinary Education and Research, Kurumbapet, Puducherry, India
Corresponding author

A B S T R A C T

Serological Proteome Analysis was performed to detect the presence of antibodies for *S.aureus* present in serum and milk isolated from mastitis affected cows. Blood and milk from animals (103 samples) suffering from mastitis were collected. Mastitis caused by *S.aureus* was identified by culture as well as by PCR. Twenty seven samples (26%) were found positive for *S.aureus*. Nine *S.aureus* isolates were obtained from these positive samples and bacterial (*S.aureus*) cell lysate was prepared by ultrasonication. The immunogenic proteins of *S.aureus* were detected by Serological proteome analysis using serum and milk whey as the source of primary antibody obtained from corresponding mastitis affected cases. The immune-blot with the serum identified six bands whereas the immune-blot with milk detected four bands. The most immunogenic protein that was detected by both serum and whey protein subjected to proteomic analysis. MALDI – TOF coupled with MASCOT search identified the protein as formate acetyltransferase or pyruvate formate lyase. This protein is involved in anaerobic respiration of *S.aureus* and its expression is crucial for the survival of the organism in the host. Now it had been proved to be immunogenic, this protein could be suggested to be a better target for vaccine and/ or for diagnostics.

Keywords

Pyruvate formate lyase; immunogenic; *S.aureus*; mastitis; serological proteome analysis; vaccine.

Introduction

Mastitis is defined as the inflammation of mammary gland. Bovine mastitis is one of the most costly diseases of dairy cattle, resulting in a great deal of economic losses, mostly because of a reduction of milk yield, decreased milk quality, and higher production costs, medication costs, discarded milk during and shortly after treatment, loss of milking days, reduced milk price, increased labour and increased recruitment costs due to culling (Cremonesi et al., 2006; Seegers et al., 2003).

The contagious pathogens are important in causing the subclinical form of mastitis. Although mastitis can be caused by 137 different microorganisms (Ranjan et al., 2006), *Staphylococcus aureus* is the etiological agent more commonly

associated to the disease and is normally related to both subclinical and chronic infections leading to severe economic loss to dairy farms (Kubota et al., 2007; Philpot and Nickerson, 2000).

In addition, Staphylococcal mastitis is very difficult to control because of high prevalence of resistance to antibiotics. They can transform into the slow growing population termed "Small Colony Variant" (SCV) that has the capacity to survive intra-cellularly and establish long-term persistence within mammalian cells (Atalla et al., 2009, 2010; Proctor et al., 2006). This ability of the bacterium to live within some cells and encapsulated nodules in mammary tissue and form biofilm may contribute to its resistance to antibiotic treatments (Almeida et al., 1996). Therefore there is urgent need for the identification of novel biomarkers which are associated with cell physiology and molecular process of pathogenecity to develop new tools for successful treatment of *S.aureus* infection and also for diagnosis (Glowalla et al., 2009; Middleton, 2008; Schaffer and Lee, 2008).

The pathogens causing the infection secrete or harbor specific proteins which act as anchoring proteins, virulence factors or antigenic proteins. Serological proteome analysis (SERPA) is a combination of Polyacrylamide gel electrophoresis (PAGE), immunoblotting and spot sequencing and can be used to shed light on host's immune response to an infection (Vytvytska et al., 2002). It can be used to identify complete virulence factor inventories, studying the response of both host and pathogen to the infection process and elucidating mechanistic actions of virulence factors as they interface with host cells (Bhavsar, 2010).

Therefore the present study was planned to detect these microbial proteins associated with *S.aureus* of clinical mastitis milk by serological proteome analysis. In this analysis the proteins that are extracted from the *S.aureus* organisms are exposed to the sera of the affected animal, so that the antigens against which the antibodies are produced can be detected. Similarly the whey protein from milk is also used to check for the presence of antibody produced against the proteins of *S.aureus.*

Materials and Methods

Collection of samples

Collection of samples from actually infected cases are much more valuable than experimentally induced conditions as the field samples truly reflect the field condition where ultimately tools developed are going to be applied. Therefore milk and blood samples were collected in sterile vials aseptically from 103 cattle (Breed – HF and Jersey cross bred and age group from 4 to 10 years) affected with clinical mastitis (based on presence of clots or flakes in milk) from 3 private dairy farms, Teaching Hospital, RIVER and Veterinary Dispensaries, located in Puducherry, India. The samples were maintained at $4^{o}C$ until further processing. The serum was collected by centrifugation of blood at 2000 rpm for 10 minutes and the serum collected was stored at $-80^{o}C$ in sterile tubes until further use.

Identification of *S.aureus* infection

The *S.aureus* infected milk samples were identified by culture and gram's staining and then confirmed by Polymerase Chain Reaction (PCR). The milk sample was streaked onto Mueller Hinton agar and

incubated at 37^0C for 24 to 48 hours. A minimum of 5 colonies of same type was recorded as causative agent and growth more than one type of colonies was determined as mixed growth. The individual colonies from mastitis samples which was caused by a single causative agent were subjected to gram staining and gram positive cocci arranged in clusters were identified and the corresponding milk samples were used for confirmation by PCR.

Separation of whey protein, Extraction of DNA and PCR for *nuc* gene of *S.aureus*

Two ml of mastitis milk maintained at 4^oC was taken in sterile tubes and centrifuged at 6000 X g for 10 minutes and whey protein was collected and stored at -80^oC until further use. The pellet was washed with PBS, 3-4 times. The final pellet contains the bacterial cells from which the DNA was isolated. The bacterial DNA from milk was extracted following the phenol chloroform method (Christensen et al., 1993).

The DNA isolated from milk samples was used as template for PCR and checked for *S.aureus* organisms using primers specific for *nuc* gene (Brakstad et al., 1992) at annealing temperature of 50^0 C for 40 seconds. The amplified products were checked on agarose (2%) gel electrophoresis at 100 volts for 45 minutes.

Western blot analysis for the detection of immunogenic protein

S.aureus that was isolated from mastitis milk sample and confirmed by PCR was cultured in luria broth (approximately 8 ml).Then the culture was centrifuged at 3000 X g for 30 minutes. The pellet was washed thrice with Phosphate Buffered Saline (PBS). Final pellet was resuspended in 10 ml of 50mM Tris EDTA (TE) buffer. The suspension was sonicated for 10 minutes with 2 minutes interval at 150 watts and stored at -80^oC overnight for lyophilization. After lyophilization the samples were suspended in 1ml ultrapure water and dialyzed overnight at 4^o C in ultrapure water to remove salts.

The protein concentration was determined by Lowry's method (Lowry et al.,1951) The samples were then subjected for sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS PAGE) with 12% resolving gel and 4% stacking gel (Laemmli, 1970). Bacterial cell lysate protein (250µg) was loaded in each well and electrophoresis was carried out at 100 volts for 90 minutes. The cell lysate after electrophoresis subjected to western blotting as per the method of Bjerrum and Schaffer-Nielsen (1986). The transfer was done at 50 volts for two hours at room temperature.

The proteins thus transferred were probed for the presence of immunogenic proteins of *S.aureus*. After overnight blocking the Nitrocellulose membrane was incubated with primary antibody (serum – 1:1000 dilution or whey protein – 1:4000 dilution in 1X assay buffer collected from the mastitis affected animals) for 1 hour at 37^oC. After washing the membrane was incubated in rabbit anti bovine IgG – HRP conjugate diluted 1:1000 in 1X assay buffer and incubated for 30 minutes at 37^oC. The membrane was then incubated in DAB system and the brown band appeared was photographed using Bio-Rad Gel documentation system.

Mass spectrometry – MALDI-TOF analysis of the immunogenic protein

The samples which were subjected for western blotting and immunodetection were again electrophoresed using similar conditions as stated above and stained using coomassie brilliant blue 250. The band corresponding to immuno dominant protein which was detected by both serum and milk whey was cut and subjected to in-gel tryptic digestion. Then the peptides were subjected to MALDI-TOF analysis. The analysis of the protein was done using the MASCOT search engine (www.matrixscience.com). The sequence search was done using the NCBInr protein database (www.ncbi.nlm.nih.gov).

Results and Discussion

Screening of clinical mastitis milk samples

The bacterial DNA isolated from culture and gram's staining positive mastitis milk samples were screened for the presence of *S.aureus* by PCR. Out of 103 samples screened, 27 (26%) were found positive for *S.aureus* organisms. Result obtained is shown in Fig.1.

Western blot analysis of immunogenic protein

Nine isolates of *S.aureus* obtained from mastitis milk were taken and lysed by ultrasonication. The bacterial cell lysate was subjected to SDS PAGE and blotting, followed by immunodetection with the serum and milk whey obtained from cows corresponding to the *S.aureus* isolates. With the serum as a source of primary antibody six intense, distinct bands were seen in seven isolates. Several lighter bands were also seen. The blot obtained is shown in Fig.2. With the whey protein as a source of primary antibody six bands were seen in three isolates and two lighter bands were seen in two isolates. The result obtained is shown in Fig.3.

Identification of immunogenic proteins

The immunodominant protein which was detected by both serum and whey protein was taken for identification. The gel band was cut and subjected to in-gel tryptic digestion. By MALDI-TOF analysis the immunogenic protein was identified as formate acetyltransferase with score 141. Out of three samples tested, two of them identified as formate acetyltransferase of *S.aureus.* Based on mascot search result, out of 49 fragments obtained with mass ranging from 813.550 to 2801.157 Da, 19 align with sequence of formate acetyltransferase of *S.aureus*. The result is shown in Fig.4 and Fig.5 and Table 1.

Bacteria have specific and general defense strategies to counter environmental changes, including detoxification of stressors, as well as protection mechanisms and repair systems. These responses are based on sophisticated pathways of signal transduction, which subsequently trigger changes in gene expression (Falko et al., 2008).

The immunodominant protein identified in this study was pyruvate formatelyase (pfl) or formate acetyltransferase (E.C.2.3.1.54) with score 141, molecular size 85kDa, pI 5.2. Pfl is a cytoplasmic protein involved in fermentation pathway for energy metabolism in microbes. The gene responsible for Pfl was found to be highly induced in *S.aureus* under anaerobic condition (Fuchs et al., 2007). In the absence of external electron acceptors like oxygen or nitrate, an induction of glycolytic enzymes was observed one of them being Pfl (Rasmussen et al., 1991).

Table.1 MASCOT search result of Immunogenic protein identified by SERPA of field isolate of *S.aureus*

Accession code	Description	Mass (Da)	No. of peptides	Score
gi|15923216	formate acetyltransferase [*S.aureus* subsp. *aureus* Mu50]	85264	19	141
gi|269939742	formate acetyltransferase [*S.aureus* subsp. *aureus* TW20	85278	19	141
gi|283469462	formate acetyltransferase [*S.aureus* subsp. *aureus* ST398]	85251	19	141
gi|161508484	formate acetyltransferase [*S.aureus* subsp. *aureus* USA300_TCH1516]	85829	18	140
gi|239637163	formate acetyltransferase [*Staphylococcus warneri* L37603]	85211	12	87
gi|289549542	Pyruvate formate-lyase [*Staphylococcus lugdunensis* HKU09-01]	85237	13	86
gi|222150684	formate acetyltransferase [*Micrococcus caseolyticus* JCSC5402]	85003	12	75
gi|27467132	formate acetyltransferase [*Staphylococcus epidermidis* ATCC 12228]	85371	12	73
gi|254451359	conserved hypothetical protein [*Octadecabacter antarcticus* 238]	6946	5	72
gi|57865733	formate acetyltransferase [*Staphylococcus epidermidis* RP62A]	85385	12	72
gi|282876701	formate C-acetyltransferase [*Staphylococcus epidermidis* SK135]	85357	12	72
gi|251811545	formate acetyltransferase [*Staphylococcus epidermidis* BCMHMP0060]	85904	12	72
gi|242243440	formate acetyltransferase [*Staphylococcus epidermidis* W23144]	85903	12	71

Figure 1

PCR Detection of *S.aureus* in Mastitis Milk Samples
Lane 1,2,5,6,9,12,14,22,23,24 and 25 - Mastitis milk samples positive for *S.aureus* (270 bp)
Lane 4,8 and 28 – positive controls
Lane 3 – 100 bp DNA ladder

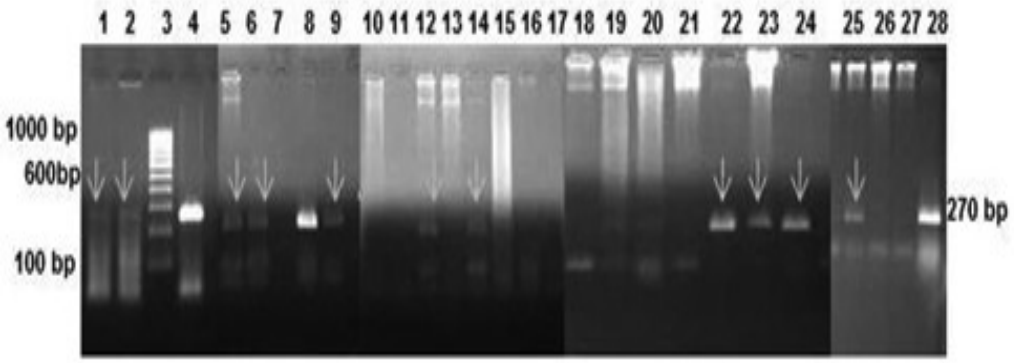


Figure 2

SERPA of *S.aureus* lysates using serum collected from mastitis affected animals as a source of Primary Antibody
Lane 1-9 – Cell lysate of *S.aureus* isolated from mastitis milk samples
M - Protein marker

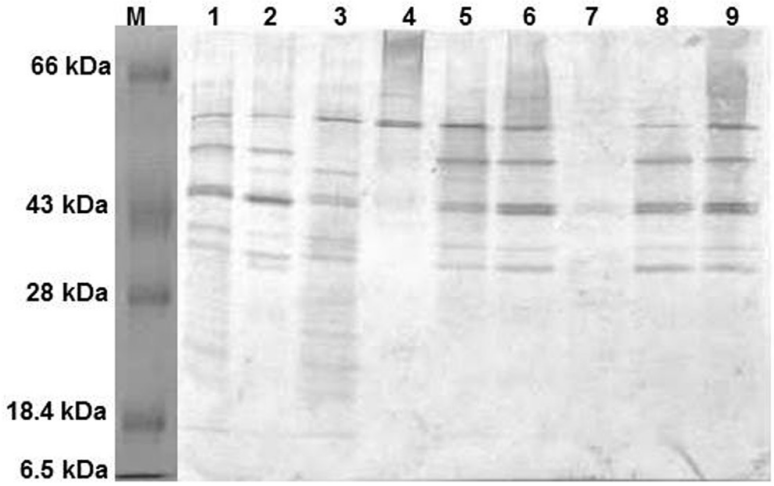


Figure 3

SERPA of *S.aureus* lysates using milk whey collected from mastitis affected animals as a source of Primary Antibody
Lane 1 to 9 – Cell lysate of S.aureus isolated from mastitis milk samples
M – Protein marker

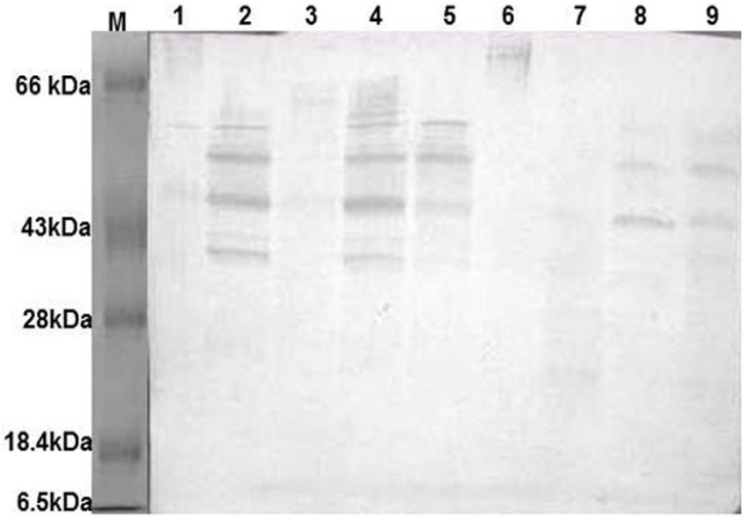


Figure 4

MALDI-TOF Spectrum Analysis Report – m/z ratio of Peptide fragments resulted from the protein of interest

a and b – protein bands from two different *S.aureus* samples isolated from mastitis milk

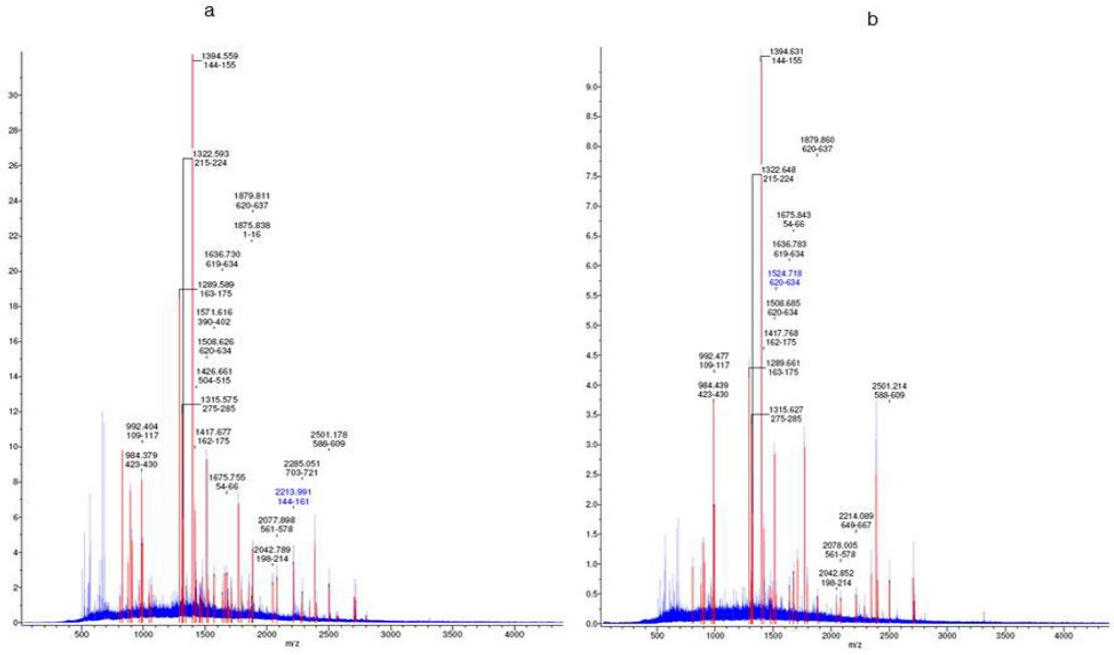
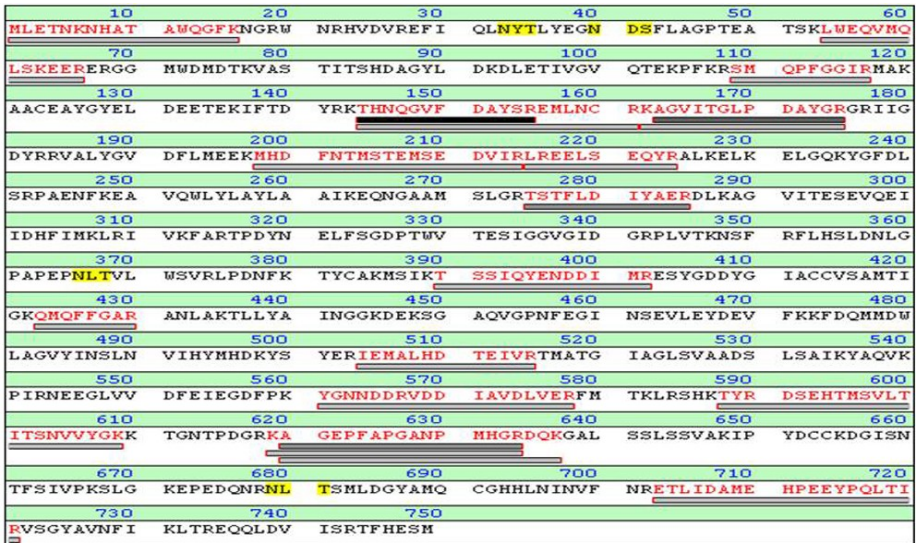


Figure 5

MALDI-TOF Spectrum Analysis Report- Alignment of Peptide fragments with Amino acid sequence of the protein – Pyruvate formate lyase



Pfl contributes significantly to the supply of formate, which is used via formyl-THF (10-formyl-tetrahydrofolate) for protein and purine synthesis under anoxic conditions. In addition, in biofilm grown *S.aureus* cells, enzymes like Pfl, Fdh (NAD - dependent formate dehydrogenase) and *fhs* (formyltetrahydrofolate synthetase) were upregulated at the transcriptional and proteome levels (Resch et al., 2005, 2006).

A biofilm is distinguished by nutrient, oxygen, and pH gradients and Pfl plays a significant role in the anaerobic layer of a biofilm. In the anaerobic layers of the mature biofilm, the Pfl converts pyruvate to acetyl-CoA and formate. The latter can be used by strictly anaerobically grown cells for the synthesis of formyl-THF and therefore for the biosynthesis of proteins, DNA, and RNA (Leibig et al., 2011). Thus pfl gene expression is associated with biofilm related anaerobic respiration involving in fermentation pathway that acts as a main source of energy, therefore it is suggested that if this enzyme is inhibited by any means the energy source is affected and the bacterial growth can be arrested and so this enzyme can be proposed as a novel drug target to treat *S.aureus* induced mastitis.

Significantly, this study proves the immunogenic nature of pfl protein associated with *S.aureus*. Therefore, this biofilm associated antigen can be a utilized as a vaccine candidate to induce early and effective antibody mediated response to prevent chronic *S.aureus* infection. More importantly, this study demonstrates the presence of antibodies for pfl associated with *S.aureus* in milk. So far there are only few studies which have been undertaken for the development of milk based immunoassay that can detect the pathogen as a definitive causative agent of mastitis, the finding of this study will definitely aid the development of such assay.

Conflict of interest

The authors declare that they have no conflict of interest.

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