

Studies on Virulence Genes of *Staphylococcus Aureus* Isolated from Mastitic Cows

A.A.Abd El-Tawab¹, A.M.Ammar², F.I.Hofy¹, S.R.Mohamed³, H.S.Abubakr³

¹Bacteriology, Immunology and Mycology Dept., Faculty of Veterinary Medicine, Benha Univ., Benha, Egypt

²Bacteriology, Immunology and Mycology Dept., Faculty of Veterinary Medicine, Benha Univ., Benha, Egypt

³Animal Health Research Institute(Dokki branch)

E-Mail: halahhableen@gmail.com

Abstract

Staphylococcus aureus is the most frequently bacterial pathogen causing clinical and subclinical mastitis in cattle. In this research the mastitic cases (103) were classified into clinical and subclinical cases (47,56) respectively.

All milk samples collected from infected cows were subjected to bacteriological examination and molecular characterization of some *Staphylococcus aureus* isolates. *Staphylococcus aureus* was isolated from clinical and subclinical mastitic cows in an incidence of (50%,17%) respectively.

The application of multiplex PCR on some *Staph.aureus* isolates (8) was effectively in detection of *Coa*, *SpA*, *TST*, *Hlg*, *CLF*, *nuc* genes by amplification at a single amplicon at (630bp, 226 bp, 326bp, 937bp, 638bp, 395bp respectively).

Keywords: *S.aureus*, cows, mastitis, Pcr, virulence genes.

1. Introduction

Milk is considered as an excellent medium for growing of many microorganisms. Milk can be contaminated with several bacteria during milking process from the milking personnel, utensils used for milking [1].

Mastitis is recognized as the most important dairy herd problems worldwide. Economic losses of mastitis include decrease in milk quantity and quality and high cost treatment. *S.aureus* is one of the most common etiological pathogens, causing intramammary infections in dairy herds leading to serve economic losses in worldwide industry [2].

The main reservoir of *S.aureus* seems to be the infected quarter, and transmission between cows usually occurs during milking. *S.aureus* produces a spectrum of extra cellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism [3] while TST-1 is a superantigenic exotoxin that causes toxic shock syndrome [4].

The *coa* gene is one of the most important virulence factors for *S.aureus*. Expression of this gene is thought to enhance bacterial growth and promote infection in the face of the host defense mechanisms, such as phagocytosis [5].

Pathogenesis of mastitis may be caused by extracellular toxins, enzymes and surface antigens [6]. Coagulase gene of *S. aureus* is considered an important virulence factor. Amplification of *S. aureus* coagulase gene (*coa*) has been recommended as an accurate method for identification of virulent strains of *S. aureus* [7]. Sequencing of the coagulase gene shows great diversity in *S. aureus* population [8].

This study aimed to throw the light on the incidence of *staphylococcus aureus* in clinical and subclinical mastitis, the genotypic characterization of some *S. aureus* strains isolated from dairy cows

suffering from mastitis and provided an overview on the distribution of virulence determinants of these *S. aureus* strains which contribute in bovine mastitis problem in the Egyptian farms.

2. Material & methods

1. Samples

A total of 412 milk samples were collected from clinically and sub clinically infected cases the samples were transferred in ice box directly with an hour to the laboratory with a minimum delay to be bacteriologically examined [9].

2. Bacteriological examination

All samples were inoculated onto blood agar base (Merck) supplemented with 5% defibrinated sheep blood and mannitol salt agar plates and incubated aerobically at 37°C for 24h. Suspected colonies were picked up for purification and subjected for identification microscopically and biochemically according to [10,9,11,12] isolates were identified by conventional methods, including Gram staining, colony morphology, hemolysis test, catalase, coagulase and anaerobic fermentation of mannitol [13].

3. Detection of virulence genes of *Staphylococcus aureus* by PCR

Primers for detection of 6 virulence genes of *Staphylococcus aureus*, these genes were *Spa*, *hlyA*, *coa*, *tst*, *nuc* and *clfA*. It was applied on 8 random isolates of *Staphylococcus aureus* following QIAamp® DNA Mini kit instructions (catalogue no. M501DP100), Emerald Amp GT PCR mastermix (Takara) with code NO. rr310A and agarose gel electrophoreses [14] sequence of primer used are illustrated in Table (1)

3. Results

Detection of virulence genes of *S.aureus* isolated from mastitic cattle

spa gene (protein A) amplified at 226bp were 5 positive, 3 negative in an incidence of (62.2%) and (37.5%) respectively as shown in Fig (1)

Nuc gene (Thermonuclease)amplified at 395bp were 3 positive and 5 negative in an incidence of (37.5%) and (62.2%) respectively also TST gene (Toxic shock syndrome gene) amplified at 326pb were 2 positive and 6 negative as (25%) and (75%) respectively as shown in Fig (2),Coa gene (Coagulase gene) amplified at 630 bp were 4 positive , 4 negative as (50%) and (50%) in Fig (3)

CLF gene (clumping factor) amplified at 638bp where 3 were positive 5 negative in an incidence of (37.7%) and (62.6%) respectively as shown in Fig (4) also HI gene hemolysin gene amplified at 937 bp where 4 were positive and 4 negative HI gene positive were (50%) and negative were (50%).

4. Discussion

Several virulence factors were produced by *S. aureus* including Coagulase protein which encoded by *Coa* gene which is important in the pathogenicity [15]. Through turn fibrinogen to fibrin which lead to abscessiation and persistence of microorganism in host tissue. Furthermore the detection of Coagulase is considered to be virulence factor in intrammary infection. Coagulase gene can be used as a simple and effective method for typing of *S. aureus* isolates from bovine mastitis . [16]. In this study *Coa* gene was detected in five isolates (62.5%) and give a single Amplicon of 630 bp as shown in fig (3). This seem to be agree with [17] who recorded a single amplicon of *Coa* genes at 600 bp of *Staphylococcus aureus* isolated from bovine mastitis. Moreover the findings reported by [18] suggesting that the amplicon of about 600 bp are predominant in bovine strains. Epidemiological studies indicates that *S. aureus* strains agents of mastitis produce a group of virulence factor and its believed that there is a relationship between severity of mastitis and the virulence factors produced by *Staphylococcus aureus* . Presence of *clfA* and *hla* gene Fig (4) and protein A considered as the staphylococcus species.

Amplification of clumping factor A(*clfA*)gene resulted in a single amplicon with a size of approximately 638bp for all (8)*S. aureus* strains indicating no size polymorphisms of this gene(*clfA*) . Amplification of *S.aureus* protein A gene spa resulted in a single amplicon with a size of approximately 226bp for 9 *S. aureus* out of 10 (90%)our results revealed that (5)isolates out of (8) tested (62.5%) have *coa* gene. Moreover coagulase gene tend to have different PCR products indicating the polymorphism of *Coa* gene. *Staphylococcus aureus* protein A (*Spa*) (Fig. 1) which encoded by the spa gene is a major important

surface proteins of bacterial cell wall product which binds with FC region of immunoglobulin G and impairs the opsonisation of serum complement and phagocytosis by polymorpho nuclear leukocytes of the host immune system, so the decrease in spa on cell surface of *S. aureus* resulted in increasing number of free receptor sites for complement C3b and phagocytosis [19].In this study amplification of spa gene of *Staphylococcus aureus* was detected at 229 bp in 6 isolates (75%), all 8 tested strain (32.6%) were positive for *Spa* gene Fig (1).[20] achieved that *spa* gene can be used for typing the isolates of *S.aureus*. The detection of genetic polymorphisms in the X region of the *spa* gene can be used for typing of *S. aureus*. [19]. Also [21]concluded that detection of *spa* gene polymorphisms with *coa*-PCR proposed as good diagnostic methods for typing of *Staphylococcus aureus* isolates which provide important results for the assessment of effective strategies against staphylococcal mastitis control.

In addition, some reports suggested that *S. aureus* strains that express SEC and TSST-1 in combination cause severe clinical mastitis that is unresponsive to treatment

The present study showed heterogeneity in the *coa* gene of *S. aureus* strains. In Pakistan similar findings were reported by [22,3]. Less variation in *coa* gene of *S. aureus* was found in the present study, which agrees with [23] The PCR amplicons variation in size of coagulase gene could be due to polymorphism among different isolates obtained from different herds and previous studies have also confirmed PCR product variation using molecular analysis of the coagulase gene [22].

S.aureus is recognized worldwide as frequent cause of intramammary infections in dairy cows. The main reservoir of *S. aureus* seems to be the infected quarter, and transmission between cows usually occurs during milking. *S. aureus* produces a spectrum of extracellular protein toxins and virulence factors which are thought to contribute to the pathogenicity of the organism [24].

The ability of *S. aureus* to adhere to extracellular matrix proteins is thought to be essential for the colonization and the establishment of infections [25].*S.aureus* possesses various adhesion genes, including *clfA*, *fnbA* [26].PCR analyses of *clfA* genes Fig (4) in the investigated 8 strains suggesting an important role of these elements in the pathogenicity of bovine mastitis. However,[27] found that some genes including *clfA* and *S. aureus* protein A gene *spa* were present in both antimicrobial resistant and susceptible isolates, statistical analysis showed there is a strong relationship with resistance patterns.

Amplification of genes encoding clumping factor (*clfA*) and thermonuclease(*nuc*) gene by polymerase chain reaction was used for the genotypic characterization of isolated *S. aureus* strains. Amplification of the clumping factor (*clfA*) gene

resulted in a single amplicon with a size of approximately 638 bp for all 8 tested *S. aureus* strains isolated from raw milk samples indicating no size polymorphisms of this gene. While the amplification of the *nuc* gene Fig (2). Produced an amplicon of 395 bp in all 8 examined *S. aureus* isolated from raw milk

samples . Specificity of the PCR products was demonstrated with 100% of the tested isolates .This specificity of *S. aureus* was agreed to the results recorded by [28] and [21].

Table (1) The results of PCR amplification of tested *S. aureus* isolates (8)

Tested genes	positive		Negative	
	No	%	no	%
Spa 5	62.2	3	37.5	
Nuc 3	37.5	5	62.2	
Tst 2	25	6	75	
Coa 4	50	4	50	
Hlg 4	50	4	50	
Clfa 3	37.5	5	62.2	

Percentage were calculated according to the no. of tested *S. aureus* isolates (8)

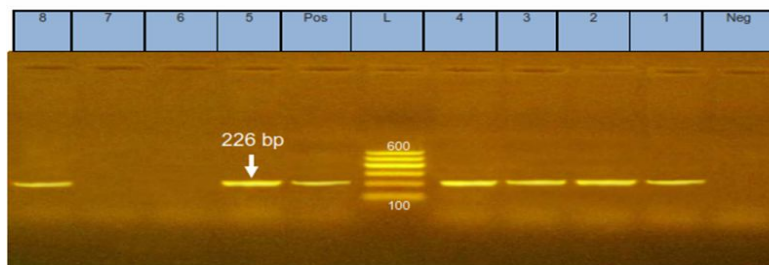


Fig (1) SPA gene. Lane M: 100-600 pb DNA ladder. Neg: Negative control. Pos: positive control 226bp. Lane: 1,2,3,4,8 pos. Lane: 6,7 Neg

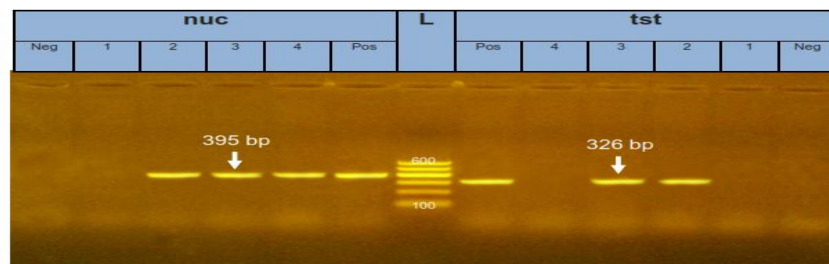


Fig (2) NUC gene. Lane M: 100-600 pb DNA ladder. Neg: Negative control. Pos: positive control 630bp. Lane: 2,3,4, pos. Lane: 1 Neg. TST gene Lane M: 100-600 pb DNA ladder. Neg: Negative control. Pos: positive control 326pb. Lane: 2,3 pos. Lane: 1,4 Neg

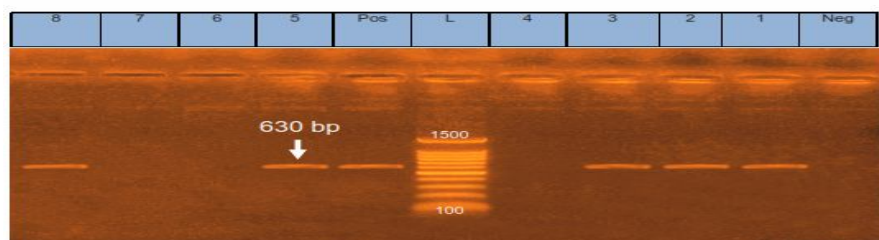


Fig (3) Co a gene. Lane M: 100-1500 pb DNA ladder .Neg: Negative control. Pos: positive control 630bp. Lane: 1,2,3, 8 pos. Lane: 4,6,7, Neg

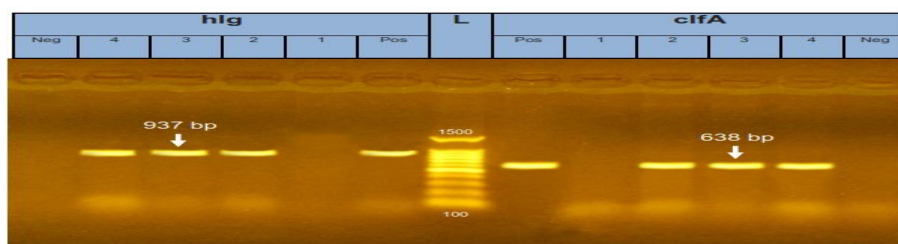


Fig (4) CLFA gene. Lane M: 100-1500 pb DNA ladder.

Neg: Negative control. Pos: positive control 638bp.Lane: 1 Neg. Hlg gene.

Lane M: 100-1500 pb DNA ladder. Neg: Negative control. Pos: positive control

937bp.Lane: 2,3,4 8 pos. Lane: 1 Neg

Conclusion

Data presented in this study showed that a broad distribution of identical or closely related *S. aureus* clones are responsible for the mastitis situation in, Egypt. Due to *S. aureus* isolates from cows with bovine mastitis were found to differ in their gene patterns, genotypic characterization provided a better understanding of the distribution of the prevalent *S. aureus* clones among bovine mastitis isolates. This can aid in the investigation and control of *S. aureus* infections in dairy herds. Further studies aim to obtain more data about different species will be conducted indifferent regions in Egypt.

References

- [1] M.U.Rehman, M.Rashid, J.A.Sheikh, M.A.Bhat Molecular epidemiology and antibiotic resistance pattern of enteropathogenic Escherichiacoli isolated from bovines and their handlers in Jammu, India. Journal of Advanced Veterinary and Animal Research, vol.1, pp.177-181, 2014.
- [2] R.G.,OldeRiekerink,H.WBarekma, D.T., Scholl, D.E.Poole, D.F Kelton management practices associated with bulk-milk prevalence of S.aureus in Canadian dairy farms. Prev.Vet.Med., vol.97(1), pp.20-28, 2010.
- [3] H.Momtaz, E.Rahimi, E.Tajbakhsh, Detection of some virulence factors in Staphylococcus aureus isolated from clinical and subclinical bovine mastitis in Iran. AfricanJ.of Biotechnology, vol.9(25), pp.3753-3758, 2010.
- [4]J.M.Fueyo, M.C.Mendoza, M.C.Martin, Enterotoxins and toxic shock syndrome toxin in *Staphylococcus aureus* recovered from human nasal carriers and manually handled foods: Epidemiological and genetic findings. *Microbes Infect.* Vol.7, pp.187-194, 2005.
- [5] F.M.Aarestrup, C.A.Dangler, L.M. Sordillo Prevalence of coagulase gene polymorphism in *Staphylococcus aureus*isolates causing bovine mastitis. Can J Vet Res vol.59, pp.124-128,1995.
- [6] K.O'Riordan, and J.C.Lee *Staphylococcus aureus* capsular polysaccharides. Clin. Microbiol. Rev., vol.17, pp.218-234,2004.
- [7] S.Morandi, M.Brasca, R.Lodi, L.Brusetti, C.Andrighetto and A.Lombardi Biochemical profiles, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and multilocus variable number tandem repeat analysis (MLVA) for typing *Staphylococcus aureus*isolated from dairy products. Res. Vet. Sci., vol.88, pp.427-435, 2010.
- [8] G.M.Costa, L.V.Paiva, H.C.P.Figueiredo, A.R.Figueira, U.P.Pereira and N.Silva Population diversity of *Staphylococcus aureus* isolated from bovine mastitis in Brazilian dairy herds. Res. Vet. Sci., vol.93, pp.733-735, 2012.
- [9] P.J.Quinn, B.K.Makey, M.E.Carter, W.J.Donnely and F.C.Leonard, Veterinary Microbiology and Microbial Diseases. Blackwell Science Ltd, 2002.
- [10] J.G.Collee, A.G.Fraser, B.P.Marmion and A.Stilmow, Practical medical microbiology. 14th Ed. Churchill, Livingstone, New York, 1996.
- [11] P.Boerlin, P.Kuhnert, D.Hussy and M. Schaellibaum, Methods for identification of S. aureus in cases of bovine mastitis. J. Clin. Microbiol, vol.41(2), pp.767-777, 2003.
- [12] F.De FreitasGuimaraes, D.B.Nobrega, V.B.Richini- Pereira, P.M.Marson, J.C.FigueiredoPantoja, and H.Langoni,. Enterotoxin genes in coagulase- negative and coagulase-positive staphylococci isolated from bovine milk. J. Dairy Sci, vol.96, pp.2866-2872, 2013.
- [13] E.W.Koneman, S.D.Allen, W.M.Janda, P.C.Schrechenberger and W.C Winn, colour Atlas and textbook of diagnostic microbiology (Fourth edition), 1979, 1983, 1988, 1992 by J. B. Lippincott Company, 1992.
- [14] J.Sambrook, E.F.Fritsgh and Mentiates Molecular cloning. A laboratory manual. Vol, Cold spring Harbor Laboratorty press, New York, 1989.
- [15] HassanMomtaz & ElaheTajbakhsh & EbrahimRahimi& Manochehr MomeniCoagulase gene polymorphism of *Staphylococcus aureus* isolated from clinical and sub-clinical bovine mastitis in Isfahan and

- Chaharma halva Bakhtiari provinces of Iran Comp ClinPathol vol.20, pp.519-522, 2011.
- [16] M.Mcadow, H.K.Kim, A.C.Dedent, A.P.Hendrickx, O.Schneewind and M.Missiakas, Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood, PLoSPatog. 7, e1002307, 2011.
- [17] M.E.Enany, S.Younes, A.M.AL-gammal, Salem and H.A.El Dieb, Prevalence of coagulase (coa) gene and mec A gene of *S. aureus* isolated from bovine clinical mastitis. Seuz Canal Veterinary Medicine Journal, vol.XVIII (1), pp.147-157, 2013.
- [18] K.G.Carbal, C.Lammler, M.Zschoc, H.Langoni, De Sa Me, C.Victoria and A. Da Silva, Pheno – and genotyping of *Staphylococcus aureus*, isolated from bovine milk samples from Sao Paulo State, Brazil. Can. J. Microbiol, vol.50(11), pp.901-9092, 2004.
- [19] J.Gao and G.C.Stewart Regulatory elements of the *Staphylococcus aureus* protein A (Spa) promoter. J.Bacteriol, vol.186, pp.3738-3748, 2004.
- [20] S.Mohammed, M.Abdelrahman and D.Abdulla, (Phenotypic and genotypic detection of virulence factors of *staphylococcus aureus* isolated from clinical and subclinical mastitis in cattle Veterinary World J., pp.2231-0916, 2015.
- [21] M.Karahan, M.N.Acik and B.Cetinkaya, B.Investigation of virulence genes by PCR in *Staphylococcus aureus* isolates originated from subclinical bovine mastitis in Turkey. Pak Vet J, vol.31(3), pp.249-253, 2011.
- [22] A.Khan, R.Hussain, M.T.Javed and F.Mahmood, Molecular analysis of virulent genes (coa and spa) of *Staphylococcus aureus* involved in natural cases of bovine mastitis. Pakistan. J. Agri. Sci, vol.50, pp.739-743, 2013.
- [23] T.Mork, T.Tollersrud, B.Kvitte, J. Jorgensen and S.Waage Comparison of *Staphylococcus aureus* genotypes recovered from cases of bovine, ovine, and caprine mastitis. J.Clin. Microbiol, vol.43, pp.3979-3984, 2005.
- [24] M.M.Salem-Bekhit, M.M.Muharram, I.M.Alhosiny.M and E.S.Y.Hashim. Molecular Detection of Genes Encoding Virulence Determinants in *Staphylococcus aureus* Strains isolated from Bovine Mastitis Journal of Applied Sciences Research, vol.6(2), pp.121-128, 2010.
- [25] SI.Salasia, Z.Khusnan, R.C.Lamme and M.Zschock Comparative studies on pheno and genotypic properties of *Staphylococcus aureus* isolated from bovine subclinical mastitis in central Java in Indonesia and Hesse in Germany. J. Vet. Sci. vol.5, pp.103-109, 2004.
- [26] El-Sayed, A.Alber, J.C.Lammler, B.Bonner, A.Huhn, E.F.Kaleta and M.Zschock. PCR based detection of genes B. Infect. Dis. Vet Public Health, vol.52, pp.38-44, 2005.
- [27] T.Brody, A.S.Yavatkar, Y.Lin, J.Ross, AKuzin, M.Kundu., Y.Fann, and W.F. Odenwald. Horizontal gene transfers link a human MRSA pathogen to contagious bovine mastitis bacteria. PLOS ONE, vol.3, pp.3074, 2008.
- [28] A.Ozkan, D.Cemil, T.Hulya, C.Zafer, E.Yaflar and D.Gokhan. Coagulase gene polymorphism of *Staphylococcus aureus* isolated from subclinical mastitis. Turk.J.Vet. Anim.Sci., vol.31(4), pp.253-257, 2007.