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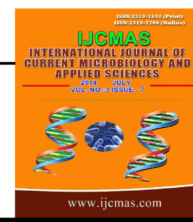

Original Research Article

Association of Bio-film formation of *S.aurues* with *PFL* Gene expression

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Keywords

S.aureus, Pyruvate formate lyase, gene expression, bovine mastitis, antibiotic resistance, biofilm

A B S T R A C T

The study of expression of genes in pathogens specific to process of infection has become essential to understand the adaptive mechanism of pathogens thereby, identification of novel targets for therapeutics. Therefore the present study was undertaken to study the expression of genes related to energy metabolism (pfl, ldh), nitric oxide stress (Hmp) and purine metabolism (PurM) in the clinical isolates of *S.aureus* obtained from mastitis affected milk. The level of expression of pfl, ldh, Hmp, and purM was significantly more when compared to the expression of same set of genes when the above *S.aureus* isolates were subjected for repeated sub culturing. At the same time the expression of pfl and ldh was persistent, but expression of Hmp and purM was totally absent in the sub-cultured *S.aureus* isolates. The persistence of pfl and ldh expression was found to be associated with bio-film forming capacity. The presence of NO stress and purine deficiency, as indicated by the increased level of expression of Hmp and purM gene in clinical isolates, could have added to the level of expression of pfl and ldh. Overall it appears that the expression of pfl and ldh was associated with the adaptive mechanism of pathogens which would have *contributed* mainly to the formation of bio-film.

Introduction

S. aureus is one of the main causative agents of bovine mastitis, the most economically important disease of dairy ruminants (Lee and Pier, 1997; Shoshani *et al.,* 2000; Zecconi*et al.,* 2003; Unnerstad *et al.,* 2009). *S. aureus* is found to be the most common isolate of bovine mastitis in all the seasons (Ranjan *et al*., 2011).Among all pathogens causing mastitis, *S.aureus* is of important concern because of its ability to cause chronic disease.

Existence of SCVs of *S.aureus* and MRSA among the *S.aureus* isolates of bovine mastitis is an increasing concern because of its zoonotic importance. *S.aureus* also exhibits resistance to antiseptics and disinfectants, such as QAC, which may aid its survival in the hospital environment.

Biofilm-associated *S.aureus* shows innate resistance to antibiotics (Ceri *et al*., 1999), disinfectants (Oie *et al*., 1996), and

clearance by host defenses (Shiau and Wu, 1998).Biofilm facilitates the adherence and colonisation of Staphylococci on the mammary gland epithelium, also contributing to the evasion of the immunological defences and to the difficulty of pathogen eradication, often resulting in persistent infections (Baselga *et al*., 1993; Arciola *et al*., 2001; Cucarella *et al*., 2002; 2004; Zadoks *et al*., 2002; Vasudevan *et al*., 2003; Brouillette *et al*., 2005; Fox *et al*., 2005; Melchior *et al*., 2006).Therefore the ability to evade and influence the host immune system becomes the most notable feature of *S.aurues* and becomes difficult to control using conventional antibiotic therapy.

The specific and general defense mechanisms of *S. aureus* are based on sophisticated pathways of signal transduction, which subsequently trigger changes in gene expression (Falko *et al.,* 2008). Tissue specific (udder and / or its secretion) gene expression studies of *S. aureus* may help to identify the antimicrobials or drug targets that can effectively treat the *S.aureus* to overcome the existing problem of multidrug resistance (Milla *et al.,* 2009; Wienrick *et al*., 2004). Therefore the following study was undertaken to study the gene expression of pfl, ldh – candidate gene related to anaerobic respiration, Hmp-indicator of NO stress and PurM- candidate gene related to purine biosynthesis. So far, studies in this direction were undertaken in *in vitro* system. But, this study was undertaken with the help of clinical isolates obtained from mastitis milk in order to understand the molecular mechanism of defense against the host in *in vivo* condition. Further, the study on naturally 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.

Materials and Methods

Collection of samples

A total of 62 milk samples were collected in sterile vials from clinical mastitis affected cattle by aseptic conditions from teaching hospital RIVER, and the dispensaries under the Department of Animal Husbandry and Animal Welfare, Puducherry. The collected samples were transported in an ice pack and stored at 4^{0}C until further processing. Samples were divided into two equal aliquots. One aliquot was immediately processed for isolation of bacterial RNA and another aliquot was used for the identification of *S.aureus* and its isolation for repeated sub-culturing. Bacterial RNA isolated from the samples which were confirmed to be infected only with *S.aureus* was used for gene expression studies.

Isolation and identification of *S.aureus* associated mastitis cases

Samples positive for the genus Staphylococcus were identified upon streaking and incubation of mastitis milk on Mueller Hinton agar with 7% sodium chloride. Gram staining was then performed and only the gram positive cocci which were arranged in clusters were considered and these individual colonies from the culture plates were streaked on Mueller Hinton agar and incubated overnight at 37°C to obtain good growth of the bacterium. A loop full of obtained culture was inoculated in 2ml Luria broth, incubated overnight at 37°C and from this final culture of Staphylococcus species, DNA was extracted following the method described by Christensen *et al*., (1993) with modifications. The quality of DNA was then checked by Agarose Gel Electrophoresis as per the method of Helling*et al*., (1974). Polymerase Chain Reaction (PCR) was then performed for the detection of *S.aureus* using the set of primers given the Table 1.

The reaction mixture for PCR detection of Staphylococcus species contained, 5.0µl of DNA template (0.2µg/µl), 1.0µl of Primer forward (20pmol/µl), 1.0µl of Primer reverse (20pmol/µl), 10.0µl of Master Mix (2X) and the final volume was made upto 20 µl using Ultrapure water. PCR conditions employed were Step 1: Primary denaturation - 95°C for 4 minutes, Step 2: Denaturation - 95°C for 45 seconds, Step 3: Annealing - 55°C for 45seconds, Step 4: Elongation - 72°C for 45 seconds, Step 5: - 30 cycles of step 2 to step 4, Step 6: Final Elongation - 72°C for 6 minutes. PCR for the detection of *S.aureus* was done the same way using species specific primers. The PCR conditions employed were Step 1: Primary denaturation - 94°C for 5 minutes, Step 2: Denaturation - 94°C for 30 seconds, Step 3: Annealing - 50°C for 40seconds, Step 4: Elongation - 72°C for 40 seconds, Step 5: - 30 cycles of step 2 to step 4, Step 6: Final Elongation - 72°C for 5 minutes. The amplified products were checked by agarose (1.5%) gel electrophoresis at 100 volts for 45minutes.

Storage of *S.aureus* isolates in glycerol broth

The bacterial isolates which were identified as positive for *S. aureus* by PCR were stored in 15% glycerol broth at -80°C for growth in *in vitro* culture.

Isolation of total RNA from bacterial cells

Mastitis milk samples (5ml) maintained at 4°C were taken in sterile centrifuge tubes (pre-cooled) and centrifuged at 6000 x g for 5 minutes at 4°C. The fat layer was removed with a sterile swab and whey protein was removed carefully not disturbing the pellet.The pellet was washed once with PBS, centrifuged at 6000 x g for 5 minutes at 4°C. The supernatant was decanted and the final pellet contains the bacterial cells from which the RNA was isolated. Isolation of total RNA from bacterial cells was carried out using TRI reagent of Medox (as per the manufacturer's protocol with slight modifications). The RNA pellet was resuspended in 80µl of DEPC treated water and incubated at 60°C for 10 min to facilitate dissolution of RNA. RNA samples were stored at -80°C until further use.

Analysis of quality of RNA isolated

The purity of RNA was measured by finding out the ratio of absorbance at 260nm and 280nm. The concentration of RNA in µg/µl was calculated by multiplying the absorbance value at 260nm by the factor 40. Formaldehyde Agarose Gel Electrophoresis of RNA was carried out to determine the integrity of RNA isolated as per Lam *et al.*, 2012 with minor modifications.

Primer designing

Complete genome sequence of *S.aureus* (Accession no. CP000046.1) available in the gene bank was used to design the primers for the genes (*pflB, ldh1, Hmp and PurM*). The primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Determining the specificity of the primers designed

The specificity of the primers designed was determined by doing PCR using these primers. DNA isolated from samples 43, M1 and LH were used as template and PCR was performed.Annealing temperatures for the amplification of *pflB*(58°C), *ldh1* (56°C), *Hmp*(57°C), *PurM*(57°C) and *16s rRNA* (56°C) were standardized. The PCR products (14 samples) obtained by the amplification of the above genes were custom sequenced by Eurofins Genomics India Pvt. Ltd., Bangalore. Finally, the sequence obtained

was subjected for sequence analysis using BLAST(http://blast.ncbi.nlm.nih.gov/Blast). With the help of BLAST score and e-value, the specificity of the sequence was ascertained.

RT-PCR amplification

Set of primers used for RT-PCR amplification of *pflB, ldh1, Hmp and PurM*genes of *S.aureus* are listed in Table 3. RNA samples used for one-step RT-PCR were adjusted to the uniform concentration of 100ng/µl. The expression of *16s rRNA* was used as positive control. The reaction mixture for one step RT-PCR was carried out in a total volume of 25 µl containing 5.0 µl of RNA(100 ng/100µl) in 0.2ml PCR tubes. RNA (5µl) (100ng/µl) was taken in a separate tube and centrifuged for 10 seconds at 10,000 rpm and was incubated at 65°C for 5 minutes in a Thermocycler (Eppendorf) to denature the RNA. The denatured RNA was immediately cooled in ice for 5 minutes. Above reaction mixture (20µl) was added to the denatured RNA so that the final volume will be 25µl. This final mixture was centrifuged again for 10 seconds at 10,000 rpm.

The final reaction mixture was subjected for PCR employing annealing temperature of 56°C (*16s rRNA*), 58°C (*pflB*), 56°C (*Ldh1),* 57°C (*Hmp*and *PurM*).Agarose Gel Electrophoresis for RT- PCR amplified products was carried out and the amplified DNA product was visualized under transilluminator and documented.

Study of gene expression in the clinical isolates of *S. aureus* grown in *in vitro* culture

The clinical isolates of *S. aureus* stored in glycerol broth and in which the the*pflB*gene expression was studied were revived and the bacteria were continuously grown *in vitro* by repeated sub-culturing for 10 times. Finally, these sub-cultured bacteria were inoculated in 5ml of luria broth and incubated over-night was used for RNA isolation. The concentration of RNA was adjusted to 100ng/µl. This RNA was used to study the gene expression of *pflB*and other genes by means of one step RT-PCR using gene specific primers. The level of gene expression in the clinical isolates grown in *in vitro* was semi-quantified using Quantity One software (Bio-Rad)

Comparison of the level of gene expression

Semi-quantification of RT-PCR amplified Products: The level of gene expression observed in the *S.aureus* isolates (Group1) and the corresponding *S.aureus* isolates grown in *in vitro* culture (Group2) were compared by semi-quantification of RT-PCR amplified products. The level of gene expression was semi-quantified using Quantity One software (Bio-Rad). The level of gene expression was expressed in arbitrary units after deducting the level of expression of *16s r RNA* gene.

Detection of bio-film formation in the clinical isolates of *S. aureus*

The clinical isolates of *S. aureus* which were stored in glycerol broth were revived and bio-film forming capacity was detected using tissue culture plate (TCP) method as per Mathur*et al*., (2006).

Statistical analysis

Students' paired t test (Nemeth *et al*., 2004) was used to calculate the difference in the level of gene expression (in terms of p value) between the two groups.

Results and Discussion

Screening of samples and identification of *S.aureus* associated mastitis samples

All the milk samples collected were subjected for bacterial culture and the microorganisms were identified up to the species level. Out of 62 samples, 29 samples (46.8%) were positive for *Staphylococcus* sp. by culture and staining. Twenty seven samples (93.1%) out of these 29 were positive for *Staphylococcus* genus by PCR using genus specific primers (*16s rRNA*).

The isolates confirmed by PCR as Staphylococciwere further subjected to PCR using primers specific for *nuc* gene of *S.aureus*. Out of 27 *Staphylococcus* sp., 15 (55.6%) were positive for *S.aureus*, of which six isolates were used for the study(Fig.1).

Confirmation of specificity of the primers designed

PCR conditions for the amplification of candidate genes selected for the study were standardized using the gene specific primers. All the primers designed were found to amplify the expected target as indicated by the size of the PCR product (*16s rRNA*-202bp; *pflB*-177bp; *ldh1*-147bp; *Hmp*-209bp; *PurM*-253bp). The specificity of the sequence was confirmed by sequence and its analysis which had detected 100% identity with the gene bank entries of corresponding genes (data not shown).

Expression of genes- 16s rRNA, pfl,ldh, Hmp and purM

Total RNA isolated from bacterial cells with proven quality based on denaturing gel electrophoresis and A260/A280 ratio were subjected for RT-PCR for 16s rRNA, pfl, ldh, Hmp and purM genes and level of gene expression was semi-quantified using 16s rRNA expression as a positive control(fig.2a & 2b).

The level of pfl and ldh is more in clinical isolates but the level of expression is significantly decreased when adopted to repeated sub-culturing. The expression of Hmp and purM gene is detected only in clinical isolates but the expression was absent in sub-cultured isolates. The results are shown in figure 3a & 3b, 4a & 4b, 5 and 6. The semi-quantified level of expression is depicted in the table 3.

Detection of Bio-film formation

Of the six isolates subjected for the detection of bio-film forming capacity, five isolates were found to be positive for bio-film forming capacity. Further those isolates found to be consistent with the expression of pfl and ldh. The results are shown in figure 7.

The adaptive response of *S.aureus* to mammary tissue while causing mastitis was studied in terms of expression of gene involved in the energy metabolism(pfl, ldh), nitric oxide stress (Hmp) and purine metabolism(purM) by comparing with the expression of above genes in the clinical isolates which were adopted to the *in vitro* culture on repeated sub-culturing.

The level of expression of pfl and ldh was more in the clinical isolates, but the level of expression was diminished in isolates adopted to *in vitro* culture. Pfl is the major enzyme that gets elevated in *S.aurues* during anaerobic conditions involving in fermentation pathway, acts as a main source of energy. *S.aureus* recycle NADH by reducing pyruvate to lactate with the help of lactate dehydrogenase enzyme (Fuchs*et al.*, 2007).

Table.1 Primers used for the detection of *S.aureus*

Organism	Target	Primer sequence (5'→3')		Expected Product Size
Genus specific *Staphylococcus*	*16S rRNA* (Zhang *et al.,* 2004)	Forward	AACTCTGTTATTAGCGAAGAACA	756bp
		Reverse	CCACCTTCCTCCGGTTTGTCACC	
S.aurues	Nuc gene (Brakstad *et al.*, 1992)	Forward	GCGATTGATGGTGATACGGT	270 bp
		Reverse	AGCCAAGCCTTGACGAACTAAAGC	

Table.2 Primers used for the RT-PCR amplification of *pflB, ldh1, Hmp, PurM* and *16s r RNA* genes of *S.aureus*

Target gene	Primer sequence(5'→3')		Expected product size
pflB	Forward	GAACGTGGCGGCATGTGGGA	177bp
	Reverse	AGCTTCACAAGCTGCTTTCGCCA	
Ldh1	Forward	CGAAGCGTTCGATGTTGCGCC	147bp
	Reverse	TGCGCTTTGCCCTCAGGACG	
Hmp	Forward	GCGTTGTCATGATGGCTTGCGA	209bp
	Reverse	GCTGCGCCTGTAGGTGGATTCG	
PurM	Forward	AGGGCTTGCGTCAAGTGGCAT	253bp
	Reverse	AGCAGCATATCCGGCTGGCAA	
16s rRNA	Forward	AAGCCTGACGGAGCAACGCC	202bp
	Reverse	TACGCGCGCTTTACGCCCAA	

Table.3 Semi-Quantified values of *16s rRNA, pflB, ldh1, Hmp and PurM* gene expression in *S. aureus* associated with mastitis milk and in *S. aureus* isolates grown in *in vitro*
(Values: Mean ± S.D)

Name of the Gene	Level of Expression of Gene		
	***S.aureus* isolated from mastitis milk (n=6)**	***S.aureus* isolates grown *in vitro*(n=6)**	**p value**
16s rRNA	142.50±3.62	143.67±5.01	NS
PflB	145.50±8.07***	74.83±10.05	*** P <0.001
ldh1	118.83±17.54***	69.33±5.96	*** P<0.001
Hmp	123.33±4.13	---	---
PurM	120.00±5.18	---	---

Fig.1 Detection of *S.aureus* based on amplification of *nuc* gene; Lane 1-4; 7 & 8: PCR Amplified products positive for *nuc* gene Lane 5: Negative control; Lane 6: 100bp DNA ladder

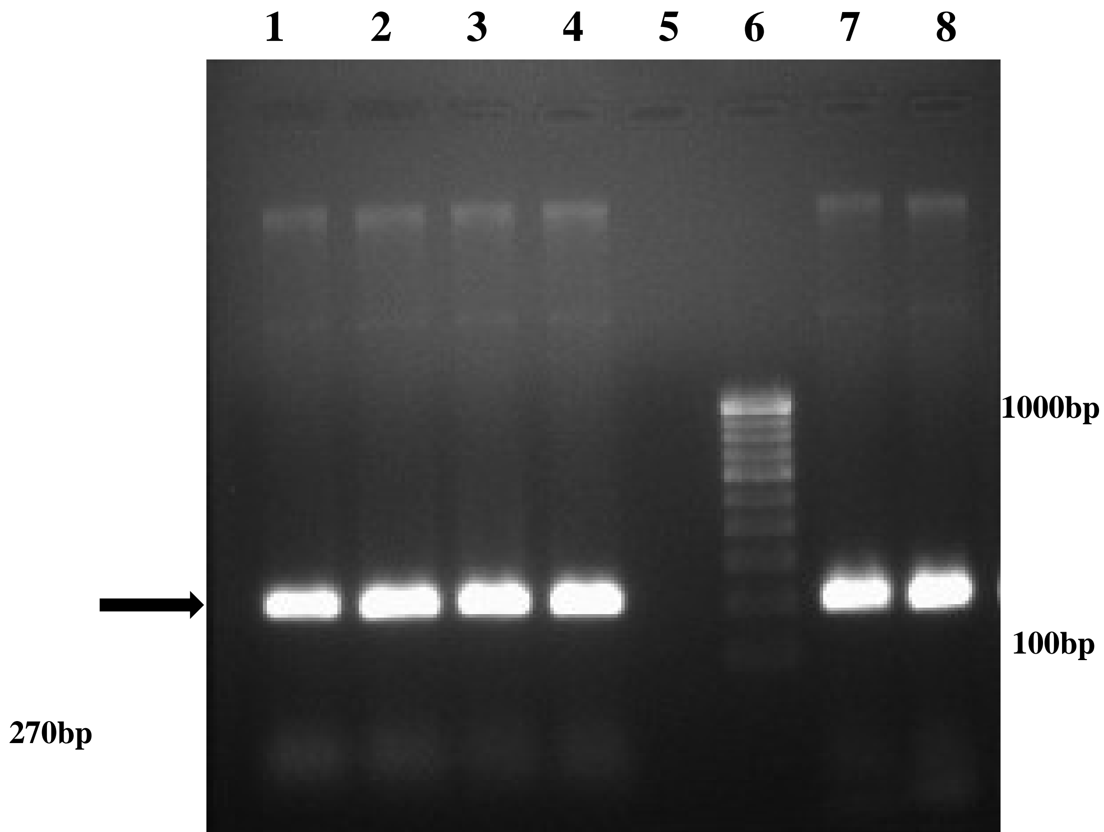


Fig.2a *16s rRNA* gene expression in *S. aureus* associated with mastitis milk
Lane 1, 3, 4, 5, 6, 7: RT-PCR product (202bp)
Lane 2: 100bp DNA ladder

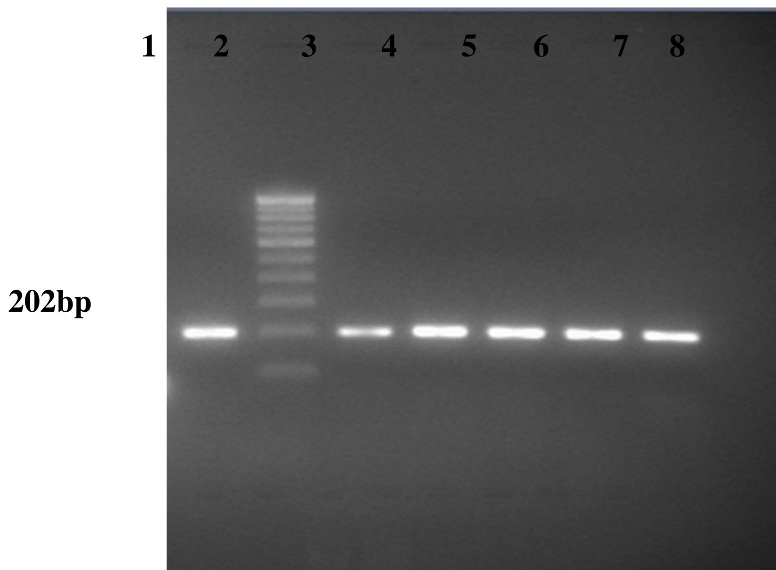


Fig.2b *16s rRNA* gene expression in *S.aureus* isolates grown in *in vitro*
Lane 1, 3, 4, 5, 6, 7: RT-PCR product (202bp)
Lane 2: 100bp DNA ladder

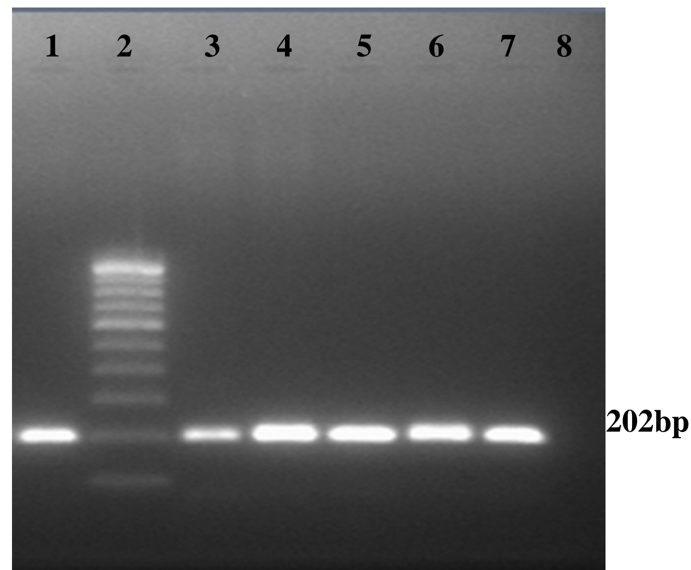


Fig.3a *pflB* gene expression in *S.aureus* associated with mastitis milk
Lane 2, 3, 4, 5, 8 & 11: RT-PCR product (177bp)
Lane 7 & 9: 100bp DNA ladder

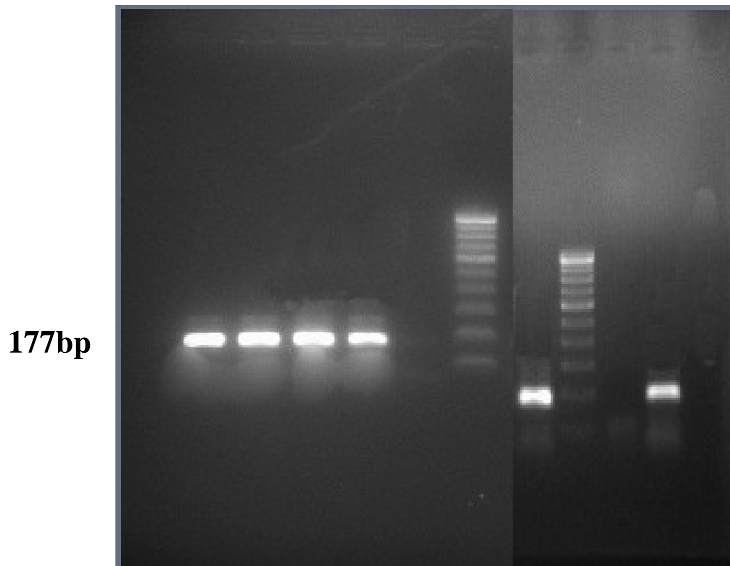


Fig.3b *pflB* gene expression in *S.aureus* isolates grown in *in vitro*
Lane 1, 2, 3, 4, 5 & 8: RT-PCR product (177bp)
Lane 6: 100bp DNA ladder

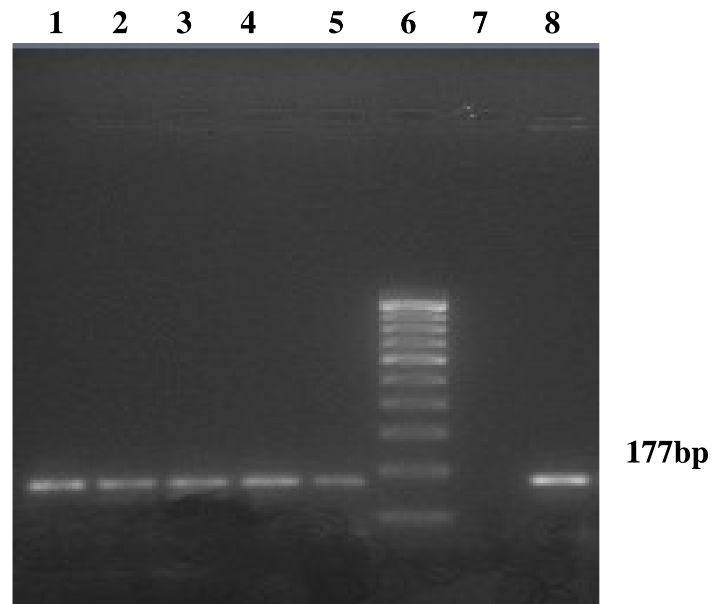


Fig.4a *Ldh1* gene expression in *S.aureus* associated with mastitis milk
Lane 1, 2, 3, 4, 5 & 8: RT-PCR product (147bp)
Lane 6: 100bp DNA ladder

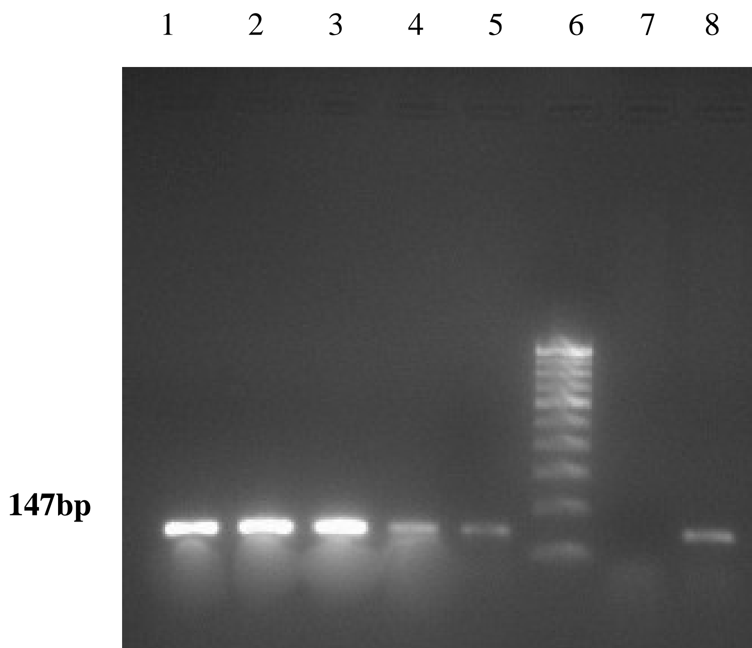


Fig.4b *Ldh1* gene expression in *S.aureus* isolates grown in *in vitro*
Lane 1, 2, 3: RT-PCR product (147bp)
Lane 6: 100bp DNA ladder

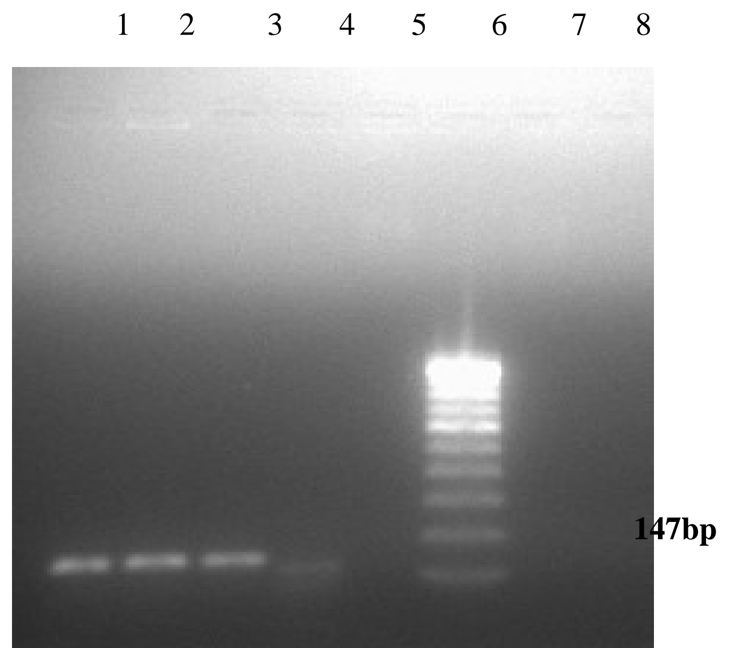


Fig.5 *Hmp* gene expression in *S.aureus* associated with mastitis milk
Lane 1, 2, 3, 4, 5, 8: RT-PCR product (209bp)
Lane 6: 100bp DNA ladder

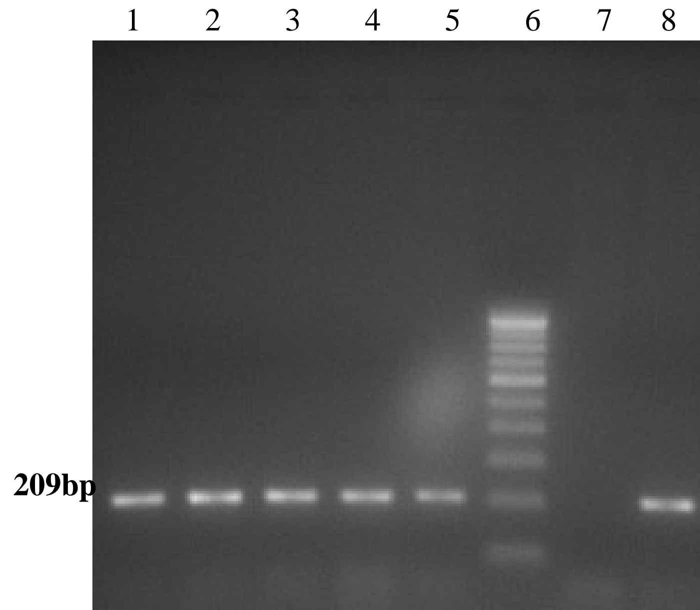


Fig.6 *PurM* gene expression in *S.aureus* associated with mastitis milk
Lane 1, 3, 6, 7, 8, 9: RT-PCR product (253 bp)

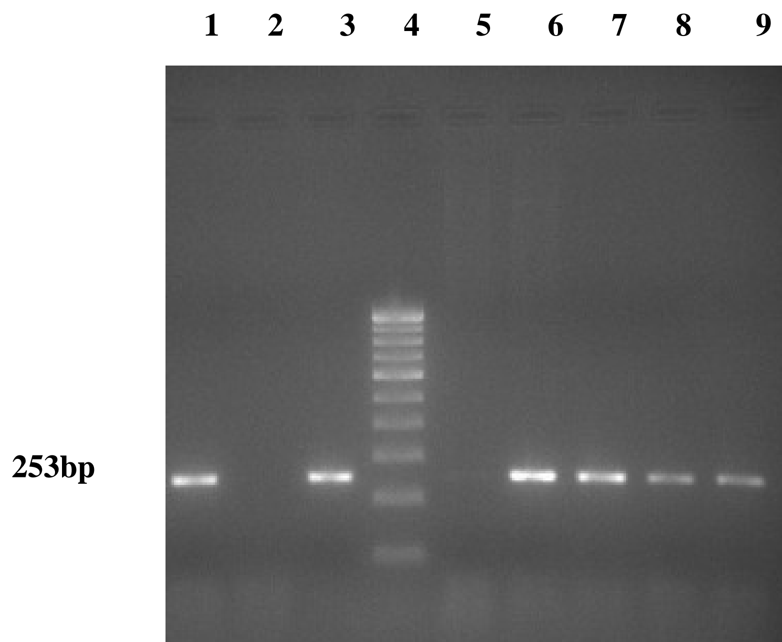
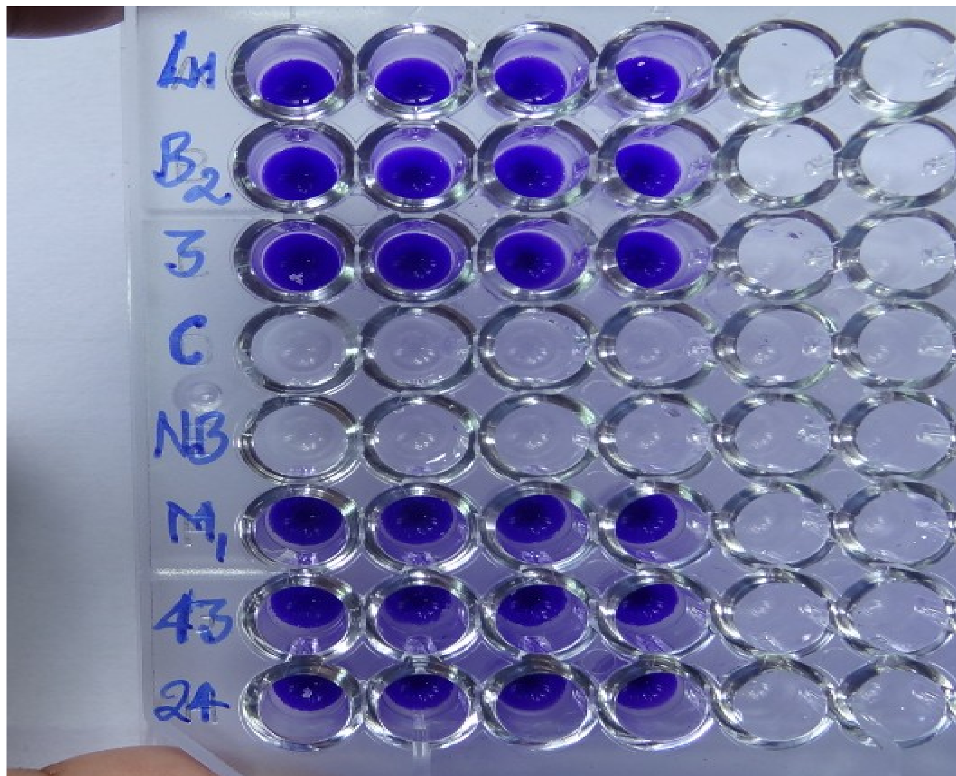


Fig.7 Screening of biofilm production by tissue culture plate (TCP) method
LH, B2, 3,M1, 43 and 24 are the samples which produced biofilm
C: Control; NB is non biofilm producing *S.aureus*



In comparative expression studies of planktonic and biofilm-grown *S.aureus* cells, it was shown that *pfl, fdh* (NAD-dependent formate dehydrogenase), and *fhs* (formyl tetrahydrofolate synthetase) are up-regulated at the transcriptional and proteome levels in bio-film (Resch*et al.,* 2005). The up-regulation of *pfl* and *fdh* appears to be an important survival strategy for *S.aureus* in biofilm (Leibeg*et al*., 2011). It is also reported that *pfl* gene expression is linked to the nitric oxide (NO) stress (Falko*et al*., 2008) and purine metabolism (Leibig*et al*., 2011). NO was found to inhibit aerobic respiration by reversible binding to cytochromes and disrupts the respiratory chain (Beltran *et al*., 2000; Moore *et al.,* 2004). *S. aureus* is capable of metabolically adapting to nitrosative stress by expressing an NO-inducible L-lactate dehydrogenase (*ldh1*, SACOL0222) divergently transcribed from the NO-detoxifying flavohemoglobin (*hmp*). L-Lactate production allows *S. aureus* to maintain redox homeostasis during nitrosative stress and is essential for virulence (Richardson *et al*., 2008). Falko *et al*., (2008) recently reported that when *S.aureus* was subjected for NO stress in *in vitro*, lactate dehydrogenase, *ldh1* and pyruvate formate lyase, *PflB* were the earliest and most strongly induced enzymes. Further it is reported that the starvation of purine nucleotides in milk stimulated the expression of pflB in *Streptococcus thermophilus* (Derzelle*et al*., 2005).

In the present study, the level of *pflB* and *ldh1* gene expression was very significant in *S.aureus* isolates obtained from mastitis milk. Associated with this, increased

expression of *Hmp* and *PurM* was also found in these isolates. *Hmp*, the gene encoding flavohemoglobin was highly induced when oxygen becomes limited (Lacelle *et al*., 1996) or NO is present (Moore *et al.,* 2004). Microaerobiosis and nitrosative stress appear to induce *hmp* expression in *S.aureus* (Goncalves *et al*., 2006) and is a candidate gene for the nitrosative stress. At the same time, when these isolates were grown in *in vitro* culture, the level of expression of *pfl* and *ldh* has come down significantly. But the expression of *Hmp* and *PurM* gene was completely lost. Further, the biofilm forming capacity was detected in almost all the clinical isolates of *S.aureus* where the *pflB* gene is highly expressed and also persisted during sub-culturing process. This suggests that the expression of *pflB* and *ldh1*is mainly due to the microaerobic environment created by biofilm formation. At the same time NO stress and deficiency of purines in milk might have added to the levels of expression of *pflB* and *ldh1*. Thus, it can be concluded that the expression of *pfl* and ldh gene is mainly as an adaptive response for the survival of the organism through the formation of biofilm.

It is reported that acetate plays a very important role in the formation of biofilm. This acetate is derived from acetyl coA. Thus, increase in the level of intracellular acetyl coA levels will cause increased biofilm amounts. For the formation of acetyl coA, bacteria depends on pyruvate formate lyase (Mugabi *et al*., 2012). Therefore, limiting the activity of pfl can control biofilm formation through acetate metabolism. Thus, *pfl* gene product can be suggested as a drug target for the limitation of the growth of *S.aureus*.

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References

Arciola,C. R., L. Baldassarri, and L. Montanaro. 2001. Presence of icaA and icaD genes and slime production in a collection of staphylococcal strains from catheter-associated infections.J. Clin. Microbiol.39: 2151–2156.

Baselga,R., I. Albizu, M. De La Cruz, E. Del Cacho, M.Barberan, and B.Amorena. 1993. Phase variation of slime production in *Staphylococcus aureus*: implications in colonization and virulence. Inf. Imm. 61: 4857–4862.

Beltran, B.A., M. R.Mathur, J.D.Duchen,Erusalimsky, and S. Moncada.2000. The effect of nitric oxide on cell respiration: a key to understanding its role in cell survival or death.Proc. Natl. Acad. Sc.97: 14602–14607.

Brakstad,O.G., K. Aasbakk, and J. A. Maeland. 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. J Clin Microbiol.30: 1654-1650.

Brouillette,E., M.Hyodo, Y. Hayakawa, D. K. R. Karaolis, and F. Malouin. 2005. 30,50-Cyclic diguanylic acid reduces the virulence of biofilm-forming *Staphylococcus aureus* strains in a mouse model of mastitis infection.Antimicrob. Agents Chemother.49: 3109–3113.

Ceri, H.,M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret.1999. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms.J. Clin. Microbiol.37: 1771–1776.

Christensen, J. P., J. E.Olsen,and M. Bisgaard.1993. Ribotypes of *Salmonella enterica*serovar*Gallinarumbiovarsgallina*

rum and *pullorum*.Avian Pathol.22: 725-38.

Cucarella,C., M. A. Tormo, E. Knecht, B. Amorena, I. Lasa, T.J. Foster, and J. R. Penades. 2002. Expression of biofilm-associated protein interferes with host protein receptors of *Staphylococcus aureus* and alters the infective process.Inf. Imm.70: 3180–3186.

Cucarella,C., M. A. Tormo, C. Ubeda., M. P. Trotonda., M. Monzon,C. Peris, B. Amorena, I. Lasa, and J. R. Penades. 2004. Role of biofilm-associated protein Bap in the pathogenesis of bovine *Staphylococcus aureus*. Inf. Imm.72: 2177–2185.

Derzelle,S., A. Bolotin, M. Y.Mistou, and F. Rul. 2005. Proteome Analysis of *Streptococcus thermophilus* Grown in Milk Reveals Pyruvate Formate-Lyase as the Major upregulated protein.Appl. Environ. Microbiol.71: 8597-8605.

Falko,H., W. Carmen, S. Fuchs, M. Liebeke, M. Lalk, S. Engelmann, andM. Hecker.2008. Nitric Oxide Stress Induces Different Responses but Mediates Comparable Protein Thiol Protection in *Bacillus subtilis* and *Staphylococcus aureus*.J. Bacteriol. 190: 4997–5008.

Fox,L. K.,R. N. Zadoks, and C. T. Gaskins. 2005. Biofilm production by *Staphylococcus aureus* associated with intramammary infection.Vet. Microbiol.107: 295–299

Fuchs,S., J. Pane-Farre, C. Kohler, M. Hecker, and S. Engelmann. 2007. Anaerobic gene expression in *Staphylococcus aureus*, J. Bacteriol.189: 4275–4289.

Goncalves, V. L., L. S. Nobre, J. B. Vicente, M. Teixeira, and L. M. Saraiva.2006.Flavohemoglobin requires microaerophilic conditions for nitrosative protection of *Staphylococcus aureus*. FEBS Lett. 580: 1817–1821.

Helling,R. B., H. M. Goodman, and H. W. Boyer. 1974. Analysis of endonuclease R-EcoRI fragments of DNA from lamboid bacteriophages and other viruses by agarose gel electrophoresis.J. Virol.14: 1235-1244.

Lacelle,M., M. Kumano, K. Kurita, K. Yamane, P. Zuber, and M. M. Nakano. 1996. oxygen controlled regulation of flavohemoglobin gene in *Bacillus subtilis*.J.bacteriology. 178:3803-808

Lam, B., M.Simkin, and Y. Haj-Ahmad.2012.Supplementary Protocol for Total RNA Isolation from*Staphylococcus aureus*-Related Bacteria and Hard-to-Lyse Bacterial Species. NorgenBiotek Corporation, Canada.

Lee,J.C., and G. B. Pier GB. 1997. Vaccine-based strategies for prevention of staphylococcal diseases, P. 631–654. In K. B. Crossley, and G. L. Archer (ed.).The staphylococci in human disease, Churchill-Livingstone. New York, N.Y.

Leibig,M., M. Liebeke, D. Mader, M. Lalk, A. Peschel, and F. Gotz. 2011.Pyruvate FormateLyase Acts as a Formate Supplier for Metabolic Processes during Anaerobiosis in *Staphylococcus aureus*. J. Bacteriol.193: 952–962.

Mariana,N. S., S. A. Salman, V. Neela, and S. Zamberi.2009. Evaluation of modified Congo red agar for detection of biofilm produced by clinical isolates of methicillin– resistance *Staphylococcus aureus.* African Journal of Microbiology Research. 3(6): 330-338.

Mathur, T., S.Singhal, S. Khan, D.J. Upadhyay, T. Fatma, and A. Rattan. 2006. Detection of biofilm formation in the clinical isolates of Staphyloccoci: An Evaluation of three different screening methods. Indian Journal of Medical Microbiology. 24 (I): 25-9.

Melchior,M. B., H. Vaarkamp, and J. Fink-Gremmels.2006. Biofilms: a role in recurrent mastitis infection.Vet.J.171: 398–407.

Milla, P., P. François, H. L. Hyyryläinen, M. Tangomo, V. Sass, H. G. Sahl, J. Schrenzel, and V. P. Kontinen. 2009. Transcriptome analysis of the responses of Staphylococcus aureus to antimicrobial peptides and characterization of the roles

of vraDE and vraSR in antimicrobial resistance. BMC Genomics. 10:429

Moore,C. M.,M. M. Nakano, T. Wang, R.W. Ye, and J. D. Helmann.2004. Response of *Bacillus subtilis* to nitric oxide and the nitrosating agent sodium nitroprusside.J. Bacteriol.186: 4655–4664.

Mugabi, R., D. Sandgren, M. Born, I. Leith, S. M. Horne, and B. M. Pruess. 2012. The Role of Activated Acetate Intermediates in the Control of Escherichia Coli Biofilm Amounts, WebmedCentral Microbiolog 3(7):WMC003577, 1-18.

Oie,S., Y. Huang, A. Kamiya, H. Konishi, and T. Nakazawa. 1996. Efficacy of disinfectants against biofilm cells of methicillin-resistant *Staphylococcus aureus*. Microbios. 85: 223-230.

Ranjan,R., M. K. Gupta, and K. K. Singh. 2011. Study of bovine mastitis in different climatic conditions in Jharkhand.Veterinary World. India. 4: 205-208.

Resch, A.,R. Rosenstein, C. Nerz, and F. Gotz. 2005. Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. Appl. Environ. Microbiol. 71:2663–2676.

Richardson, A. R. 2008. Nitric Oxide Inducible Lactate Dehydrogenase Enables *Staphylococcus aureus* to Resist Innate Immunity.Science AAAS.319: 1672.

Shiau, A. L, and C. L. Wu. 1998. The inhibitory effect of *Staphylococcus epidermidis* slime on the phagocytosis of murine peritoneal macrophages is interferon-in dependent.Microbiol. Immunol.42:33–40.

Shoshani,E. G.,B. Leitner, A. Hanochi, N. Y. Saran,Shpigel, and A. Berman. 2000. Mammary infection with *Staphylococcus aureus* in cows: progress from inoculation to chronic infection and its detection.J. Dairy Res. 67: 155–169.

Unnerstad,H.,A. Lindberg, K. P. Waller, T. Ekman, K. Artursson, M. Nilsson-Ost, and B. Bengtsson. 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet. Micro.137: 90–97.

Vasudevan,P., M. K. M. Nair, T. Annamalai, and K. S. Venkitanarayanan. 2003. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation.Vet. Microbiol.92: 179–185.

Weinrick B., P. M. Dunman, and R. P. Novick. 2004. Effect of Mild Acid on Gene Expression in *Staphylococcus aureus*. J. Bacteriol. 186(24): 8407–8423.

Zadoks,R. N., W. B. Van Leeuwen, D. Kreft, L. K. Fox, H. W. Barkema, Y. H. Schukken, and A. Van Belkum.2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin,milking equipment, and bovine milk by phage typing, pulse-field gel electrophoresis, and binary typing.J. Clin. Microbiol. 40: 3894–3902.

Zecconi, A., R. Piccinini, and K. L. Fox.2003. Epidemiologic study of intramammary infections with *Staphylococcus aureus* during a control program in nine commercial dairy herds. JAVMA. 223: 684–688.

Zhang,K., J. Sparling, B. L. Chow, S. Elsayed, Z. Hussain, D. I. Chruch, D. B. Gregson, T. Louie, and J. M. Conly. 2004. New Quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of *S. aureus* from coagulase- negative staphylococci.J. Clin.Microbiol.42: 4947-4955.