

**PCR FOR DETECTION OF VIRULANCE AND ANTIBIOTIC RESISTANCE
GENES OF STAPHYLOCOCCUS AUREUS ISOLATED FROM
SUBCLINICAL MASTITIS AT AL-GHARBIA GOVERNORATE**

By

Marwa N. Sayed; Elesawy H.A*. ; Salwa A.M* and Gamal R.H****

Department of food hygiene and control, Faculty of veterinary medicine, Cairo University*

** Animal health research institute- Tanta Branch.

ABSTRACT

Three hundred and fifty quarter milk samples were collected from apparently healthy cows at Al-Gharbia governorate, Egypt. Samples were tested using California Mastitis Test (CMT) and somatic cell count, of which 235 samples were detected as positive. Overall, 168-quarter milk samples (71.48%) were found to be contaminated with coagulase positive *Staphylococcus aureus* with mean count of $64 \times 10^3 \pm 19 \times 10^3$ cfu/ml. Isolated *Staphylococcus aureus* strains were tested for methicillin to identify Methicillin resistant strains (MRSA). Antibiotic sensitivity test was carried out by using two antibiotic disks against forty six identified *Staphylococcus aureus* isolates. The obtained results indicated that resistance against cefoxitin was 34.78% and sensitivity was 65.21%, while the resistance against vancomycin was 26.08% and sensitivity was 73.91%. PCR technique was used to detect presence of *mecA* gene that coded for penicillin-binding protein 2a. The results on sixteen positive isolates, which suspected to have *mecA* gene by antibiotic sensitivity test were 93.75%. The total of subclinical mastitis cases infected with MRSA was 6.38%. The results provided evidence that the presence of coagulase positive *Staphylococcus aureus*, as well as Methicillin-resistant strains have become remarkably widespread in subclinical mastitis quarter milk samples. This calls for better control of the sources of milk contamination as well as spread of antimicrobial resistance organisms.

Key words:

Subclinical mastitis, California mastitis test, *Staphylococcus aureus* and MRSA.

INTRODUCTION

Raw milk is an excellent medium for growth of several types of microorganisms. Milk and its products are considered vehicles of *Staphylococcus aureus* for infection of humans. It is an important food borne pathogens causing a wide variety of diseases in humans and animals

ranging in severity from mild skin infections to are more severing diseases such as pneumonia and food borne illness. *Staphylococcus aureus* intoxication ranked third of food poisoning cases all over the world (Asao et al., 2003), as it is mediated by the ingestion of enterotoxins produced by enterotoxigenic strains of *Staphylococcus aureus* (Strommenger et al., 2018). *Staphylococcus aureus* is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. In dairy cattle, *Staphylococcus aureus* is frequently associated with subclinical mastitis (Fagundes et al., 2010). Cows with subclinical *Staphylococcus aureus* infections can shed a large number of the organism in their milk that can pose an elevated health hazard, resulting in reduction of milk yield by 10 to 20% with undesirable effect on its constituents and nutritional value rendering it of low quality and unfit for processing. Although there are no visible or palpable external changes, the infection is present and inflammation occurred in the udder (Zdunczyk et al., 2003 and Abdel - Rady and Sayed 2009). *Staphylococcus aureus* produce a large number of potential virulence factors, which have an important role in the pathogenesis of mastitis (Kalorey et al., 2007). These include, Coagulase, which is considered the most important virulence factors that clot plasma and coats the bacterial cell, so prevent the phagocytosis (Panizzi et al., 2004). Currently, the main therapy for treatment of subclinical mastitis is the administration of antibiotics; but, this approach is associated with a risk of the development of antimicrobial resistant bacteria. *Staphylococcus aureus* has been reported frequently to show multiple antimicrobial resistant patterns. It is detected adverse increasing trend worldwide prevalence of methicillin- resistant strains of staphylococci (MRSA). The infections from methicillin-resistant staphylococci have turned into one of the major problem in antibiotic treatment. The *mecA* gene, encoding the penicillin binding protein 2a, mediates methicillin- resistance in staphylococci (PBP2a), which has reduced affinity for β -lactamase. The MRSA with mentioned gene is resistant to many other types of antibiotics, this makes the treatment of microorganisms related diseases too hard and causes a greater spread of it in the society (Pereira et al., 2009). As MRSA may be present in raw milk and traditional dairy products, this insufficiently hygienic handling of these contaminated foods may lead to transmission of MRSA to human and possible colonization of nostrils, skin and gastrointestinal tract (Mirzaei et al., 2011). Many recent MRSA clones have been shown to transmit between animals and humans. Furthermore, MRSA is now invading the hospitals. The MRSA- infected individuals transmit these strains in the hospital setting and result in nosocomial infections (Kennedy

and Deleo 2009). Therefore, the aim of the present study was to illustrate the prevalence of subclinical mastitis at El-Gharbia Governorate as well as determination the prevalence of MRSA in the collected subclinical mastitic raw milk samples.

MATERIAL AND METHODS

1- Collection of milk samples according to Andrews *et al.*, 1992:

Three hundred and fifty quarter milk samples were collected from apparently healthy lactating cows in Al-Gharbia Governorate.

2- California Mastitis Test (C.M.T.) According to A.P.H.A., 1992.

Equal volumes of quarter milk sample and CMT reagent (Schalm *et al.*, 1971) were mixed thoroughly in a cup of plastic paddle. The mixture was gently swirled by circular motion of the paddle. The results were recorded after 10 seconds and judged. The results were classified into four scores: 0= negative, 1 = slightly positive (+), 2 = positive (++) and 3= highly positive (+++).

3-Making Laboratory measurement of milk somatic cells According to Zecconi *et al.*, 2002.

Each positive CMT quarter milk sample was collected under aseptic conditions in a sterile screw capped bottle and sent directly to the laboratory with a minimum of delay to measure the somatic cell count.

4- Phenotypic characterization: Through culturing positive CMT samples and also showing high somatic cell count onto Baired parker agar medium for demonstrating characteristic shape of staphylococci according to ISO (2003), as well as its biochemical and virulence activities was conducted according to A.P.H.A, 2004.

5- Detection of *mecA* gene of *Staphylococcus aureus* by in vitro antibiotic sensitivity test:

Sensitivity to antibiotics was determined by agar diffusion test on Muller Hinton agar using the following antibiotic impregnated discs: cefoxitine (30 µg) and vancomycin (30 µg), Oxoid Germany. Zones of growth inhibition were evaluated according to Clinical Laboratory Standard Institute (CLS I, 2014).

6- PCR detection of *mecA* gene of *Staphylococcus aureus*.

DNA Molecular weight marker

The ladder was mixed gently by pipetting up and down. 6 µl of the required ladder were directly loaded.

Agarose gel electrophoreses (Sambrook et al., 1989)

Electrophoresis grade agarose (1.5 g) was prepared in 100 ml TBE buffer in a sterile flask, it was heated in microwave to dissolve all granules with agitation, and allowed to cool at 70°C, then 0.5µg/ml ethedum bromide was added and mixed thoroughly.

RESULTS

Table (1): The prevalence of subclinical mastitis in quarter milk samples collected from lactating cows according to the results of California mastitis test (CMT) (N=350).

Total quarter milk samples	Negative by CMT		Subclinical mastitis by CMT							
			Total positive		Score +		Score ++		Score +++	
	No.	%	No.	%	No.	%	No.	%	No.	%
350	115	32.86	235	67.14	28	11.92	78	33.19	129	54.89

Table (2): Statistical analytical results of milk somatic cell count (SCC) / ml in examined 235 quarter milk samples.

No. of examined samples	Minimum	Maximum	Mean	± S.E.M.
235	10x10⁴	3.0x10⁶	46.0x10⁴	3.7x10⁴

Table (3): Frequency distribution of the examined 235-quarter milk samples according to their milk somatic cell count (SCC) / ml.

Interval	NO. of samples	%
<200x10³	89	37.87
≥200 x10³-<400 x10³	66	28.09
≥400 x10³-<600 x10³	21	8.94
≥600 x10³-<800 x10³	20	8.51
≥800 x10³-<1000 x10³	16	6.81
≥1000 x10³	23	9.78
Total	235	100.00

Table (4): Prevalence and statistical analysis of *Staphylococcus aureus* and MRSA in the examined quarter milk samples.

No. of examined samples	Coagulase positive Staphylococci samples		Mean (Cfu/ml)	±SEM (Cfu/ml)	Positive Staphylococcus aureus samples		Positive MRSA Samples	
	NO.	%			NO.	%	No.	%
235	168	71.48	64x10 ³	19x10 ³	45	19.14	14	31.11

Table (5): Results of Mannitol Salt Agar test for differentiation between Coagulase positive Staphylococci isolates.

Tested coagulase positive staphylococcus isolates	<i>S. aureus</i>		<i>S. intermedius</i>		<i>S. hyicus</i>	
	No.	%	No.	%	No.	%
383	46	12.01	222	57.96	115	30.03

Table (6): Detection of *mecA* gene by Antibiotic Sensitivity Test.

Tested <i>Staphylococcus aureus</i> isolates	Cefoxitine				Vancomycin			
	Resistant		Sensitive		Resistant		Sensitive	
	No.	%	No.	%	No.	%	No.	%
46	16	34.78	30	65.22	12	26.09	34	73.91

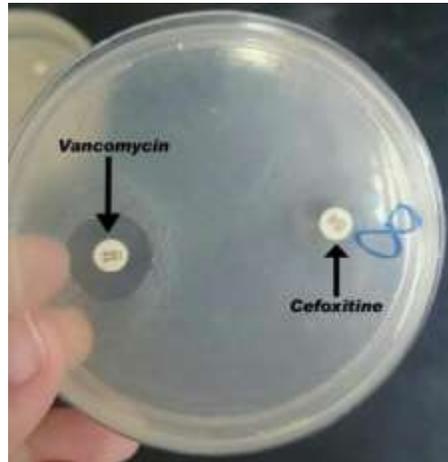


Fig. (1): Antibiotic sensitivity test showing resistance to cefoxitine and sensitivity to vancomycin which suspect to have *mecA* gene of *Staphylococcus aureus*.

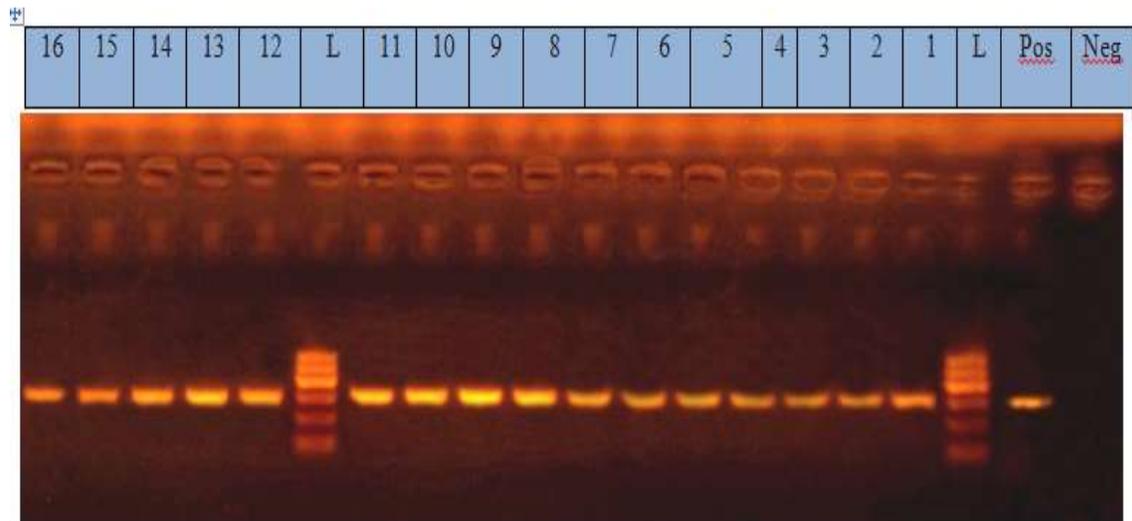


Fig. (2): Results of PCR indicate the presence of *mecA* gene in the identified *Staphylococcus aureus* isolates.

DISCUSSION

Mastitis in dairy cows is a serious problem as it is an economically devastating disease causing great economic losses in the dairy industry in Egypt (Seleim *et al.*, 2002). Subclinical mastitis is more serious than clinical one because visible abnormalities such as udder swelling, hardness of the affected quarter, pain, and watery milk remain absent. It results in reductions of milk yield and undesirable changes in the milk composition, as well as increased costs associated with control strategies (Halasa *et al.*, 2007). Data represented in (Table 1) revealed that, the results of examined 350 quarter milk samples by California Mastitis Test was 115 (32.86%) samples were negative, while 235 (67.14%) samples were positive. Among the positive samples the highest incidence was recorded in CMT (+++) as 129 (54.89%) samples; while the lowest one lied in CMT (+) as 28 (11.91%) samples. These results totally agree with those reported by Mulei (2000) and Kerro and Tareke (2003) while lower incidences were obtained by Elbably *et al.* (2013); Lucia *et al.*, (2017) and Xavier *et al.*, (2017). Kalorey *et al.* (2007) recorded higher incidence of subclinical mastitis using CMT (90.76%). The difference in prevalence of subclinical mastitis observed may be due to differences in management practices, use of different methods for diagnosing subclinical mastitis, breeds of the animals, immune responses and climatic conditions. Other factors that could influence the prevalence of subclinical mastitis could be attributed to variation in hygienic standards of the dairy environment and milking conditions, as well as genetic variation in disease resistance amongst the breeds maintained in the systems (Saidi *et al.* 2013). (Table 2) showed that somatic cell count of the examined quarter milk samples was in the range of 10×10^4 to 3.0×10^6 with a mean value of $46.0 \times 10^4 \pm 3.7 \times 10^4$. Increase in somatic cell count in milk leads to the release of lipolysis (lipases) and proteolysis (plasmin) enzymes, which can degrade the triglycerides of milk fat and casein contents of milk. Leading to poor quality milk in the mastitis-affected animals (Ondiek *et al.* 2013). The highest frequency distribution of milk somatic cell count was 65.96% (155 samples), lied within the range $< 200 \times 10^3$ - $< 400 \times 10^3$ cells/ml (Table 3); which substantiate what have been reported by Nam *et al.*, (2010) and Dieser *et al.*, (2014). Statistical analytical result of Coagulase Positive Staphylococci was present in a percentage of 71.48 % of the examined raw milk samples with a mean value of $64 \times 10^3 \pm 19 \times 10^3$ (Table 4). Lucia *et al.*, (2017), recorded lower results. The production of the coagulase enzyme becomes the pathogenic factor of *Staphylococcus*

aureus, differentiating it from other types of Staphylococci, which is able to clot blood plasma, since it resembles prothrombin, which can convert fibrinogen to fibrin (Lucia et al., 2017). In addition, results in (Table 4) showed that, the incidence of *Staphylococcus aureus* was 19.14% from the examined raw milk samples. Sharma et al., (2015), obtained nearly similar results; Fagundes et al., (2010) and Umaru et al. (2017) recorded lower results, while higher incidences were recorded by Abdel-Rady and Sayed (2009). According to the results reported in table 4 , out of 45 coagulase positive *Staphylococcus aureus* samples detected, 14 (31.11%) were found to contain *mecA* gene which is indication of presence of methicillin resistant *Staphylococcus aureus* (MRSA) by using PCR technique, Guimarães et al. (2017) recorded higher results. Data represented in (Table 5) shows that, out of 383 tested isolates of Staphylococci, 46 were *Staphylococcus aureus* in apercentage of 12.01%, whileCoagulase Positive *Staphylococcus intermedius* was found in percentage of 57.96%. Coagulase Positive *Staphylococcus hyicus* was found in a percentage of 30.03 %. The prevalence of *Staphylococcus aureus* can most likely be attributed to the wide distribution of the organism inside the mammary glands, as well as on the skin of teats and udders (Anueyiagu et al., 2016). The data obtained in (Table 6) showing in vitro antibiotic sensitivity test of the examined isolates to different antibiotics, which revealed that 16 isolate (34.78%), were resistant to cefoxitine. Similar results obtained by Sharma et al., (2015), while lower results were obtained by Hamid et al., (2017). Dorgham et al., (2013) recorded very high results of resistance to cefoxitine. The resistances of isolates to vancomycin were present in a percentage of 26.09 % while 34 samples (73.91%) were sensitive. Al-Ashmawy and Sallam (2016) obtained similar results. Fourteen isolates were resistant to cefoxitine and sensitive to vancomycin, which were suspected to have *mecA* gene, while two isolates only were resistant to cefoxitin, and vancomycin. In other studies carried out on cow's milk, MRSA were most frequently isolated from milk of animals showing signs of subclinical mastitis (Lee 2003). Antimicrobial resistance represents a serious problem in the treatment of infectious diseases including mastitis. In recent years, an increasing antimicrobial resistance rate has been recognized in *Staphylococcus aureus* from bovine mastitis (Saini et al., 2012 and Wang et al., 2013). Moreover, there is an increased incidence of Methicillin resistant *Staphylococcus aureus* (MRSA) all over the world, which seems to be widely spread among *Staphylococcusa ureus* isolates from bovine milk. MRSA first emerged as a serious pathogen in human medicine during late 1970s and has been reported in animals during the past 10

years (Leonard and Markey, 2008). With the emergence of MRSA, methicillin became ineffective against them while vancomycin became the drug of choice for MRSA (Ng *et al.*, 2011). By using PCR for detecting the *mecA* genes in 16 *Staphylococcus aureus* isolates, the results showed that 15 out of 16 positive isolates by antibiogram method (93.75%) contained *mecA* gene. El-seedy *et al.* (2010) and Hamid *et al.* (2017) obtained lower results. In conclusion, the results obtained in this study confirmed that *Staphylococcus aureus* was implicated in subclinical mastitis in the examined quarter milk samples, of which high incidence of MRSA are present representing major threats for transmission to human beings. Thus to safe guard consumers from being infected, improving the hygienic conditions and careful handling of the producing animal during milking must be applied.

REFERENCES

- Abdel-Rady, A., and Sayed, M. (2009):** Epidemiological Studies on Subclinical Mastitis in Dairy cows in Assiut Governorate. *Veterinary World*, 2 (10).
- Al-Ashmawy, M. A., and Sallam, K. I. (2016):** Prevalence, Detection of Marker and Virulence Genes of Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolated form Milk and Dairy Products and their Antimicrobial Susceptibility. *Zagazig Veterinary Journal (Zag. Vet. J.)*, 42(1).
- Alouf, J.E., and Müller-Alouf, H.(2003):**Staphylococcal and streptococcal superantigens: molecular, biological and clinical aspects. *International journal of medical microbiology*, 292 (7-8), 429 - 440.
- Andrews, A.; Blowey, R.; Boyd, W. and Edy, R. (1992):** *Bovine Medicine. Diseases and Husbandry of cattle.* Blackwell Scientific Publications.
- Anueyiagu, K. N. and Isiyaku A. W. (2016):** Isolation, identification of *Staphylococcus aureus* from bovine milk and its antibiotics susceptibility. *International Journal of Livestock Production*. Vol.6 (6), pp74 -77, June 2015.
- APHA "American Public Health Association" (1992):** *America's public health report card: A state-by-state report on the health of the public.* American Public Health Association.
- APHA "American Public Health Association" (2004):** *Standard methods for the examination food dairy products*, 17th Ed. American public health association. Washington D.C.
- Asao, T., Kumeda, Y., Kawai, T., Shibata, T., Oda, H., Haruki, K., and Kozaki, S. (2003):** An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk. *Epidemiology and infection*, 130 (1).

- CLSI (2014):** Clinical and Laboratory Standards Institute” Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement. Approved Standard M100 - S23, Clinical and Laboratory Standards Institute, Wayne, Pennsylvania, USA, 2014.
- Dieser, S. A., Vissio, C., Lasagno, M. C., Bogni, C. I., Larriestra, A. J., and Odierno, L. M. (2014):** Prevalence of pathogens causing subclinical mastitis in Argentinean dairy herds. *Pak Vet J*, 34 (1), 124-126.
- Dorgham, S. M., Hamza, D. A., Khairy, E. A., and Hedia, R. H. (2013):** Methicillin-resistant staphylococci in mastitic animals in Egypt. *Global Veterinaria*, 11 (6), 714 -720.
- Elbably, M. A., Emeash, H. H. and Asmaa, N. M. (2013):** Risk factors associated with mastitis occurrence in dairy herds in Benisuef, Egypt. *World's Veterinary Journal*, 3 (1), 5-10.
- El-Seedy, F.R., El-Shabrawy, M., Hakim, A. S., Dorgham, S.M., Ata, S. Nagwa Bakry, M.A., and Osman, N. M. N. (2010):** Recent Techniques used for Isolation and Characterization of *Staphylococcus Aureus* from Mastitic Cows. *Journal of American Science*, 6 (12).
- Fagundes, H., Barchesi, L., Nader Filho, A., Ferreira, L. M., and Oliveira, C. A. F. (2010):** Occurrence of *Staphylococcus aureus* in raw milk produced in dairy farms in São Paulo state, Brazil. *Brazilian Journal of Microbiology*, 41(2), 376-380.
- FDA (U.s. Food And Drug administration) (2001):** food borne pathogenic microorganisms and natural toxins. Available at [http:// www.cfsan.fda.gov](http://www.cfsan.fda.gov). Accessed 12jul.2007.
- Guimarães, F. F., Manzi, M. P., Joaquim, S. F., Richini-Pereira, V. B., and Langoni, H. (2017):** Outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA)-associated mastitis in a closed dairy herd. *Journal of dairy science*, 100 (1), 726-730
- Halasa, T., Huijps, K., Østerås, O., and Hogeveen, H. (2007):** Economic effects of bovine mastitis and mastitis management: A review. *Veterinary Quarterly*, 29 (1), 18-31.
- Hamid, S., Bhat, M. A., Mir, I. A., Taku, A., Badroo, G. A., Nazki, S., and Malik, A. (2017):** Phenotypic and genotypic characterization of methicillin-resistant *Staphylococcus aureus* from bovine mastitis. *Veterinary world*, 10 (3), 363.
- ISO, International Standard Organization (2003):** ISO standard DIS 6888:2003(E). Horizontal for the enumeration of Coagulase Positive Staphylococci (*Staphylococcus aureus* and other species).
- Kalorey, D.R.; Shanmugam, Y.; Kurkure, N.V.; Chousalkar, K.K. and Barbuddhe, S.B. (2007):** PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases. *J Vet Sci*, 8: 151-154.
- Kennedy, A.D. and Deleo, F.R. (2009):** Epidemiology and virulence of community-associated MRSA. *Clinical microbiology Newsletter*. 31: 153 - 60.
- Kerro, D.O. and Tareke, F., (2003):** Bovine mastitis in selected areas of Southern Ethiopia. *Tropical Animal Health and Production*, 35, 197-205.

- Le Loir, Y., Baron, F., and Gautier, M. (2003):** Staphylococcus aureus and food poisoning. *Genet Mol Res*, 2(1), 63-76.
- Lee, J.H. (2003):** methicillin (Oxacillin) -resistant Staphylococcus aureus strains isolated from major food animals and their potential transmission to humans. *Applied and Environmental Microbiology*. 69:6489 - 6494.
- Leonard, F.C. and B.K. Markey, (2008):** Methicillin-resistant Staphylococcus aureus in animals: A review. *Vet. J.*, 175: 27-36.
- Lucia, M., Rahayu, S., Haerah, D., and Wahyuni, D. (2017):** Detection of Staphylococcus Aureus and Streptococcus Agalactiae: Subclinical Mastitis Causes in Dairy Cow and Dairy Buffalo (Bubalus Bubalis). *American Journal of Biomedical Research*, 5 (1), 8-13.method
- Mirzaei, H., Tofighi, H.; karimi, S. and Mahdi, F. (2011):** Prevalence of methicillin resistant Staphylococcus aureus in raw milk and dairy products in sarab by culture and PCR techniques. *Journal of Animal and Veterinary Advances*. 10: 3107-3111.
- Mulei, C. M. (2000):** Micro-organisms associated with non-functional mammary gland quarters in dairy cows in small-scale farms in Kenya. *Indian Journal of Animal Sciences*, 70 (9), 897-898.
- Nam, H. M., Kim, J. M., Lim, S. K., Jang, K. C., and Jung, S. C. (2010):** Infectious etiologies of mastitis on Korean dairy farms during 2008. *Research in veterinary science*, 88 (3), 372-374.
- Ng, S.T., C.Y. Lim, C.S. Tan, A.A. Karim, H. Haron, N.S. Ahmad and V. Murugaiyah, (2011):** Emergence of Vancomycin-Resistant Staphylococcus aureus (VRSA). *Webmed Central Infect. Dis.*, Vol. 2. 10.9754/journal.wmc.2011.002773.
- Normanno, G.; La Salandra, G.; Dambrosio, A.; Quaglia, N.C.; Corrente, M.; Parisi, A.; Santagada ,G.; Firinu, A.; Crisetti, E. and Celeno, G.V. (2007):** Occurrence, characterization and antimicrobial resistance of enterotoxigenic Staphylococcus aureus isolated from meat and dairy products. *Int. J. Food Microbial*. 115: 290 -296.
- Ondiek, J. O., Ogore, P. B., Shakala, E. K., and Kaburu, G. M. (2013):** Prevalence of bovine mastitis, its therapeutics and control in Tatton Agriculture Park, Egerton University, Njoro District of Kenya. *Basic Research Journals of Agricultural Science and Review*, 2 (1), 15-20.
- Panizzi, P.; Friedrich, R.; Fuentes-Prior, P.; Bode, W. and Bock, P.E. (2004):** The staphylocoagulase family of zymogen activator and adhesion proteins. *Cell Mol Life Sci* 61: 2793-2798.
- Pereira, V.; Lopes, C.; Castro, A.; Silva, J.; Gibbs, P. and Teixeira, P. (2009):** Characterization for enterotoxin production. Virulence factors and antibiotic susceptibility of Staphylococcus aureus isolates from various foods in Portugal. *Food Microbial*. 26: 278-282.
- Presscott, L.M.; Harley, J.P and Klein, D.A. (2002):** text book of Microbiology. Brown Publishers. 5th ed.: 441-442.

- Saidi, R., Khelef, D., and Kaidi, R. (2013):** Subclinical mastitis in cattle in Algeria: Frequency of occurrence and bacteriological isolates. *Journal of the South African Veterinary Association*, 84 (1), 00-00.
- Saini, V., J.T. McClure, D.T. Scholl, T.J. De Vries and H.W. Barkema,(2012):** Herd-level association between antimicrobial use and antimicrobial resistance in bovine mastitis *Staphylococcus aureus* isolates on Canadian dairy farms. *J. Dairy Sci.*, 95: 1921-1929.
- Sambrook, J.; Fritsch, E.F.; and Mentiates (1989):** Molecular cloning. A laboratory manual. Vol., Cold spring Harbor Laboratory press, New York.
- Schalm, O. W., Carroll, E. J., and Jain, N. C. (1971):** Bovine mastitis. *Bovine mastitis*.
- Seleim, R.S.; Rashed, Amany Y.M. and Fahmy, B.G.A. (2002):** Mastitis pathogens: attachment-related virulence features, whey protein markers and antibiotic efficacy in cows. *Vet. Med. J. Giza*, 50:405.
- Sharma, L., Verma, A.K, Kumar, A., Rahat, A.Neha and Nigam, R. (2015):** Incidence and Pattern of Antibiotic Resistance of *Staphylococcus aureus* Isolated from Clinical and Subclinical Mastitis in Cattle and Buffaloes. *Asian Journal of Animal Sciences*, 9 (3), 100 -109.
- Strommenger, B., Layer, F., and Werner, G. (2018):** *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* in Workers in the Food Industry. In *Staphylococcus aureus* (pp. 163-188).
- Umaru, G. A., Kabir, J., Umoh, V. J., Bello, M., and Kwaga, J. K. (2017):** Methicillin-resistant *Staphylococcus aureus* (MRSA) in fresh and fermented milk in Zaria and Kaduna, Nigeria. *International Journal of Drug Research and Technology*, 3 (3), 8.
- Wang, S., C. Wu, J. Shen, Y. Wu and Y. Wang, (2013):** Hypermutable *Staphylococcus aureus* strains present at high frequency in subclinical bovine mastitis isolates are associated with the development of antibiotic resistance. *Vet. Microbiol*, 165: 410 - 415.
- Xavier, A. R. E. O., Almeida, A. C., Souza, C. N., Silva, L. M. V., Ruas, A. X. A., Sanglard, D. A. and Xavier, M. A. S. (2017):** Phenotypic and genotypic characterization of *Staphylococcus aureus* isolates in milk from flocks diagnosed with subclinical mastitis. *Genetics and molecular research: GMR*, 16 (2).
- Zdunczyk, S.; Zerbe, H. and Hoede maker, M. (2003):** Importance of oestrogen and oestrogen-active compounds for udder health in cattle: A review. *Dtsch Tierarztl Wochenschr.* 110:461.
- Zecconi, A.; Casirani, G.; Binda, E. and Piccinini, R. (2002):** The importance to assess the effects of voluntary milking system on teat tissues, intramammary infections and somatic cell counts. Dept. Anim. Path.-Infect. Dis. Lab., University of Milan. Delaval Hygiene, Technology center, Inaugural Symposium, May 15-16, 2002.