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RESEARCH ARTICLE

PREVALENCE OF STAPHYLCOCCUS AUREUS IN RAW MILK: CAN IT BE A POTENTIAL PUBLIC HEALTH THREAT?

Sukumar Bharathy [1*], Lakshmanasami Gunaseelan [2], Kannan Porteen[3] and Munnisamy Bojiraj[4]

1.Teaching Assistant, Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, Tirupati- 517 502.

2.Professor and Head, Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai-07.

3.Assistant Professor, Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai-07.

4.MVSc Research Scholar, Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai-07.

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Corresponding Author

SukumarBharathy

Background:*Staphylococcus aureus* is important milk borne pathogen and causes a wide variety of diseases in humans and animals and it is frequently associated with subclinical mastitis in dairy animals and may contaminate milk and other dairy products which act as vehicles for *S. aureus* infection in humans. **Aim:** The present study was aimed to identify the prevalence of *S. aureus* in raw milk samples from small holder dairy units in Chennai city and to relate the results with public health implication of the infection levels in dairy animals and the milk. **Methodology:** A total of 100 individual raw milk samples (50 cows, 10 buffaloes, 40 goats) were collected. Standard protocol was followed for the isolation of *S. aureus* from milk in Baird Parker Agar with 2% egg yolk tellurite emulsion. Colonies suggestive for *S. aureus* (black surrounded by white halo) were submitted for the morphological characteristics by Gram's staining and biochemical tests such as catalase and coagulase tests for confirmation. The positive presumptive isolates were further confirmed by Polymerase Chain Reaction (PCR) by targeting *Nuc* gene. **Results:** The overall prevalence rate of *S. aureus* in milk samples based on conventional techniques was 61 per cent (cow's milk 68%, goat's milk 62.5% and buffaloes milk 40%) and 65.57% *S. aureus* presumptive isolates were positive by PCR which includes 73.53 % of cow's milk, 52 % of goat's milk and 50% of buffaloes milk. **Conclusion:** In this study examined raw milk samples contains *S. aureus* indicates a potential route of *S. aureus* transmission may occur to consumers via contaminated milk or through contaminated dairy products. Hence, to improve the quality of milk and to prevent *S. aureus* contamination more hygienic preventive measures should be implemented.

INTRODUCTION

A variety of agents predominantly bacteria are considered major causes of milk borne illnesses worldwide. Milk understandably an important constituent of human diet and raw milk is an ideal growth medium for several microorganisms. Milk and its derivate are considered vehicles for *Staphylococcus aureus* infection in humans (Zecconi and Hahn, 2000). *S. aureus* causes a wide variety of diseases in humans and animals, ranging in severity from a mild skin infection to more severe diseases, such as pneumonia and septicemia (Fagundes et al., 2010).

In dairy cattle, *S. aureus* is frequently associated with subclinical mastitis and may contaminate milk and other dairy products (Jones et al., 2006). Although pasteurization is likely to destroy all pathogens, there is concern when raw milk is consumed or when pasteurization is incomplete or faulty. *S. aureus* produces several staphylococcal virulence factors, including enterotoxins (SEA to SEE and SEG to SEQ), and other toxins, such as exfoliative toxin A and B, and toxic shock syndrome toxin (TSST-1) (Fagundes and Oliveira, 2004) and milk of the infected animal is the main source of enterotoxigenic*S. aureus* of animal origin and these toxins are known to cause nausea, vomiting and abdominal cramps when ingested by human and are responsible for staphylococcal food poisoning outbreak (Loncarevic et al., 2004 and Kerouanton et al., 2007).

Staphylococcus aureus in raw milk generally comes from cows with mastitis, from handlers or from deficient hygiene. When found in milk, high level of contamination can be reached quickly under favorable conditions. Its presence in food can be a risk to human health, causing a public health problem, as these bacteria produces toxins that can cause food intoxication (Quintana and Carneiro, 2006).

The objective of this study was to detect *S. aureus* in milk samples by conventional (Colony morphology, Gram's staining and Biochemical tests) and Molecular (polymerase chain reaction) methods to identify the prevalence of *S. aureus* in raw milk from small holder dairy units in Chennai city and to relate the results with the public health implication of the infection levels in dairy animals and the milk.

MATERIALS AND METHODS

Sample collection: A total of 100 individual raw milk samples (50 cows, 10 buffaloes, 40 goats) were collected from Madras Veterinary College Teaching Hospital, Chennai city. Each milk samples (5 – 10 mL) was collected from apparently health udder in a sterile screw cap bottle aseptically from all quarters after discarding the initial 1-2 mL of milk during milking, and the samples were refrigerated at 4˚C until analysis.

Isolation and Identification of *Staphylococcus aureus*: Standard protocol was followed for the isolation of *S. aureus* from milk (Singh and Prakash, 2008). One mL of milk sample diluted with 9 ml sterile of buffered peptone water and enriched for 24hrs at 37˚C. The selective medium used for isolation of *S. aureus* was Baird parker Agar (BPA) with 2% egg yolk tellurite emulsion (HiMedia Pvt. Ltd). A loopful of inoculation from enrichment were streaked on BPA and incubated for 24-48 hours at 37˚C. Typical black colonies surrounded by white halo were considered to be presumptive *S. aureus*.

Colonies suggestive for *S. aureus* were submitted for the morphological characteristics by Gram's staining and Biochemical test by catalase and coagulase test for confirmation.

Morphological characteristics: The smear was prepared from the isolated culture on clean grease free microscopic glass slide and stained with Gram's method of staining. Smear shown Gram positive, spherical cells arranged in irregular clusters resembling to bunch of grapes.

Tube Coagulase test: Five typical colonies were selected for seeding in the tubes containing Brain Heart Infusion (HiMedia Pvt. Ltd) and the incubation was done at a temperature of 37˚C for 24 hours. From each tube containing Brain Heart infusion, 0.3 ml was transferred to sterile tube containing 0.5 mL of rabbit plasma (HiMedia Pvt. Ltd). The incubation was done at a temperature of 37˚C for 6 hours. Results were noted based on the degree of clotting. The reaction was considered as positive if the clot was immovable when the tube tilted.

Catalase test: Presumptive colonies were subjected to catalase test by using 3% hydrogen peroxide (H_2O_2), in positive reaction bubbles of oxygen were liberated within a few seconds.

DNA Extraction: DNA extraction was carried out by alkaline polyethylene glycol (AL - PEG) method according to Piotrchomczynskiand Michal Rymaszewski, 2006. In this method a few colonies were dissolved in 100µl of distilled water and 500µl of AL-PEG reagent (60g PEG 200 + 0.93ml 2M KOH +39 ml water) was added and incubated at 60˚C for 10mins. From this 2-3µl of supernatant was used as a template in PCR.

PCR amplification and gel – electrophoresis: PCR assay targeting *Nuc* gene (*S. aureus* species specific gene) was performed using the primer set; forward *Nuc*1: 5'-GCG ATT GAT GGT GAT ACG GTT-3' and reverse *Nuc* 2: 5'-AGC CAA GCC TTG ACG AAC TAA AGC-3' targeting a DNA amplicon of 270-bp.(Zhang et al., 2004). Amplification was carried out as follows: an initial denaturation of 94°C for 5 min; 35 cycles of 94°C for 1min, 55˚C for 40sec and 72°C for 1 min and a final extension step at 72° C for 10 min according to Zhang *et al* (2004) with slight modification. The amplified PCR products were electrophoresed in 1.5% agarose using sodium borate buffer (pH 8.2) (Fisher Scientific, India) with a constant voltage of 100v for 2h. The DNA fragments were stained with ethidium bromide (1mg/mL) and were visualized using UV- transilluminator. The size of the amplified product was compared by 100-bp DNA ladder (GeNei^{TM}, Bangalore, India).

RESULTS AND DISCUSSION

On the basis of conventional methods (Colony morphology, Gram staining and Biochemical tests) (Fig.1), 61 suspected *S. aureus* isolates were identified from a total of 100 milk samples which includes 50 cow's milk, 40 goat's milk and 10 buffaloes milk (Tab.1). The overall prevalence rate of *S. aureus* in milk sample that were collected from Chennai (South India) based on conventional techniques was 61 per cent (61/ 100) was almost similar to other reports in India, 61% by Lingathurai and Vellathurai (2010) in Madurai (South India), 62.34% by Singh *et al*., 2011 in of NDRI, Karnal, Haryana (North India) and 53.7 % by Sadashiv and Kaliwal, 2013 in North Karnataka (South India).

As compared to present findings lower level of incidence of *S. aureus* have been reported in other countries. In Palestine, 48 (36.9%) milk samples were positive for *Staphylococcus aureus* from total of 130 samples (Farhan and Salk, 2007). Park et al. (2007) analyzed 30,019 samples of raw milk in Korea and detected 104 (0.35%) samples contaminated with *Staphylococcus aureus* and Daka et al. (2012) reported 40.6 % in South Ethiopia. The difference in the prevalence rate was due to variation in the sanitary condition of udder, size of sampling and geographic region (Sadashiva and Kaliwal, 2013 and Shopsin et al., 2000).

The species wise prevalence of *S. aureus* were 68 per cent (34/ 50) for cow's milk, for goat's milk 62.5 per cent (25/40) and for buffaloes milk 40 per cent (4/10). Concerning the results of cow's milk, it is higher than that recorded by Mohamed and El Zubeir, 2007 (46.7%), Ibrahem, 2010 (16.47%), Alnakip, 2009 (16.7%), Amer*et al*., 2007 (30%) and Suelam et al., 2012 (30%). Ekici et al. (2004) isolated *S. aureus* from 3 (12%) out of 25 goat milk samples, it is lower when compared with our results. Results of buffaloes milk is lower than that previously reported by Tahoun, 2009 (78%) and higher than that of Bayoumi, 2003 (28%) and Suelam et al., 2012 (25%). The variation in prevalence of *S. aureus* in raw milk may be due to the improper hygiene and poor animal management (Ateba et al., 2010).

DNA was isolated from all the *S. aureus* suspected presumptive culture colonies by alkaline polyethylene glycol method and the presence of the *Nuc*gene was detected by polymerase chain reaction. The *Nuc* gene amplicon of 270 bp (Fig. 2) obtained from 40 samples out of 61 positive culture isolates, which includes 25 cow's milk, 13 goat's milk and 2 buffaloes milk. *S. aureus* was isolated by species specific *Nuc* gene targeted PCR from 73.53 per cent (25/34) of cow's milk, 52 per cent (13/25) of goat's milk and 50 per cent (2/4) of buffaloes milk with overall prevalence rate of *S. aureus* in milk sample based on PCR was 65.57 (40/61) (Tab. 1).

In the present study, we used oligonucleotide primer set which recognizes *Nuc* gene which encodes the TNase produced by *S. aureus.* By using *Nuc* gene set of primer 65.57 % culture isolates were identified as *S. aureus*. In previous study by Kuzma et al. (2003) in mastitis milk detected *S. aureus* in all 29 positive isolates by PCR. A *Nuc* gene targeted PCR was able to identify all *S. aureus* strains from intramammary infection (Brakstad et al.,1992 and Kuzma et al.,2003). In this study we were able to detect 65.57% of *S. aureus* isolates. Inhibitors of PCR are common in milk which may contain variety of substances that inhibit the polymerase chain reason and they are the reason of false negative results (Kim et al., 2001)

The PCR technique based on the *nuc* gene was able to detect *S. aureus* faster than traditional culturing. Although antibiotics in milk may inhibit growth in culture, they should not affect the results of the PCR assay and conventional culture techniques may not yield positive results from truelysubclinically infected glands due to the presence of very low numbers of pathogens (Lowy, 1998). An unidentified infected animal act as reservoirs of infection. Consumption of raw milk has always been common among farm families, it is varying from 35% to 60% (Jayarao and Henning, 2001). Milk and milk products are very essential in the Indian diet so their contamination can cause varied health hazards.

Table 1. Prevalence of *Staphylococcus aureus* in examined raw milk

Source	No. of samples examined	Presumptive culture Method		Polymerase chain reaction	
		No. of positive	%	No. of positive	%
Cow's milk	50	34	68	25	73.53
Goat's milk	40	25	62.5	13	52
Buffaloes milk	10	4	40	2	50
Total	**100**	**61**	**61**	**40**	**65.57**

Figure 1: *Staphylococcus aureus* colony on Baird- Parker agar

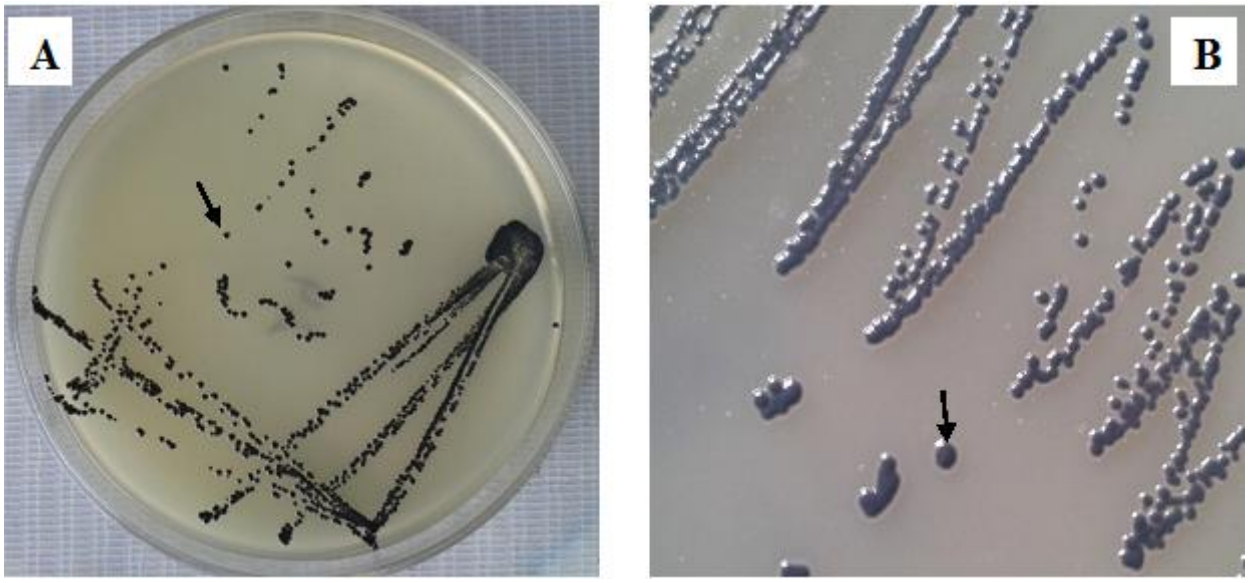

Fig 1. A and B showing typical jet black colonies surrounded by halo zone of *S. aureus* on Baird- Parker agar.

Figure 2: *Staphylococcus aureus* species specific Polymerase chain reaction

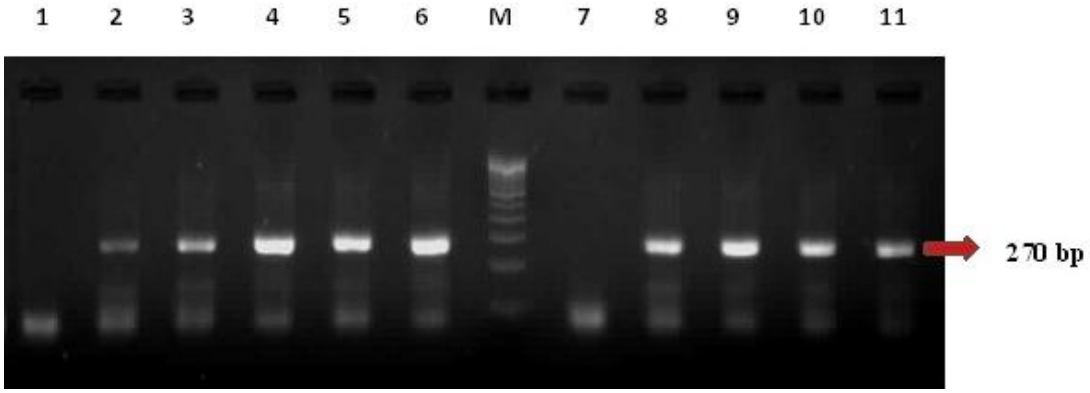

Fig 2. PCR amplicons (270- bp) using *Nuc* gene primers in raw milk samples. M: 100 bp ladder; Lane 1-5: Cow's milk samples; Lane 6: Positive control; Lane 7: Negative control; Lane 8-10: Goat's milk samples; Lane 11: Buffalo milk sample.

CONCLUSION

In this study, all the milk samples were collected form apparently healthy udder (animals did not showed any sign of mastitis). With severe clinical mastitis, abnormalities of milk are easily observed and milk is discarded by the producer. Such milk normally would not enter the food chain but milk of cows with sub-clinical mastitis, *i.e.* with no visible changes, can be mixed into bulk milk, such kind of milk may act as a potential route of *S. aureus* transmission to consumers via consumption of contaminated milk or through contaminated dairy products which produced from milk obtained from animals affected by subclinical mastitis.

Our study result indicates that samples of examined raw milk contained *S. aureus*. The presence of *S. aureus* in milk represents a public health threat. Hence, there is an urgent need for more strict and hygienic preventive measures to reduce the bacterial contamination, so as to increase the wholesomeness and quality of these milk and milk based products for the good health of all consumers. It is essential to prevent risks of contamination of *S .aureus* from the point of production to the point of consumption of milk.

REFERENCE

1. Alnakip, M. E. (2009): Prevalence of gram positive bacteria in milk and some dairy products. M.V.Sc thesis, Faculty of Veterinary Medicine, Zagazig University, Egypt.
2. Amer,I. H., Abdel Aal, S. F. and Awad, E. I. (2007): Prevalence of bacterial content and food borne organisms in raw cows milk. Alexandria J. Vet. Sci., 1: 153-164.
3. Ateba, C. N., Mbewe, M., Moneoang, M. S. and Bezuidenhout, C. C. (2010): Antibiotic resistant *Staphylococcus aureus* isolated from milk in the Mafikeng area, North West province, South Africa. S. Afr. J. Sci., 106: 1-6.
4. Bayoumi, M. A. (2003): Studies on some food poisoning microorganisms in milk and some dairy products. M.V.Sc thesis, Faculty of Veterinary Medicine, Zagazig University, Egypt.
5. Brakstad, O. G., Aasbakk, K. and Maeland, J. A. (1992): Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. J. Clin. Microbiol., 30: 1654- 1660.
6. Daka, D., Gsilassie, G. and Yihdego, D. (2012): Antibiotic resistance *Staphylococcus aureus* isolated from cow's milk in the Hawassa area, South Ethopia. Ann. Clin. Microbiol and Antimicrob., 11: 26-31.
7. Ekici, K., Bozkurt, H. and Isleyici, O. (2004): Isolation of some pathogens from raw milk of different milch animals. Pak. J. Nutr., 3: 161- 162.
8. Fagundes, H. and Oliveira, C.A.F. (2004): *Staphylococcus aureus*intramammary infections and its implications in public health. Cienc. Rural., 34: 1315-1320.
9. Fagundes, H., Barchesi, L., Filho, A. N., Ferreira, L. M. and Oliveira (2010): Occurrence of *Staphylococcus aureus* in raw milk produced in dairy farms in Sao Paulo state, Brazil. Braz. J. Microbiol., 41: 376-380.
10. Farhan, M. and Salk, S. (2007): Evaluation of Bacteriological contamination in raw (unprocessed) milk sold in different regions of Lahore (Pakistan). J. Agr. Soc. Sci., 3: 1813-2235.
11. Ibrahem, M. A. (2010): Microbiological status of raw and pasteurized milk in Sharkia Governorate, Ph.D. thesis. Faculty of Veterinary Medicine, Zagazig University, Egypt.
12. Jayarao, B. M. and Henning, D. R., (2001): Prevalence of foodborne pathogens in bulk tank milk. J. Dairy Sci., 84: 2157 -2162.
13. Jones, F. T., Creech, B.C., Erwin, P., Baird, G. S., Woron, A. M. and Schaffner, W. (2006): Family outbreaks of invasive community associated methicillin resistant *Staphylococcus aureus infection*. Clin. Infect. Dis., 42: 76-78.
14. Kerouanton, A., Hennekinne, J. A., Letertre, C., Petit, L., Chesneau, O., Brisabois, A., De Buyser, M. L. (2007): Characterization of *Staphylococcus aureus* strains associated with food poisoning outbreaks in France. Int. J. Food Microbiol., 115: 369 -375.
15. Kim, C. H., Khan, M., Morin, D. E., Hurley, W. L., Tripathy, D.N., Kehrli, Jr. M., Oluoch, A. O. and Kakoma, I. (2001): Optimization of the PCR for detection of *Staphylococcus aureus*nuc gene in bovine milk. J. Dairy Sci., 84: 74-83.
16. Kuzma, K., Malinowski, E., Lassa, H. and Klossowska, A. (2003): Specific detection of *Staphylococcus aureus* by PCR in Intramammary infection. B. Vet. I. Pulawy., 47: 183-190.
17. Lingathurai S and Vellathurai P. 2010. Bacteriological quality and safety of raw cow milk in Madurai, South India. Webmed Central Microbiol., 1: WMC00 1029.

18. Loncarevic, S., Jorgensen, H. J., Lovseth, A., Mathisen, T. and Rorvik, L. M. (2004): Diversity of *Staphylococcus aureus* enterotoxin types within single samples of raw milk and raw milk products. J. Appl. Microbiol., 98: 344-350.

19. Lowy F.D. (1998): *Staphylococcus aureus* infection. New Engl. J. Med., 339:520-532.

20. Mohamad, N. I. and El Zubeir, I. E. (2007): Evaluation of the hygienic quality of market milk of Khartoum State (Sudan). Int. J. Dairy Sci. 1: 33-41.
21. Park, Y. K., Koo, H. C., Kim, S. H, Hwang, S. Y. and Jung, W. K. (2007): The analysis of components and pathogenic bacteria isolated from bovine raw milk in Korea. J. Dairy Sci. 90: 5405-5414.
22. Piotrchomczynski and Michal Rymaszewski, (2006): Alkaline polyethylene glycol based method for DNA isolation from bacteria, tissue samples and blood. Biotechiques., 40: 454-458.
23. Quintana, R. C. and Carneiro, L. C. (2006): Avaliacao do leite in naturacomercializadoclandestinamente no municipo de Morrinhos, GO. Revista do Instituto Adolfo Lutz., 65: 194-198.
24. Sadashiv, S. O. and Kaliwal, B. B. (2013): Antibiotic resistance of *Staphyloccocus aureus* and Coagulase negative *Staphylococci* (CNS) isolated from bovine mastitis in the region of North Karnataka, India. World J. Pharm. Res., 3: 571-586.
25. Shopsin, B., Mathema, B., Martinez, J. Ha. E., Campo, M. L., Fierman, A., Krasinski, K., Kornblum, J., Alcabes, P., Waddington, M., Riehamn, M. and Kreiswirth, B. N. (2000): Prevalence of methicillin resistant susceptibility of drug resistant *Staphylococcus aureus* in raw milk of dairy cattle. Int. Res. J. Microbiol., 2: 466-470.
26. Singh, R. S., Kumar, R. and Yadav, B. R. (2011): Distribution of Pathogenic factors in *Staphylococcus aureus* strains isolated from intra-mammary infections in cattle and buffaloes. Indian J. Biotechnol., 10: 410-416.
27. Singh, P. and Prakash, A. (2008): Isolation of *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*from milk products sold under market conditions at Agra Region. Acta Agri. Slov. 92: 83-88.
28. Suelam, I. I. A., Raslan, A. R. A. and Mohamed, M. E. M. (2012): Isolation of *Staphylococcus aureus* from milk and Human with reference to its survival on surfaces. World J Dairy and Food Sci. 7: 142-145.
29. Tahoun, A. B. M. (2009): Hygienic studies of raw milk in Sharkia Governorate markets. M.V.Sc thesis, Faculty of Veterinary Medicine, Zagazig University, Egypt.
30. Zecconi, A. and Hahn, G. (2000): *Staphylococcus aureus* in raw milk and human health risk. Bull. Int. Dairy Fed. 345:15-18.
31. Zhang, K., Sparling, J., Barbara, L., Chow, Sameer, E., Hussain, Z., Deirdre, L., Daniel, B. G., Louie, T. and John, M. C. (2004): New Quadriplex PCR Assay for Detection of Methicillin and Mupirocin Resistance and Simultaneous Discrimination of *Staphylococcus aureus* from Coagulase-Negative *Staphylococci*. J. Clin. Microbiol. 42: 4947-4955