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Methicillin-Resistant Staphylococci in Mastitic Animals in Egypt

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Abstract: Twenty six staphylococci strains were isolated from 40 milk samples with aprevalence 11(16) 68.75% and 15(24) 62.5 % of clinical and sub-clinical mastitis cases respectively. The samples were collected from (cows, buffaloes and goats). All the isolates were fully identified phenotypically to coagulase positive and negative staphylococci. Antibiotic sensitivity test was carried out by using (10) antibiotic disks (Oxoid) *in vitro* against (26) staphylococci strains. Resistance against pencillinG, oxacillin, cefoxitine, erythromycin, gentamycin, tetracycline and amickacin showed with an incidence 97.00%, resistance against ciprofloxacin with an incidence 92%. sulph/trimetho and amoxy/fluclox with an incidence 94. %.According to PCR results on (10) staphylococci strains, all of them carried 16SrRNA at 228bp. Three strains carried *nuc* gene at 279bp and (5) strains carried *mecA* gene at 147bp.

Key words: MRSA · Coagulase Positive · Coagulase Negative Staphylococci And PCR

INTRODUCTION

Staphylococcus aureus represents an important etiologic agent Causingmastitis in cows, goats and sheep [1]. On the other side, Coagulase-negative staphylococci (CNS) are considered emerging agents of subclinical or mild clinical mastitis [2], Multi-drug resistance (MDR) *S. aureus* and CNS were isolated from mastitic cases [3]. MRSA disseminates intra-mammary that often produces incurable severe intra-herd infections [3].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen in human medicine [4].

MRSA strains acquired gene gives them resistance to methicillin and essentially all other beta-lactam antibiotics (multi-drug resistant) such as aminoglycosides, macrolides, lincosamides, streptogramins, tetracyclines, which are often used in the treatment of mastitis [5]. MRSA strains show pathogenic and epidemiologic characteristics via mutation, clonal evolution and horizontal gene transfer [6, 7]. These evolutionary processes enhance pathogenicity and antimicrobial-resistant properties of *S. aureus* strains [8].

Resistance to methicillin (and β-lactams) is associated with the *mecA* gene, which codes for penicillin-binding protein 2a (PBP2a), a cell wall synthetic protein with low affinity in binding to β-lactams [9].

The mec genes are embedded in large mobile elements called staphylococcal cassette-chromosome-mec (SCCmec) [10], Methicillin-resistant coagulase-negative staphylococci (MR-CoNS) generally carry a vast variety of SCCmec elements and are currently considered to be the likely reservoir of the different types of *mecA* gene in MRSA [11].

The aim of the study is isolation and identification of methicillin-resistant staphylococci strains either coagulase positive or negative through phenotypic and genotypic characters from mastitic farm animals (cows, buffaloes and goats) in Egypt.

MATERIALS AND METHODS

Sample Collection: A total of (40) milk samples were collected from cattle (no=20), buffaloes (no=15) and goats (no=5). All samples were collected in clean and sterile tubes. Milk samples were examined by California Mastitis Test (CMT), then transported to the laboratory on ice packs without delay.

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Table 1: primers sequencesfor the *Staphylococus* genus (16SrRNA), thermostable nuclease gene for S. aureus (*nuc*) and methicillin resistance gene (*mecA*) and product size

Gene	Primer sequence	Product size	Reference
16SrRNA	F.5`GTAGGTGGCAAGCGTTAT3`		
	R.5`CGCACATCAGCGTCAG3`	228bp	Monday and Bohach. 1999[17].
nuc	F.5`GCGATTGATGGTGATACGGTT3`		
	R.5`AGCCAAGCCTTGACGAACTAAAGC3`	279 bp	Brakstad*et al*., 1992[18].
mecA	F.5`GTGAAGATATACCAAGTGATT3`		
	R.5`ATGCGCTATAGATTGAAAGGAT3`	147 bp	Zhang *et al.*,2005[19].

Isolation and Identification of Staphylococci: About 10 µL of collected milk samples was spread over mannitol salt and sheep blood agar plates then incubated at 37°C for 24 h. Suspected colonies were examined by Gram's stain according to Cruickshank *et al.*, [12]. Biochemical identification was carried out according to Collee *et al.* [13] and CDC [14] that including: catalase, coagulase, gelatin liquefaction and sugar fermentation for glucose, maltose, lactose and sucrose.

Antibiotic Sensitivity Test: Twenty six Staphylococci strains were examined *in vitro* against (10) different antibiotics. It was carried out by using agar diffusion antibiotic sensitivity according to Beaney *et al*., [15]. Interpretation was carried out according to NCCLS, [16]. Antibiotic discs were obtained from Oxoid which including: penicillin-G (10 units), erythromycin (15 µg/ml), gentamicin (10 µg/ml), ciprofloxacin (5 µg/ml), tetracycline (30 µg/ml), sulpha/ trimetho (23.75+1.25 µg/ml), amikacin (30µg/ml), amoxy/fluclox (25 µg/ml) and oxacillin (1µg/ml) and cefoxitine (30 µg/ml).

DNA Extraction and PCR Amplification: Genomic DNA of staphylococci strains were extracted by using an extraction kit (QIA amp mini kit,Qiagen). Specific oligonucleotide primers for the [*Staphylococus* genus (16SrRNA), thermostable nuclease gene for *S.aureus* (*nuc*) and methicillin resistance gene (*mecA*)] genes were used. The amplification conditions included initial denaturation step at 94 °C for 4 min and 35 cycles of denaturation at 94 °C for 60 sec, primer annealing at 55 °C for 60sec, extension at 72 °C for 60 s and final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis through 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed. Primers sequences and amplified product size were showed in table (1).

RESULTS

All milk samples were collected and classified as clinical or subclinical mastitic cases according to the results of California mastitis test. A total of (26) staphylococci strains isolated were from (40) mastitic milk samples from cows, buffaloes and goats.The suspected colonies were circular, smooth and glistening. Gram's stain revealed Gram positive, non-spore forming cocci, arranged in grapes or irregular clusters. The results of biochemical tests proved that suspected colonies to be *staphylococci* spp.

The numbers of samples and type of mastitis were illustrated in Table (2).

Antibiotic sensitivity test was carried out using (10) antibiotic disks (Oxoid) *in vitro* against (26) Staphylococci strains showed resistance against pencillin–G, oxacillin, cefoxitine, erythromycin, gentamycin, tetracycline and amickacin with an incidence 97%, resistance against ciprofloxacin with an incidence 92%. sulph/trimethoandamoxy/fluclox with an prevalence 94.%.

Table 2: Number of samples and type of mastitis cases

		Type of mastitis	
Host	No. of samples	Clinical	Subclinical
Cows	20	8	12
Buffalo	15	6	9
Goats	5	2	3
Total	40	16	24

Coagulase test was used to identify the isolates to coagulase positive (CPS) and CNS as showed in table (3)

Table 3: Number and incidence of coagulase positive (CPS) and coagulase negative staphylococci (CNS)

	No.% (CPS)		No. % (CNS)	
Host	Clinical	Sub-clinical	Clinical	Sub-clinical
Cow	3(8)37.5%	3(12)25%	2 (8)25%	5(12)41.66%
Buffalo	1(6)16%	3(9)33.33%	2(6)33.33%	2(9)22.22%
Goat	2(2)100%	- 0, 0%	1(2)50%	2(3)66.66%
Total *Staphylococci* strains No. and %				
Clinical				Sub-clinical
11 (16) 68.75%				15(24) 62.5%

Table 4: Relationship between (CPS) and (CNS) and 16SrRNA, *nuc*and *mecA* genes

Strain host	CPS	CNS	16SrRNA	*nuc*	*mecA*
Goat	+	-	+	+	+
Goat	-	+	+	-	-
Buffalo	-	+	+	-	-
Buffalo	+	-	+	+	-
Buffalo	+	-	+	-	+
Cow	-	+	+	-	-
Cow	+	-	+	+	+
Cow	+	-	+	-	+
Cow	+	-	+	-	-
Cow	+	-	+	-	+

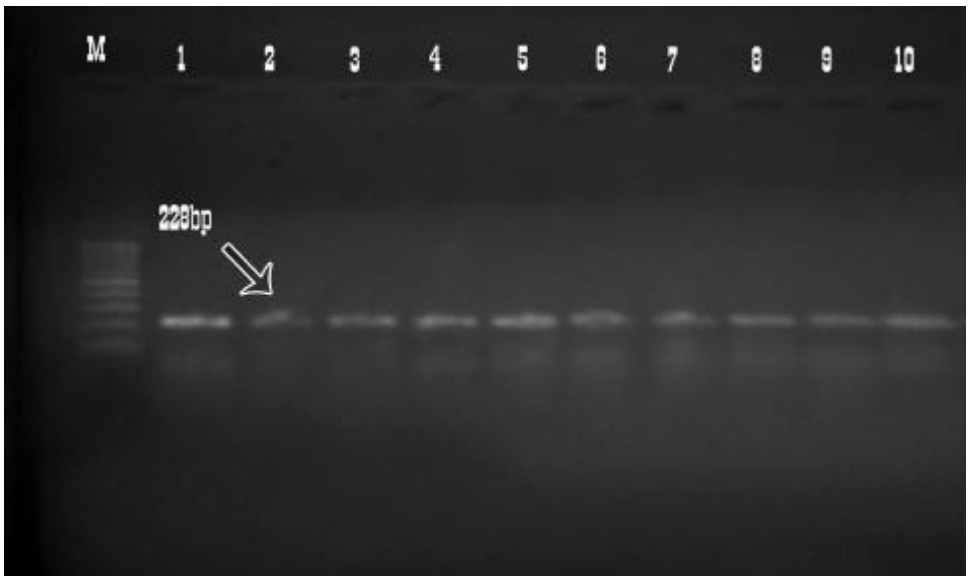

Photo 1: Amplified PCR product of (16SrRNA) gene at (228 bp). Lane M: 100bp ladder, Lane 1-10 positive to *genus staphylococci*.

Atotal of (10) staphylococci strains which showed 100% resistance to the previous antibiotics were chosen for PCR. Among (10) staphylococci strains, (3) strains (cows, buffaloes and goats) are (CNS) and the rest of strains are (CPS).The result of detection to (16SrRNA, *nuc* and *mecA*) genes and their relationship with CPS and CNS were illustratedin table (4).

DISCUSSION

Mastitis faces dairy production industry. Economic significances of the mastitis are related to the reduced milk quantity and quality, veterinary expenses and milk loss due to the antibiotic withdrawal time post treatment [20].

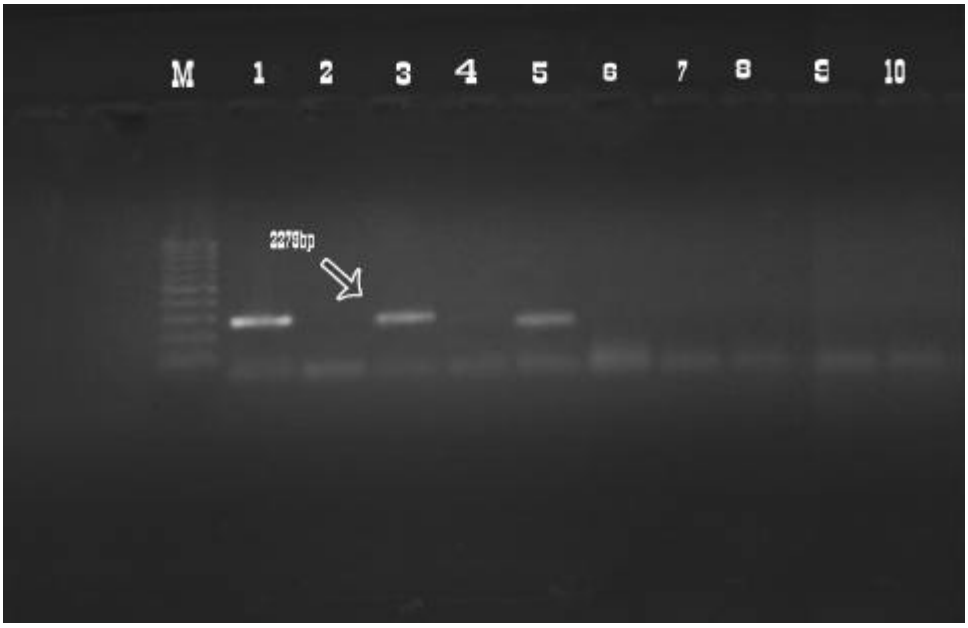

Photo 2: Amplified PCR product of (*nuc*) gene at (279bp). Lane M: 100bp ladder, Lane 1,3,5 positive to *S.aureus*.

Photo 3: Amplified PCR product of (*mecA*) gene at (147bp). Lane M: 100bp ladder, Lane 1, 2, 5,6,10 positive to MRSA.

Staphylococci are common etiological agents of mastitis [21]. In our study, 26 staphylococci strains [11 (16) 68.75% and 15 (24) 62.5%] were isolated from clinical and sub-clinical mastitis milk samples respectively, this isolation prevalence nearly agreed with [22] Asfour and Darwishwho isolated Staphylococci with prevalence of 52% and 67% from clinical and sub-clinical bovine mastitis milk samples respectively.

The number and prevalence of CPS strains in clinical and subclinical mastitis cases in cows were 37.5 and 25% while CNS strains in clinical and subclinical mastitis cases were 25 and 41.6% respectively. In buffaloes, CPS strains were 16 and 33.33% and CNS strains were 33.33 and 22.22% in clinical and sub-clinical mastitis cases respectively.

In goats, CPS strains inclinical and subclinical mastitis were 100 and 0 %, while CNS strains were 50 and 66.66% in clinical and subclinical mastitis cases respectively. According to the previous results, the incidence of isolation of CNS is higher than CPS in sub-clinical mastitis cases of cows' and goats' milk, these result was in harmony with Taponen and Pyorala [2] who stated that CNS became the most common bovine mastitis isolates in many countries and are regarded as emerging mastitis pathogens. CNS occasionally causes sub-clinical or clinical mastitis [23].

In addition it elevates somatic cell count in infected quarters [24], hurts udder tissue, decreases milk quality and quantity [25] so identification of CNS species is essential to determine their pathogenicity and to develop management practices to prevent mastitis.

The results of antibiotics sensitivity showed that staphylococci strains were resistant to pencillin-G, oxacillin, cefoxitine, erythromycin, gentamycin, tetracycline and amickacin with 97.00%; ciprofloxacin with 92%; and sulph/trimetho and amoxy/fluclox with 94%. This finding was agreed with Abd El-Moez *et al*. [26] who reported that staphylococci strains resistance against cefotaxime, sulph/trimetho and amoxy/fluclox was 94.40%. Resistance against pencillin–G, erythromycin, gentamycin, tetracycline and amickacin was 89.00%. Resistance against ciprofloxacin was 83.30%. These results mentioned that our study staphylococci strains are multidrug-resistantandconfirmed to be methicillin-resistant staphylococci (MRS), Karska-Wysocki *et al.,* [27] explained that MRSA is a multidrug-resistant microorganism. Attention with MRSA is very important because infections caused by methicillin-resistant staphylococci (MRS) are more difficult to treat and may pose a public health risk [28]. Some MRSA isolates from bovine mastitis are thought to be bovine and some of human origin, clonal transmission between farmers and dairy cows has been shown to occur, Haenni *et al*. [29].

Conventional identification of MRSA requires between 24-48 hours after sampling, in addition to that accreditation on identification of MRSA by antibiotic susceptibility testing alone isn't enough so rapid and sensitive method of identification as PCR for detection of *mec-A* gene that codes for penicillin-binding protein (PBP2a) or 2(PBP2) are recommended. The 16SrRNA gene was detected at (228bp) in all tested strains which confirmed to be in *Staphylococus* genus, this result agreed with Asfour and Darwish [22].Out of (3) strains of (10) were carried *nuc* gene at (279bp) which confirmed to be *Staphylococcus aureus*. Five isolates were confirmed to carry *mec* Ageneat (147bp) which responsible for β-lactams resistance.

In our study all staphylococci strains used in PCR were oxacillin resistsnt but (5) strains only carried *mec*A gene, this can explained by presence of other β-lactama resistance gene which is *bla*Z gene that can affect the expression of *mec*A gene [30]. This result also agreed with Olsen *et al.* [31] who stated that there are two genetic mechanisms are associated with resistance to the β-lactam class of antibiotics in *Staphylococcus* spp, the most important is production of β-lactamase that may bemediated by the *bla*Z gene and *mec* Agene. On the other side, disk diffusion test gives false positive and negative results due to variation in inoculums size and growth condition [32].

There are (2) strains carried both *nuc* and *mec A*genes, this finding stated that Methicillin-resistant *Staphylococcus aureus* (MRSA) were detected with an incidence (2/5) 40%,on the other side, (3) strains CPS other than *S. aureus* were carried *mec A*gene with an incidence (3/5) 60%.

This result was agreed with Park *et al.*[33] who stated that CPS other than *S. aureus* was classified as contagious pathogens infecting milk samples. While *S. aureus*is the most common coagulase-positive staphylococcus isolated in the clinical laboratory, *S. intermedius*, *S. delphini*, *S. schleiferi* sub sp. *coagulans*, *S. lutrae* and some strains of *S. hyicus* are also coagulase positive and have public health important so attention to CPS other than *S. aureus* must be considered.

CONCLUSIONS

Coagulase negative and coagulase positive staphylococci strains other than *S.aureus* are multidrug resistant representing a serious problem which need more attention as *S.aureus,* so further studies were recommended as detection of virulence genes and sequence analysis to methicillin resistance genes which able these strains to be multiple multidrug resistance.

REFERENCES

1. Mørk, T., T. Tollersrud, B. Kvitle, H.J. Jørgensen and S. Waage, 2005. Comparison of *Staphylococcus aureus* genotypes recovered from cases of bovine, ovine and caprine mastitis. J. Clin. Microbiol., 43: 3979-3984.
2. Taponen, S. and S. Pyorala, 2009. Coagulase-negative *Staphylococcias* cause of bovine mastitis—not so different from *Staphylococcus aureus*? Vet. Microbiol., 134: 29-36.
3. Kumar, R., B.R. Yadav and R.S. Singh, 2010. Genetic determinants of antibiotic resistance in *Staphylococcus aureus* isolates from milk of mastitic crossbred cattle. Curr. Microbiol., 60: 379-386.
4. Weese. J.S. and E. van Duijkeren, 2010. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseud intermedius* in veterinary medicine. Vet. Microbiol., 140: 418-429.

5. Wang, Y., C.M. Wu, L.M. Lu, G.W. Ren, X.Y. Cao and J.Z. Shen, 2008. Macrolide-lincosamide-resistant phenotypes and genotypes of *Staphylococcus aureus* isolated from bovine clinical mastitis. Vet. Microbiol., 130: 118-125.
6. Fitzgerald, J.R., D.E. Sturdevant, S.M. Mackie, S.R. Gill and J.M. Musser, 2001. Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of Methicillin–resistant strains and the toxic shock syndrome epidemic. Proc. Natl. Acad. Sci. USA., 98: 8821-8826.
7. Brody, T., A.S. Yavatkar, Y. Lin, J. Ross, A. Kuzin, M. Kundu, Y. Fann and W.F. Odenwald, 2008. Horizontal Gene Transfers Link a Human MRSA Pathogen to Contagious Bovine Mastitis Bacteria. PLoS ONE., 3: e3074.
8. Moon, J.S., A.R. Lee, H.M. Kang, E.S. Lee, Y.S. Joo, Y.H. Park, M.N. Kim and H.C. Koo, 2007. Antibiogram and coagulase diversity in staphylococcal enterotoxin-producing *Staphylococcus aureus* from bovine mastitis. J. Dairy Sci., 90: 1716-1724.
9. Katayama, Y., D.A. Robinson, M.C. Enright and H.F. Chambers, 2005. Genetic background affects stability of mecA in *Staphylococcus aureus*. J. Clin. Microbiol., 43: 2380-2383.
10. Katayama, Y., T. Ito and K. Hiramatsu, 2000. A new class of genetic element, *staphylococcus* cassette chromosome mec, encodes methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother., 44: 1549-1555.
11. Hanssen, A.M. and J.U. Sollid, 2007. Multiple staphylococcal cassette chromosomes and allelic variants of cassette chromosome recombinases in *Staphylococcus aureus* and coagulase-negative staphylococci from Norway. Antimicrob. Agents Chemother., 51: 1671-1677.
12. Cruickshank, R., J.P. Duguid, B.P. Marmion and R.H. Swain, Med. Micro, 1975. The Practice of Medical Microbiology. 12th Ed. vol., 11 Edinburgh, London and New York.
13. Collee, J.G., A.G. Fraser, B.P. Marmion and A. Simmons, 1996. Practical Medical Microbiology, 14thed.Churchill, Living Stone, NewYork.
14. Centers for Disease Control and Prevention [CDC], 2005. Health care-associated methicillin resistant *Staphylococcus aureus* (HA-MRSA) [online]. CDC; June. Available at: http://www.cdc.Beaney, N., N.G. Goodwin, R.V. Jones, R. Winter and G.R. Sippe, 1970. Antibiotic sensitivity testing. Med. J. Aust., 1: 483-484.
15. Beaney, N., N.G. Goodwin, R.V. Jones, R. Winter and G.R. Sippe, 1970. Antibiotic sensitivity testing. Med. J. Aust., 1: 483-484.
16. NCCLS, 2002. Performance standards for antimicrobial susceptibility testing. Informational supplement. M100-S12. Villanova (PA).
17. Monday, S.R. and G.A. Bohach, 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. J. Clin. Microbiol., 37: 3411-3414.
18. 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: 1654-1660.
19. Zhang, K., J.A. McClure, S.T. Elsayed, T. Louie and J.M. Conly, 2005. Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette chromosome mec types I toV in methicillin-resistant *staphylococcus aureus*. J. Clin. Microbiol., 43: 5026-5033.
20. Cunha, A.P., L.B.G. Silva, J.W. PinheiroJúnior, D.R. Silva, A.A. Oliveira, K.P.C. Silva and R.A. Mota, 2006. Perfil de sensibilidadeantimicrobiana de agentescontagiosos e ambientaisisolados de mastiteclínica e subclínica de búfalas. Arqs Inst. Biológico, São Paulo, 73(1): 17-21.
21. Wang, Y., C.M. Wu, L.M. Lu, G.W. Ren, X.Y. Cao and J.Z. Shen, 2008. Macrolide-lincosamide-resistant phenotypes and genotypes of *Staphylococcus aureus* isolated from bovine clinical mastitis. Vet. Microbiol., 130: 118-125.
22. Asfour, H.A.E. and S.F. Darwish, 2011. Phenotypic and Genotypic Detection of Both *mecA*- and *blaz*-Genes Mediated β- Lactam Resistance in Staphylococcus Strains Isolated from Mastitis. Global Veterinaria, 6: 39-50.
23. Resch, M., V. Nagel and C. Hertel, 2008. Antibiotic resistance of coagulase-negatives taphylococci associated with food and used in starter cultures. Int. J. Food Microbiol., 127: 99-104.
24. Kudinha, T. and C. Simango, 2002. Prevalence of coagulase–negative staphylococci in bovine mastitis in Zimbabwe. J.S. Afr. Vet. Assoc., 73: 62-65.
25. Quinn, P.J., B.K. Markey, M.E. Carter, W.J. Donnelly, F.C. Leonard and D. Maguire, 2002. Staphylococcus species.In Veterinary Microbiology and Microbial Disease.1edition. Edited by: Quinn PJ. USA: Blackwell Science Ltd; pp: 43-48.

26. Abd El-Moez, S.I., S.M. Dorgham and E. AbdEl-Aziz, 2011. Methicillin Resistant *Staphylococcus aureus*-Post surgical Infections in Egyptian Hospital.Life Science Journal, 8: 520-526.
27. Karska-Wysocki, B., M. Bazo and W. Smoragiewicz, 2010. Antibacterial activity of *Lactobacillus acidophilus* and *Lactobacillus casei* against methicillin-resistant *Staphylococcus aureus* (MRSA). Microbiol. Res., 165: 674-686.
28. Juhász-Kaszanyitzky, E., S. Jánosi, P. Somogyi, A. Dán, L. van der Graaf-van Bloois, E. van Duijkeren and J.A. Wagenaar, 2007. MRSA transmission between cows and humans. Emerg Infect Dis., 13: 630-632.
29. Haenni, M., L. Galofaro, C. Ponsin, M. Bes, F. Laurent and J.Y. Madec, 2011. Staphylococc albovine mastitis in France: enterotoxins, resistance and the human Geraldine methicillin-resistant *Staphylococcus aureus* clone. J. Antimicrob. Chemother., 66: 216-218.
30. Berger-Bächi, B. and S. Rohrer, 2002. Factors influencing methicillin resistance in staphylococci. Arch. Microbiol., 178: 165-171.
31. Olsen, J.E., H. Christensen and F.M. Aarestrup, 2006. Diversity and evolution of blaZ from *Staphylococcus aureus* and coagulase negative staphylococci. J. Antimicrob. Chemother., 57: 450-460.
32. Livermore, D.M., 1995. beta-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev., 8: 557-584.
33. Park, Y.K., H.C. Koo, S.H. Kim, S.Y. Hwang, W.K. Jung, J.M. Kim, S. Shim, R.T. Kim and Y.H. Park, 2007. The analysis of milk components and pathogenic bacteria isolated from bovine raw milk in Korea. J. Dairy Sci., 90: 5405-5414.