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THE RELATIONSHIP BETWEEN BIOTYPE, SEROTYPE, ANTIBIOTIC SUSCEPTIBILITY AND *COA* GENE POLYMORPHISM IN STAPHYLOCOCCUS AUREUS ISOLATED FROM BOVINE

BY

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دراسة العلاقة بين الانماط الحيوية والمصلية والحساسية الدوائية مع تعدد الاشكال الجينى للجين Coa فى جرثومة staphylococcus المعزولة من الابقار

بواسطة

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درست ٤٠ عزلة من انماط مختلفة من سلالات staphylococcus aureus المعزولة من الابقار. تم التحرى عن وجود جين coa بأستخدام تقنية RCR . وقد اظهر هذا الجين تعدد اشكال بشكل واضح وبأحجام مختلفة تراوحت بين ٥٠٠و ٦٠٠و٦٥٠و٨٠٠ و٨٥٠ زوج قاعدى .وقد تم دراسة العلاقة بين الانماط الجينية و الحيوية والمصلية لهذه الجرثومة. لقد وجد ان التواجد الاكثر للنمط المصلى IV فى النمط الحيويC . ولم يسجل تواجد للانماط المصلية I و II فى النمط الحيوي A . كما لم يسجل تواجد للنط مصلى V فى اى من العزلات.اما ما يخص العلاقة مع النمط الجينى فقد وجد ان العزلات من نمط الحيوى A و C لها نفس النمط الجينى بحجم ٥٠٠ و ٨٠٠ زوج قاعدى من الجين coa وتقع فى نفس الخط المصلى II وIV. اما عن دراسة العلاقة بين تواجد وحجم الجين coa مع المقاومة للاوكساسيلين والفانكومايسين فقد وجد ان ٥٠% منها كانت مقاومة بالكامل للاوكساسيلين ووجدت اعلى مقاومة عندما كان الجين بحجم ٨٥٠ زوج قاعدى يليه الجين بحجم ٥٠٠ زوج قاعدى . اما يخص المقاومة للفانكومايسين فوجدت المقاومة فى ٤٠ عزلة كانت المقاومة تامة فى ٥٠% علماً ان اعلى مقاومة كانت عندما كان الجين بحجم ٨٥٠ زوج قاعدى.

SUMMARY

Forty Staphylococcus aureus strains of different biotypes and serotypes, previously isolated from bovine, were used in this study. The presence of coagulase gen (coa) were investigated using PCR assay. coa gene shows polymorphism since it appear in 500, 600, 650, 800 and 850 bp. The relation between genotype, biotypes and serotypes of S. aureus were also investigated. The relation between biotype and serotype of S. aureus isolates revealed that the high occurrence of serotype IV was recorded in the biotype C. No serotype I or II were recorded in biotype A isolates. Serotype V not recorded in any strain. The relation between the biotype, genotype and serotype in the same forty S. aureus isolates, showed that the isolates from biotypes C and A, revealed that the same product of coa gene (500 and 800 bp) share in the same serotype, II and IV. The study of the relation between the coa product with oxacillin showed that the total resistance in genotypes to oxacillin was 50%. The high percentage of resistance was found in isolates with coa product 850 bp followed by 500 bp. The relations appeared between the coa product with vancomycine resistance in 40 bovine isolates were also recoded. The total resistance in genotypes to vancomycine was 50%. The high percentage of resistances was found in isolates with coa gene product 850 bp.

INTRODUCTION

Staphylococcus aureus acts as opportunistic pathogens and cause infections of urinary tract, respiratory tract, and intestinal tract (Olorunfemie et al., 2005). S. aureus is considered as pathogenic bacteria when appearing in suitable condition. It is able to infect any part of the body causing some diseases in human and animal that start with skin infection, end to food poisoning and brain abscesses with outbreak in the post operative wound infection (Kenneth, 2008). In the cows, it causes some important diseases for example mastitis (clinical and sub clinical) and respiratory tract infection, skin sepsis, Tic pyemia in lamb and contagious skin necrosis (Kinight, 1999). The phenotypical characterization of S. aureus biotypes establishes the presumptive origin of isolation (Ordonez et al., 2005). Biotype has been stated that biotypes S. aureus strains may give an indication of the origin contamination in food products as the biotype correlates well with the animal host (Lamperll et al., 2004).

S. aureus serotypng is epidemic indicator that depend on molecular immunity possess organism and ability of B-lymphocyte to produce antibody, this typing standing and stabile of epidemic indicator but it does not have properties that be handled to differently. Some serological method agglutination test, complement fixation tests, immunoelectrophresis, radioimmuno assay are also found. The coagulase protein is an important phenotypic determinant and accepted as a major virulence factor of S. aureus. Analysis of coagulase encoding S. aureus DNA coa gene has demonstrated variable sequences in the 3´ end coding region (Goh et al., 1992; Kawaguchiya et al., 2013). This region contains a polymorphism repeat region that can be used to differentiate S. aureus isolates. This characteristic has been used to type S. aureus isolates of human and bovine origin (Guler et al., 2005). The coa gene polymorphism was used for typing and differentiation of S. aureus strains isolated from bovine mastitic milk sample but using internal transcribed spacer-PCR proved to be a useful and inexpensive procedure for conducting epidemiological surveys of S. aureus (Ciftci et al., 2009; Karakulska et al., 2011; Silveira-Filh et al.,, 2014) .

MATERIALS AND METHODS

Bacterial Strain: Forty *S. aureus* previously isolated from bovine were used through this study.

Biotyping and serotyping: Biotyping of *S aureus* isolates were determined according to Isigidi *et al*., (1990). All isolates were biotyped by using pigment production, haemolysin, coagulation of bovine plasma and growth on crystal violate. All isolates were serotyped to I, II, III, IV, V, VI, VII, VIII according to coagulase serotyping method.

Antibiotic susceptibility test : This test was done according to method of Kirby and Bauer, (1966) using disc diffusion method.

PCR analysis: The *coa* gene was studied according to protocol of (Hookey *et al*., 1998). Oligonucleotide primers sequences used for PCR amplification of *coa* gene as follow:

Coa1 5´-ATA GAG ATG CTG GTA CAG G-3´.

Coa2 5´-GCT TCC GAT TGT TCG ATG C-3´.

RESULTS

Biotyping and Serotyping of S. aureus

The results of biotyping of forty *S. aureus* isolates revealed that 83.33% (34/40) were biotype C and 16.66% (6/40) were biotype A. There were significant differences ($P < 0.05$) between biotype C and A of *S. aureus* isolates. Coagulase enzyme used for serotyping of the same forty isolates. The results obtained showed that the serotype IV had the high occurrence (9 isolates) followed by VI and VII serotypes (6 isolates for each). There were significant differences ($P < 0.05$) between serotypes in bovine isolates. The relation between biotype and serotype of *S. aureus* isolates revealed that the high occurrence of serotype IV was recorded in the biotype C. No serotype I or II were recorded in biotype A isolates. Serotype V not recorded in any strain (Table 1).

Molecular genetics study results (PCR on *coa* gene)

The results of DNA amplification of *coa* gene in *S. aureus* isolates revealed that PCR products were found in 38 isolates at molecular size 500, 600, 650, 800, and 850 bp in percentage22.5%, 12.5%, 15%, 25%, and 25% Respectively. There were no significant differences ($p > 0.05$) between different *coa* gene products in bovine isolates (table 1). All isolates with single band except two isolates showed 500 & 600 bp bands (Fig. 1).

Relationship between biotypes, genotypes and serotypes

The relation between the biotype, genotype and serotype in the same forty *S. aureus* isolates, showed that the isolates from biotypes C and A, revealed that the same product of *coa* gene (500 and 800 bp) share in the same serotype, II and IV (Table 1).

Antibiotic susceptibility of S. aureus

The sensitivity results of forty isolates to thirteen antibiotics discs were studied. Results showed susceptibility to tobramycin 65%, ciprofloxacin 62.5%, chloramphenicol 60%, clindamycin 52.5%, oxacillin 52.5% and vancomycin 52.5%. The less susceptible results were showed to erythromycin, gentamycin, nitrofurantoin, streptomycin, tetracycline, and penicillin 45%-12.5%, as well as resistance to cloxacillin, that had significantly differences ($P < 0.01$) between different antibiotics to susceptibility of *S. aureus* isolates (Figure 2).

Relationship between biotypes, serotypes and ORSA and VRSA

The relationship between biotype A and C with ORSA and VRSA in *S. aureus* isolates revealed high resistance of biotype C to oxacillin and less

to vancomycin, while in the biotype A showed high resistance to vancomycin compared to oxacillin (Table 2).

The results also showed high resistance of serotypes to oxacillin were recorded in serotype IV and VΠ, while the high resistance to vancomycine recorded in the serotype VΠ also (Table 2).

Relationships between coa gene polymorphism and and resistance to oxacillin (ORSA)and vancomycine (VRSA):

The study of the relation between the *coa* product with oxacillin resistance in the 40 bovine isolates showed that the total resistance in genotypes to oxacillin was 50%. The high percentage of resistance was found in isolates with coa product 850 bp followed by 500 bp The relations appeared between the *coa* product with vancomycine resistance in 40 bovine isolates were also recoded. The total resistance in genotypes to vancomycine was 50%. The high percentage of resistances was found in isolates with *coa* gene product 850 bp (Table 3).

DISCUSSION

In this study the results showed the specific biotype C to bovine isolates predominant in bovine isolates. Bendahou et al., (2008) showed that the four biotypes A, B, C and unspecific of the S. aureus isolates from milk and milk production appeared bovine origin biotype C recorded and more dominant than the other biotypes. In the present study the bovine isolates showed high percentage of recorded in serotype IV, VII ,VI and Π. Many other studies on serotyping of animals isolates were carried out. Hata et al., (2006) tested 231 S. aureus isolates (161clinical mastitis and 70subclinical mastitis) collect from 214 farms. Moon et al., (2007) who found 4 main serotypes comprising I ,VIII , II and VII when used ten coagulase serotype patterns on S. aureus isolates. The isolates of S. aureus from raw milk sample for coagulase serotyping revealed, serotype Π , ΠI ,IV, VΠ, VΠI recorded while the serotype I, V, VI was not recoded (Pork et al., 2001). The serotypes were recorded similar in biotype A isolates were Π, IV, VI VΠ, VΠI, while the serotypes recorded similar in biotype C isolates were Π ΠI, IV VI. In the two aforesaid studies revealed the serotype recorded in the animals able to record in the human and vice versa. Coagulase serotyping method has been used successfully in the epidemiological investigation staphylococcal food poisoning, coagulase serotypes VII, accounted for about 70% of the total outbreak. Coagulase serotypes III, 12%, serotypes II, 11% and serotypes

VI, 3%, of coagulase responsible for food poisoning incidence (Shimizu et al., 2000).

The results of this study on coa gene revealed polymorphism phenomena in this gene resulted of different molecular weight 500 bp, 600 bp, 650 bp, 800 bp and 850 bp. Testing for multiple gene polymorphisms is more useful for local epidemiologic studies (Omer et al., 2014). In the bovine isolates the present study recorded 500 bp, 600 bp, 650 bp, 800 bp and 850 bp of coa gene from S. aureus which isolated from different tested samples. This region contains a polymorphism repeat region that can be used to differentiate S. aureus isolates. This characteristic has been used to type S. aureus isolates of human and bovine origin (Guler et al., 2005; Hooky et al., 1998). Other study showed that S. aureus isolates of bovine mastitic milk origin from herds at different locations were revealed three different types of coa gene products 400bp, 510bp, 600 bp, 680 bp, 700bp and 850 bp (Salasia et al., 2004; Maslankova et al., 2009; Coelho et al., 2009).

In this study in bovine isolates the total resistance to oxacillin and vancomycine were 50%. The antibiotics resistance pattern revealed resistance to oxacillin and vancomycin in 25% and 17% respectively (Coelho et al., 2009). In the study of (Ordonez et al., 2005), the percentage of resistance of S. aureus isolated from mastitis is 19%. The S. aureus isolated from different patients from clinical specimens the molecular typing of MRSA appeared 54 isolates with 64.28%, while MSSA recorded 30 isolates with 35.71%, by used restriction digestion of coa PCR product (Tiwari et al., 2008). Enterotoxin genes were more prevalent in MRSA than in MSSA and the MSSA strains were genetically more diverse than the MRSA strains (Lim et al., 2012).

The relationship between isolates origin with genotype and serotype, bovine biotypes C and A, were appeared the same product of coa gene (500 and 800 bp) share in the same serotype, II, IV. Six hundred nightly six isolates of S. aureus isolated from milk mastitis cows. The test of coa gene polymorphism showed ranging between 620-809 bp for each S. aureus. The serotype recoded serotype I 25.4% followed by VIII 17.8%, serotype II 13.9% and serotype VII 13.2% (Moon et al., 2007). The coagulase serotypes showed serotype I 1.46%, serotype II 64.30%, serotype III 5%, serotype IV 7.37, serotype V 3.68%, serotype VI 0.44%, serotype VII 13.27% and serotype VIII 1.91% while recorded non specific 2.5%. The coa gene polymorphism recoded different products 350 bp, 431 bp, 512 bp, 593 bp, 674 bp, 755 bp, 836 bp and 917 bp, in the isolates from nosocomal infection in human (Ishino et al.,

2007,). Amplification of the coagulase gene from 86 S. aureus strains isolates by specific primers showed 31 specimens contained 970 bp fragment, and 11 strains contained 730 bp fragment relevant to coa gene (coagulase) in PCR (Momtaz et al., 2011).

CONCLUSION

The relation between S. aureus biotype, genotype and serotype showed that the isolates from biotypes C and A have the same product of coa gene (500 and 800 bp) and found in the same serotype. coa gene shows polymorphism since it appear in different size. Fifty percent of isolates having coa gene showed resistance to oxacillin and vancomycine.

Table (1): Relationship between biotype, *coa* gene product and serotype in the bovine isolates

***Coa* bp**	***Iisolates No (%)***	***Biotype C***	**Serotype**	**Biotype A**	**Serotype**
500	9(22.5)	7	I, (II) 2, (III) 2, IV, VI	2	II, IV
600	5 (12.5)	5	(VI) 2, II, III, IV.	0	/
650	6(15)	4	II, VI, (IV)2	2	VII, VIII
800	10(25)	8	I, II,(III)2, VI, IV, (VII)2	2	II, IV
850	10(25)	10	(I)2 ,(IV) 2, VI, (VII)3, (VIII)2	/	/
Total	40	34	I=4, II=5, III=5, IV=7, VI=6, VII=5, VIII=2	6	II=2, IV=2, VII=1, VIII=1

Table (2): Relationships between serotypes and resistance to ORSA and VRSA of *S. aureus*

Serotype	Milk	Vaginal swabs	Nasal Swabs	Total	(%)VRSA	(%)ORSA
I	1	1	2	4	2 (50)	2 (50)
II	5	1	0	6	3 (50)	1 (33.3)
III	3	1	0	4	1 (25)	1 (25)
IV	6	2	2	10	7	7(70)
V	0	0	0	0	0(0.0)	0(0.0)
VI	3	2	1	6	3(50)	3(50)
VII	2	2	3	7	4(57.1)	4(57.1)
VIII	0	1	2	3	1(33.3)	1(33.3)
Total	20	10	10	40	18(45)	19(47.5)
Biotype						
A	4	2	-	6	3(50)	2(33.33)
C	16	8	10	34	16(47)	17(50)
Total	20	10	10	40	19(47.5)	19(47.5)

Table (3): Relationships between ORSA, VRSA and *coa* gene polymorphism of *S. aureus* isolates

Sample/No.	Resistance	500 bp	600 Bp	650 bp	800 bp	850 bp	% Resistance
Milk /20	ORSA VRSA	6 6	0 0	0 1	4 5	0 0	50 55
Vaginal swabs/10	ORSA VRSA	0 0	1 1	2 1	0 0	0 0	30 20
Nasal swabs/10	ORSA VRSA	0 0	0 0	0 0	0 0	7 6	70 60
Total / (%)	ORSA VRSA	6(15) 6(15)	1(2.5) 1(2.5)	2(5) 2(5)	4(10) 5(12.5)	7(17.5) 6(15)	50 50

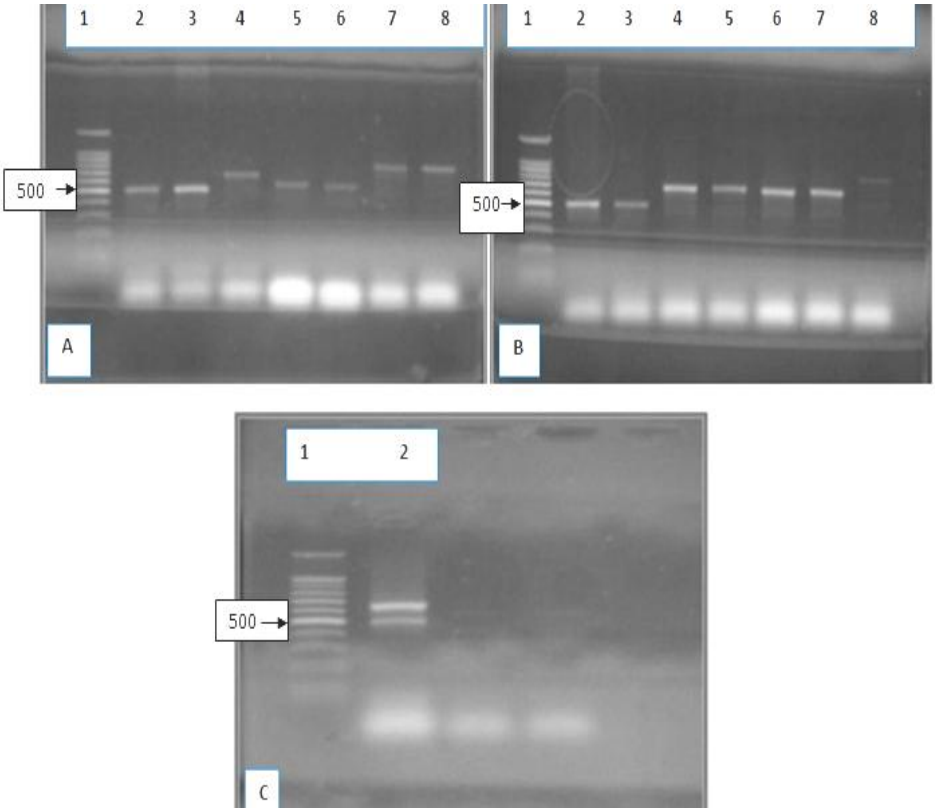

Fig. (1) Electrophoresis of *coa* gene PCR products in 1% agarose. A; Lane 1= 100bp DNA ladder, 2&3= 500bp, 4= 650bp, 5&6= 600bp, 7&8 =850bp. B; Lane 1= 100bp DNA ladder, 2&3= 500bp, 4-7= 650bp, 8= 800bp. C; Lane 1= 100bp DNA ladder, 2= double band of 500 & 650 bp.

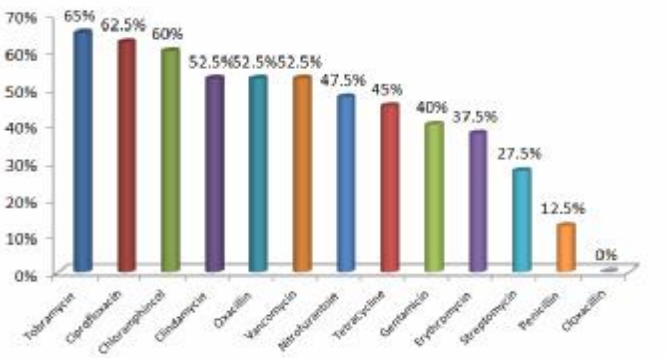

Fig. 2: The percentage of antibiotic susceptibility of Staphylococcus aureus agaivst different antibiotics

REFERENCES

Bendahou A, M Lebbadi L Ennanei, Z Essadqzui and M Abid, 2008. Characterization of Staphylococcus species isolated from raw milk and milk products (Iben and jben) in North Morocco. J. Infect. Developing Countries. 2(3):218-225.

Ciftci A, EE Onuk, A Findik, T Yildirim, MU Sogut, 2009. Molecular typing of Staphylococcus aureus strains from ovine mastitis by pulsed-field gel electrophoresis and polymerase chain reaction based on coagulase and protein A gene polymorphisms. J Vet Diagn Invest. 21(6):849-853.

Coelho S, E Reinoso, I Pereira, L Soares, M Demo, C Bogni and M Souza, 2009. Virulence factors and antimicrobial resistance of Staphylococcus isolates from bovine mastitis in Riode Janeiro. Pesq. Vet. Bras. 29(5):369-374.

Goh SH, SK Byrne, JL Zhang and AW Chow, 1992. Molecular typing of Staphylococcus aureus on the basis of coagulase gene polymorphisms. J. Clin. Microbiol. 30:1642-1645 .

Güler L, U Ok, K Gündüz, Y Gülcü and HH Hadimli, 2005. Antimicrobial susceptibility and coagulase gene typing of Staphylococcus aureus isolated from bovine clinical mastitis cases in Turkey. J. Dairy Sci. 88: 3149–3154.

Hata E, K Katsuda, H Kobayanhi, T ogawa, T Endo and M Eguch, 2006. Characteristic and epidemiological genotyping of Staphylococcus aureus isolates from bovine mastitic milk in hakkaido, Japan. Japan J. Vet. Med. Sci. 68(2):165-170.

Hookey JV, JF Richardson and BD Cookson, 1998. Molecular typing of Staphylococcus aureus based on PCR restriction fragment length polymorphism and DNA sequence analysis of the coagulase gene. J. Clin. Microbiol. 36:1083–1089.

Ishino K, N Tsuchizaki, J Ishikawa and K Hotta, 2007. Usefuless of PCR-Restriction Fragment Length Polymorphism Typing of the Coagulase Gene To Discriminate Arbekacin-Resistant Methicillin-Resistant Staphylococcus aureus Strains. J. Clin. Microbiol. 45(2):607-609.

Isigidi BK, LA Dveriese, C Godared and J Van Hoof, 1990. Characteristics of Staphylococcus aureus associated with meat products and meat workers. Appl. Microbiol, 11:145-147.

Karakulska J, A Pobucewicz , P Nawrotek , M Muszyńska, AJ Furowicz, D Czernomysy-Furowicz, 2011. Molecular typing of Staphylococcus aureus based on PCR-RFLP of coa gene and RAPD analysis. Pol J Vet Sci. 14(2):285-286.

Kawaguchiya M, N Urushibara, S Ghosh, O Kuwahara, S Morimoto, M Ito, K Kudo, N Kobayashi, 2013. Genetic diversity of emerging Panton-Valentine leukocidine/arginine catabolic mobile element (ACME)-positive ST8 SCCmec-IVa meticillin-resistant Staphylococcus aureus (MRSA) strains and ACME-positive CC5 (ST5/ST764) MRSA strains in Northern Japan. J Med Microbiol. 62(Pt 12):1852-1863

Kenneth T, 2008. Staphylococcus aureus Text book of Bacteriology. University of Wisconsin-Medison. Department of bacteriology.

Kinight C, 1999. Oxytocin : analternative to antibiotic for treating mastitis, Hannuch Research Insitute, Year book.

Kirby WM and AW Bauer, 1966. Antibiotic susceptibility testing by a standardized single disc method .The American. J. Clin. Path. 45 (4): 493-496.

Lamperll, L.; Villard, L.; Chamba, F.; Bewier, E.; Borges, E. Maurin, F.; Mazerolles, G.; Noel, Y. and Khudaier, B. Y. Abbas, B. A. and Khudaier, A. M. (2013). Detection of Methicillin Resistant Staphylococcus aureus Isolated from Human and Animals in Basrah Province / Iraq**.** MRVSA 2(3), 12-21.

Lim KT, CC Yeo, Z Suhaili, KL Thong, 2012 **.**Comparison of methicillin-resistant and methicillin-sensitive Staphylococcus aureus strains isolated from a tertiary hospital in Terengganu, Malaysia. Jpn J Infect Dis. 65(6):502-509.

Maslankova J, I Pilipcincova and L Tkacikova, 2009. Phen- and Genotyping Staphylococcus aureus isolates of sheep origin. ACTA. VET. BRNO. 78:345-352.

Momtaz H, E Tajbakhsh, E Rahimi and M Momeni, 2011. Coagulase gene polymorphism of Staphylococcus aureus isolated from clinical and sub-clinical bovine mastitis in Isfahan and Chaharmahal va Bakhtiari provinces of Iran. Comp Clin Path. 20(5):519-522.

Moon JS, AR Lee, HM Kang, ES Lee, YS Joo, YH Park, MN Kim and HC Koo, 2007. Antibiogram and coagulase Diversity in Staphylococcal Enterotoxin-producing Staphylococcus aureus from Bovine Mastitis. J. Dairy. Sci. 90:1716-1724 .

Olorunfemi OB, AA Onasanya and FC Adetuyi, 2005. Genetic variation and relationship in Staphylococcus aureus isolates from human and food samples using random amplified polymorphic DNAs. African Journal of Biotechnology. 4(7):611-614.

Omar NY, HA Ali, RA Harfoush and EH ElKhayat, 2014. Molecular typing of methicillin resistant staphylococcus aureus clinical isolates on the basis of protein A and coagulase gene polymorphisms. Int J Microbiol. 2014:650328.

Ordonez VV, MUA Fresan, SL Bernabe, MT Rojas and JS Oaxaca, 2005. Human and bovine Staphylococcus aureus biotypes associated with haemolisin production and resistance to Oxacillin in cows with subclinical mastitis in family dairy farms. J. ISAH. Warsaw. Poland. 1:330-333.

Pork JK, JH Lim, YD Sea, NY Kin, DY Lim, SJ Yoon, JS Choi and HB Koh, 2001. Studies on the enterotoxin production and coagulase serotyping of Staphylococcus aureus isolates from cows in Chonnom province Korean. J. Vet. Serv. 23(4):313-320.

Salasia SIO, Z Khusnan, C Lammer and M Zsch,O,ck, 2004. Comparetive studies on phenol-and genotypic properties of staphylococcus aureus isolated from bovine sub-cilincal mastitis in central Java in Indonesia Hesse in Germany. J. Vet. Sci.5:103-109 .

Shimizu A, M Fujita, A Igarashi, A Sasaki and J Kawano, 2000. Characterization of Staphylococcus aureus coagulase type VIII isolates from Staphylococcal food poisoning outbreak (1980-1995) in Tokyo-Japan, by pulsed-filed Gel electrophoresis. J. Clin. Microbiol. 38:3746-3749.

Silveira-Filho VM, IS Luz, AP Campos, WM Silva, MP Barros, ES Medeiros, MF Freitas, RA Mota, MJ Sena, TC Leal-Balbino, 2014. Antibiotic resistance and molecular analysis of Staphylococcus aureus isolated from cow's milk and dairy products in northeast Brazil. J Food Prot. 77(4):583-591.

Tiwari HK, D Sapkota, A Gaur, JP Mathuria, A Singh and MR Seu, 2008. Molecular typing of clinical Staphylococcus aureus isolates from Northern India using coagulase gene PCR-RFLP. Southeast Asian J. Trop. Med. Public Health. 39(3): 467-473.