International Journal of Livestock Research, Vol. 11 (4) Apr' 2021
http://www.ijlr.org
eISSN: 2277-1964

Original Research

Standardization of mPCR for Biocide Resistance Genes and their Prevalence in *Staphylococcus aureus*

Tawheed Ahmad Shafi*, Baljinder Kumar Bansal, Shukriti Sharma, Dhiraj Kumar Gupta and Swaran Singh Randhawa

Department of Veterinary Medicine, Guru Angad Dev Veterinary and Animal Sciences University Ludhiana, Punjab, INDIA

*Corresponding Author: tawheed78@gmail.com

How to cite this paper: Shafi, T., Bansal, B., Sharma, S., Gupta, D., & Randhawa, S. (2021). Standardization of mPCR for Biocide Resistance Genes and their Prevalence in *Staphylococcus aureus*. *International Journal of Livestock Research*, 11(4), 133-139. https://doi.org/10.5455/ijlr.20200729084437

Received : Jul 12, 2020
Accepted : Mar 15, 2021
Published : Apr 30, 2021

Abstract

Biocides are commonly used in infection control programs to prevent the new infections of the udder and colonization of microorganisms on other contact surfaces. In the present study, a multiplex PCR (mPCR) assay simultaneously amplifying 04 biocide resistance genes was standardised. The mPCR was applied on 30 S. aureus isolates, isolated from milk of mastitis affected cows and prevalence of these genes in S. aureus was studied. High resistance mediated by sepA (83.33%), norA (80%) and mepA (80%) resistance genes was observed while resistance mediated by qacA/B (3.33%) gene was found very low. Genetic resistance against biocides is a widespread problem that has been extensively studied in food industry and hospitals particularly targeting methicillin resistant S. aureus, however, limited studies on biocide resistance in S. aureus of animal origin have been undertaken.

Keywords: Biocides resistance, Bovine mastitis, *sep*A, *nor*A, *mep*A, *qac*A/B

Introduction

Antiseptics and disinfectants have been commonly used as teat dips to maintain udder health and prevent the transmission of microbes between dairy cattle (National Mastitis Council, 1999). Biocides such as quaternary ammonium compounds (QACs) or biguanides, are commonly used to control and prevent the new infections of the udder, colonization on the teat surfaces, milking machines, milk tanks and other milk processing equipment (Ucuncu, 2015). *S. aureus* is a major mastitis pathogen in dairy animals, and also of special public health concern as it causes many serious and life-threatening infections in humans (Bansal *et al*., 2019; Shrivastava and Shrivastav, 2019). Genetic resistance against biocides has been extensively studied in food industry and hospital associated methicillin resistant *S. aureus* (MRSA) (Mayer *et al*., 2001; Zhang *et al*., 2011; McGann *et al*., 2013). The resistance against QACs is primarily encoded in the *qac* genes that operate via deferent mechanisms (Jaglic and Cervinkova, 2012; Cervinkova *et al*., 2013; Ergun *et al*., 2017), and their acquisition by staphylococci has resulted in high resistance to chlorhexidine, one of the most frequently used biocides (Horner *et al*., 2012). Multidrug resistance efflux pumps such as *NorA, NorB, NorC, MdeA* and *SdrM* (chromosomally encoded) and *qacA/B* (plasmid-encoded) have been reported in *S. aureus* of human, animal and environmental origin resulting in cross-resistance to both antibiotics and biocides. (Sierra *et al*., 2000; Noguchi *et al*., 2005; Hassan *et al*., 2007; Kosmidis *et al*., 2012; Costa *et al*., 2013; Couto *et al*., 2015). Other types of resistance pumps include, *Smr* (belongs to small multidrug resistance family), *MepA* (belongs to multidrug and toxic compound extrusion family) and *SepA* (Paulsen *et al*., 1995; Narui *et al*., 2002; *Kaatz et al*., 2005). Most of these pumps have got potential to extrude compounds from different chemical classes and therefore, help the organism to develop multidrug resistance (MDR).

This study highlights the importance of emerging biocide resistance in dairy sector in which in-depth studies over this emerging issue have not been undertaken. Therefore, it is need of the hour to thoroughly study the emergence of biocide resistance in microorganisms of animal origin that are also having greater implications on human health. Therefore, this study was aimed to develop and standardize a multiplex PCR (mPCR) for detecting simultaneously 04 biocide resistance genes, and thereafter applying this to estimate their prevalence in *S. aureus* of bovine mastitis origin.

Materials and Methods

Isolation and Identification of *S. aureus*

The study involved 30 *S. aureus* isolated from bovine mastitis from different agro-climatic regions of Punjab, India. *S. aureus* was identified on the basis of colony characteristics on blood agar, Gram staining, clumping factor, growth characteristics on mannitol salt agar, DNase agar, Baird parker agar, tube coagulase test and final confirmation by species specific PCR. Individual isolates were stored at −20 °C in trypticase soy broth containing 30% glycerol for future use. *S. aureus* (ATCC 25923, ATCC 33591) and *S. epidermidis* (MTCC 3382) were used as standard positive control.

DNA Extraction

After overnight incubation of an individual bacterial colony in brain heart infusion (BHI) broth (HiMedia, India), 1 ml growth was pelleted at 7500 rpm for 5 minutes (min) in refrigerated centrifuge (Heraeus Biofuge Primo R, Thermo Scientific). The pellet was suspended in 180 µl lysis solution (lysozyme enzyme solution) and incubated at 37 ºC for 30 min. Bacterial DNA was extracted using QIAamp DNA mini kit (Qiagen) as per manufacturer guidelines. Eluted *genomic* DNA was stored at −20 °C until use.

PCR Confirmation of *S. aureus*

Duplex PCR was carried out for the detection of *Staphylococcus* genus (16S rDNA i.e., ribosomal DNA), and *S. aureus species* (*nuc* gene) using primers given in Table 1 (Brakstad *et al*., 1992; Strommenger *et al*., 2003). The amplification was carried out in a total reaction volume of 25 µl containing 12.5 µl 2X PCR Master Mix (Qiagen), 10 pmol/µl of each primer set containing forward and reverse primers, 0.01 µg- 0.2 µg template and sterilized nuclease free water to make up the reaction volume. Master cycler Gradient Thermocycler (Bio-rad, USA) was used to perform the amplification reaction involving initial denaturation at 94 °C for 5 min; followed by 30 cycles each of denaturation at 94°C for 30 seconds; annealing at 57.7 °C for 40 seconds and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 5 minutes and followed by hold at 4 °C.

Multiplex PCR Standardization

Multiplex PCR for amplification of biocide resistance genes was standardized using *S. aureus* (ATCC 33591) for *sepA* gene, *S. hemolyticus* (gene bank accession number MW296867) from department culture storage for *qacA/B* gene and *S. aureus* (gene bank accession numbers MW254363 and MW289832) from this study for *norA* and *mepA* genes. The mPCR was repeated several times for reagent optimization, reproducibility and specificity. The primers for mPCR for amplifying 04 biocide resistance genes were selected from the published literature given in Table 1. The mPCR comprised of 100 pg of DNA template in a 30 µl reaction mixture with 15 µl of 2X Taq DNA Master Mix (Qiagen) and 0.5µl (20 nM) of each of the forward and reverse primers (Bioserve Pvt. Ltd.). The reaction mixture was amplified in a thermocycler (Bio-rad, USA); initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 45 seconds, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. PCR products were analysed using conventional agarose gel electrophoresis in 1.5% agarose (Genaxy) in 1x tris-borate-edta (TBE) buffer (Genaxy) containing ethidium bromide at 0.1 µg/ml and using 50 bp DNA Ladder-DM1100 (SMOBio) for comparison. The DNA bands were visualized and imaged using the Molecular Imager® ChemiDoc™ XRS+ imaging system (Bio-Rad).

Table 1: Target genes, sequences and amplicon size of primers used in the study

Target genes	Primer sequences (5'-3')	Product size (bp)	References
16S rDNA	Forward-CAG CTC GTG TCG TGA GAT GT	420	Strommenger *et al*., 2003
	Reverse- CAT TTG TCC CAC CTT CG		
Nuc	Forward-GCGATTGATGGTGATACGGTT	280	Brakstad *et al*., 1992
	Reverse- AGCCAAGCCTTGACGAACTAAAGC		
qacA/B	Forward-TCCTTTTAATGCTGGCTTATACC	220	Ergun *et al*., 2017
	Reverse- AGCCKTACCTGCTCCAACTA		
norA	Forward-TTCACCAAGCCATCAAAAAG	620	Couto *et al*., 2008
	Reverse- CTTGCCTTTCTCCAGCAATA		
mepA	Forward-ATGTTGCTGCTGCTCTGTTC	718	
	Reverse- TCAACTGTCAAACGATCACG		
sepA	Forward-GCAGTCGAGCATTTAATGGA	103	
	Reverse- ACGTTGTTGCAACTGTGTAAGA		

Results and Discussion

S. aureus was confirmed using duplex PCR consisting of *Staphylococcus* genus (16S rDNA), and *S. aureus* species (*nuc*) specific genes (Figure 1) and thereafter, a mPCR for amplification of 04 biocide resistance genes was standardised successfully (Figure 2). mPCR was applied on 30 *S. aureus* isolates for validation of the protocol and estimation of biocide resistance genes. It was observed that there is high resistance mediated by *sepA* (83.33%), *norA (80%) and mepA* (80%) resistance genes while as resistance mediated by *qacA/B* (3.33%) gene was found very low (Table 2).

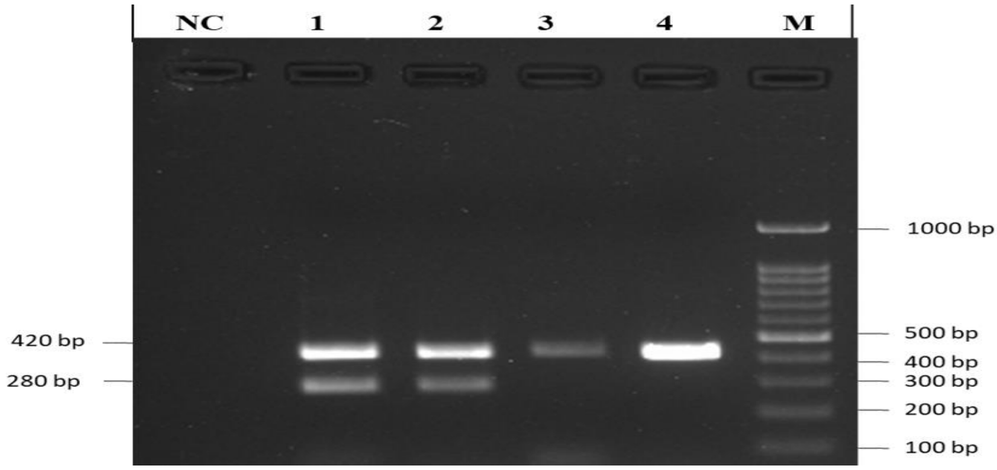

Figure 1: Electrophoresis image of duplex PCR for detection of 16SrDNA (420bp) and nuc (280bp) genes. Lanes indicate NC: negative control-nuclease free water, 1: S. aureus 2: S. aureus positive control (ATCC 25923) 3: Staphylococcus spp. 4: S. epidermidis positive control (MTCC 3382). M indicate ExcelBand 100 bp DNA ladder (DM2300, SMOBio).

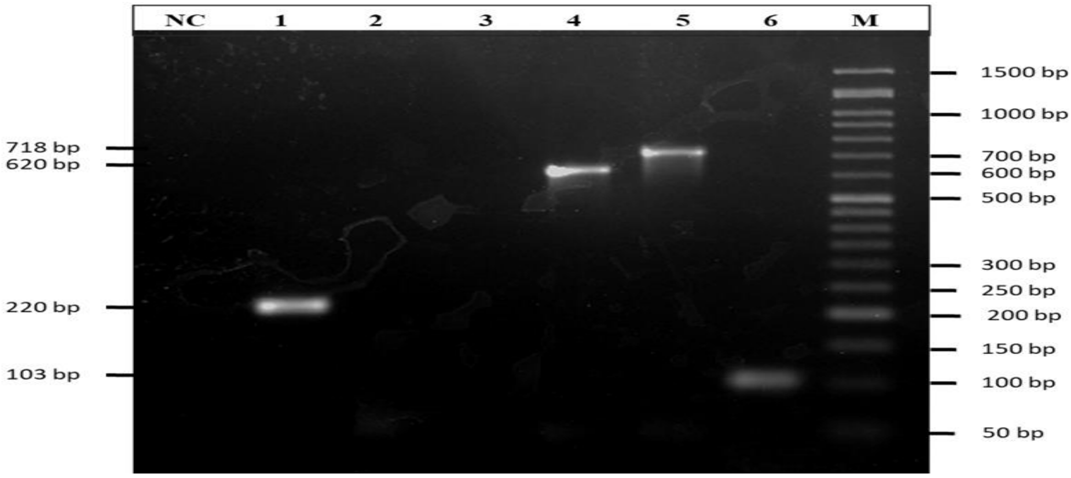

Figure 2: Electrophoresis image of mPCR for amplification 4 biocide resistance genes. Lanes indicate NC: negative control-nuclease free water, 1, 4, 5, 6: qacA/B (220 bp), norA (620 bp), mepA (718 bp) and sepA (103 bp); 2, 3 no amplification (negative for biocide resistance). M indicate ExcelBand 50 bp DNA ladder-DM1100 (SMOBio).

Table 2: Frequency of biocide resistance in *S. aureus*

Biocide resistance gene	Frequency	Percentage
qacA/B	1	3.33
norA	24	80
mepA	24	80
sepA	25	83.33

In the present study resistance mediated by *qacA/B* gene was found very low, possibly due to its plasmid origin, however, in this study chromosomal DNA was used for standardisation of mPCR as well as screening of resistance genes in *S. aureus*. The prevalence of *qacA/B* resistance in MRSA has been reported as low as 1% in a few Asiatic countries to as high as 80% in Brazil, exhibiting great variation in geographical locations (Mayer *et al.*, 2001, Wang *et al.*, 2008a, Wang *et al.*, 2008b, Ho *et al.*, 2012). Acquisition of *qacAB* gene family (located on transferable plasmids and transposons harbouring antibiotic resistance genes as well) by staphylococci has resulted resistance to chlorhexidine, one of the most frequently used biocides and also against aminoglycosides, penicillin and trimethoprim (Berg *et al.*, 1998; Horner *et al.*, 2012).

High resistance mediated by *norA, mepA and sepA* was observed in this study, which belong to multidrug resistance efflux pumps. Resistance mediated by multidrug resistance efflux gene pumps such as *qacA/B*, *smr*, *norA*, *mepA*, *sepA and lmrS* have also been reported in *S. aureus* of human, animal or environmental origin (Sierra *et al.*, 2000, Naguchi *et al.*, 2005, Hassan *et al.*, 2007, Kosmidis *et al.*, 2012, Costa *et al.*, 2013, Couto *et al.*, 2015; Senn *et al.*, 2016). High genetic resistance against biocides in MRSA has been attributed to selective pressure caused by use of antiseptics or acquisition of antibiotic resistance gene determinants or horizontal transfer of plasmids (Thomas *et al.*, 2000, Sidhu *et al.*, 2002, McGann *et al.*, 2013; LaBreck *et al.*, 2018).

Conclusion

The emergence of biocide resistance mediated by multidrug resistance efflux pumps result in cross-resistance to antibiotics, and dissemination of these resistance genes among bacteria result in demand for newer antibiotics and biocides to contain the infections and contaminations. To mitigate this threat thorough studies involving standard testing for biocide susceptibility and genotypic estimation of biocide resistance must be undertaken. The mPCR developed in this study will be helpful for estimation of resistance mediated via these important resistance genes and therefore, will be helpful in deciding the management strategies against dissemination of these genes to other bacteria of clinical importance.

Acknowledgement

The authors are highly thankful to Director of Research for providing the necessary facilities to carry out the research work.

Conflict of Interests

There is no conflict of interest.

Publisher Disclaimer

IJLR remains neutral concerning jurisdictional claims in published institutional affiliation.

References

1. Bansal, B., Gupta, D., Sharma, S., Shafi, T., Filia, G. (2019). Phenotypic antibiotic resistance pattern and presence of *mecA* in *Staphylococcus aureus* isolated from bovine mastitis. *International Journal of Livestock Research*, 9(9): 65-79. doi: 10.5455/ijlr.20180516043453
2. Berg, T., Firth, N., Apisiridej, S., Hettiaratchi, A., Leelaporn, A., Skurrayet, R.A. (1998). Complete nucleotide sequence of pSK41: evolution of staphylococcal conjugative multi-resistance plasmids. *Journal of Bacteriology*, 180: 4350-4359.
3. Brakstad, O.G., Aasbakk, K., Maeland, J.A. (1992) Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. *Journal of Clinical Microbiology*, 30(7): 1654-1660.
4. Cervinkova, D., Babak, V., Marosevic, D., Kubikova, I., Jaglic, Z. (2013). The role of the *qacA* gene in mediating resistance to quaternary ammonium compounds. *Microbial Drug Resistance*, 19(3): 160-167.
5. Conceição, T., Coelho, C., de Lencastre, H., Aires-de-Sousa, M. (2016). High prevalence of biocide resistance determinants in *Staphylococcus aureus* isolates from three African countries. *Antimicrobial Agents and Chemotherapy*, 60: 678-681. doi:10.1128/AAC.02140-15.
6. Costa, S.S., Viveiros, M., Amaral, L., Couto, I. (2013). Multidrug efflux pumps in *Staphylococcus aureus:* an update. *The open Microbiology Journal*, **7**: 59-71. doi: 10.2174/1874285801307010059
7. Couto, I., Costa, S.S., Viveiros, M., Martins, M., Amaral, L. (2008). Efflux-mediated response of *Staphylococcus aureus* exposed to ethidium bromide. *Journal of Antimicrobial Chemotherapy*, 62: 504-513. doi: 10.1093/jac/dkn217
8. Couto, N., Belas, A., Couto, I., Perreten, V., Pomb, C. (2015). Genetic relatedness, antimicrobial and biocide susceptibility comparative analysis of methicillin-resistant and susceptible *Staphylococcus pseudintermedius* from Portugal. *Microbial Drug Resistance*, 20(4): 364-371. doi: 10.1089/mdr.2013.0043

9. Ergun, Z., Cantekin, K., Gurturk, H., Solmaz, I., Ekin, H., Ozturk, D. (2017). Distribution of antiseptic resistance genes in Staphylococcus spp. from bovine mastitis Y. *Veterinarni Medicina*, 62(04): 200-203. doi: 10.17221/265/2015-VETMED
10. Hassan, K.A., Skurray, R.A., Brown, M.H. (2007). Active export proteins mediating drug resistance in staphylococci. *Journal of Molecular Microbiology and Biotechnology*, 12: 180-196. doi: 10.1159/000106673
11. Ho, C.M., Li, C.Y., Ho, M.W., Lin, C.Y., Liu, S.H., Lu, J.J. (2012). High rate of *qacA* and *qacB* positive methicillin-resistant *Staphylococcus aureus* isolates from chlorhexidine-impregnated catheter-related bloodstream infections. *Antimicrobial Agents and Chemotherapy*, 56(11): 5693-5697.
12. Horner, C., Mawer, D., Wilcox, M. (2012). Reduced susceptibility to chlorhexidine in staphylococci: is it increasing and does it matter? *Journal of Antimicrobial Chemotherapy*, 67: 2547-2559. doi: 10.1093/jac/dks284
13. Jaglic, Z., and Cervinkova, D. (2012). Genetic basis of resistance to quaternary ammonium compounds--the qac genes and their role: a review. *Veterinarni Medicina*, 57(6): 275-281. doi: 10.17221/6013-VETMED
14. Kaatz, G.W., McAleese, F., Seo, S.M. (2005). Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. *Antimicrobial Agents and Chemotherapy*, 49: 1857-1864. doi: 10.1128/AAC.49.5.1857-1864.2005
15. Kosmidis, C., Schindler, B.D., Jacinto, P.L., Patel, D., Bains, K., Seo, S.M., Kaatz, G.W. (2012). Expression of multidrug resistance efflux pump genes in clinical and environmental isolates of *Staphylococcus aureus*. *International Journal of Antimicrobial Agents*, 40(3): 204-209. doi: 10.1016/j.ijantimicag.2012.04.014
16. LaBreck, P.T., Rice, G.K., Paskey, A.C., Elassal, E.M., Cer, R.Z. Law, N.N., Schlett, C.D., Bennett, J.W., Millar, E.V., Ellis, M.W., Hamilton, T., Bishop-Lilly, K.A., and Merrell, D.S. (2018). Conjugative Transfer of a Novel Staphylococcal Plasmid Encoding the Biocide Resistance Gene, *qacA*. *Frontiers in Microbiology*, 9: 1-17. doi: 10.3389/fmicb.2018.02664
17. Mayer, S., Boos, M., Beyer, A., Fluit, A.C., Schmitz, F.J. (2001). Distribution of the antiseptic resistance genes *qacA*, *qacB* and *qacC* in 497 methicillin-resistant and susceptible European isolates of *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 47: 896-897. doi: 10.1093/jac/47.6.896
18. McGann, P., Kwak, Y.I., Summers, A., Cummings, J.F., Waterman, P.E., Lesho, E.P. (2011). Detection of *qacA/B* in clinical isolates of methicillin-resistant *Staphylococcus aureus* from a regional healthcare network in the eastern United States. *Infection Control and Hospital Epidemiology*, 32: 1116-1119. doi: 10.1136/bmj.327.7425.1177
19. Narui, K., Noguchi, N., Wakasugi, K., Sasatsu, M. (2002). Cloning and characterization of a novel chromosomal drug efflux gene in Staphylococcus aureus. *Biological and Pharmaceutical Bulletin*, 25: 1533-1536. doi: 10.1248/bpb.25.1533
20. National Mastitis Council (1999).. Laboratory Handbook on Bovine Mastitis. Revised ed. National Mastitis Council, Verona: WI.
21. Noguchi, N., Suwa, J., Narui, K., Sasatsu, M., Ito, T., Hiramatsu, K., Song, J.H. (2005). Susceptibilities to antiseptic agents and distribution of antiseptic-resistance genes *qacA/B* and smr of methicillin-resistant Staphylococcus aureus isolated in Asia during 1998 and 1999. *Journal of Medical Microbiology,* 54: 557-565.
22. Paulsen, I.T., Brown, M.H., Dunstan, S.J., Skurray, R.A. (1995). Molecular characterization of the staphylococcal multidrug resistance export protein *QacC*. *Journal of Bacteriology*, 177: 2827-2833. doi: 10.1128/jb.177.10.2827-2833.1995
23. Senn, L., Clerc, O., Zanetti, G., Basset, P., Prod'hom, G., Gordon, N.C., Sheppard, A.E., Crook, D.W. (2016). The stealthy superbug: the role of asymptomatic enteric carriage in maintaining a long-term hospital outbreak of ST228 methicillin-resistant *Staphylococcus aureus*. *MBio,* 2016; 7(1): e02039-15. doi:10.1128/mBio.02039-15
24. Shrivastava, N., Shrivastav, A. (2019). MRSA in Dairy Cattle: A Review. International Journal of Livestock Research, 9(11): 38-55. doi: 10.5455/ijlr.20190611070925
25. Sidhu, M.S., Heir, E., Leegaard, T., Wiger, K., Holck, A. (2002). Frequency of disinfectant resistance genes and genetic linkage with beta-lactamase transposon Tn552 among clinical staphylococci. *Antimicrobial Agents and Chemotherapy,* 46: 2797-2803.
26. Sierra, J.M., Ruiz, J., Jimenez De Anta, M.T., Vila, J. (2000). Prevalence of two different genes encoding *NorA* in 23 clinical strains of *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 46: 145-146.
27. Strommenger, B., Kettlitz, C., Werner, G., Witte, W. (2003). Multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*. *Journal of Clinical Microbiology*, 41(9): 4089-4094.

28. Thomas, L., Maillard, J.Y., Lambert, R.J.W., Russell, A.D. (2000). Development of resistance to chlorhexidine di acetate in *Pseudomonas aeruginosa* and the effect of a ‘residual’ concentration. *Journal of Hospital Infection*, 46: 297-303. doi: 10.1053/jhin.2000.0851
29. Ucuncu, M. (2015). Dairy Products and Technologies (in Turkish). Meta Press. Bornova, Izmir. 94-96.
30. Wang, C., Cai, P., Zhan, Q., Mi, Z., Huang, Z., Chen, G. (2008b). Distribution of antiseptic-resistance genes *qacA/B* in clinical isolates of methicillin resistant *Staphylococcus aureus* in China. *Journal of Hospital Infection*, 69: 393-394.
31. Wang, J.T., Sheng, W.H., Wang, J.L., Chen, D., Chen, M.L., Chen, Y.C., Chang, S.C. (2008a). Longitudinal analysis of chlorhexidine susceptibilities of nosocomial methicillin-resistant *Staphylococcus aureus* isolates at a teaching hospital in Taiwan. *Journal of Antimicrobial Chemotherapy*, 62: 514-517.
32. Zhang, M., O’Donoghue, M.M., Ito, T., Hiramatsu, K., Boost, M.V. (2011). Prevalence of antiseptic-resistance genes in *Staphylococcus aureus* and coagulase-negative *Staphylococci* colonising nurses and the general population in Hong Kong. *Journal of Hospital Infection*, 78: 113-117. doi: 10.1016/j.jhin.2011.02.018

****