

Serum Amyloid A4 and Ceruloplasmin Evaluated Mastitic Cattle with *Escherichia coli* or *Staphylococcus aureus* including Resistant Genes

Verginia Mohamed El-Metwally Farag^{1*}, Amany Mohamed Abd-El-Moaty², Nermin Awad Ibrahim², Samar Magdy Atwa³ and Mohamed Abd-EL-Naeem EL-Beskawy³

¹Department of Clinical Pathology, Mansoura University, Egypt

²Department of Bacteriology, Mycology and Immunology, Mansoura University, Egypt

³Department of Internal Medicine, Infectious and Fish Diseases (Infectious Diseases), Mansoura University, Egypt

Abstract

This study investigated the prevalence of an important Gram-negative and a Gram-positive bacterial strain, as well as the test on a few antibiotic resistant genes and sensitivity on the two bacteria. Moreover, the relations of the two microorganisms with some Acute Phase Proteins (APPs) inflammatory marker such as Serum Amyloid A (SAA) and Ceruloplasmin (Cp) in serum and milk were inspected. Out of 120 samples of Mastitic cow's milk were identified 19.2% *Escherichia coli* (*E. coli*) and 14.2% *Staphylococcus aureus* (*S. aureus*) single isolates. Isolates of *E. coli* were completely sensitive to imipenem followed by meropenem then amikacin, enrofloxacin, gentamicin and cefoprazone. Two out of four isolates of multidrug resistance (against cefotaxime, penicillin, cefoprazone and gentamicin) showed positive amplification for beta lactam encoded gene blaTEM. Furthermore two isolates (out of four) resisted tetracycline were positive for TetA (A) gene. In *S. aureus* isolates, resistance was gradually increased from amikacin, tetracycline, and cefotaxime to oxacillin till reached 100% against penicillin. All analyzed eight isolates of penicillin resistance carried blaZ gene. Likewise, Methicillin A (*mecA*) gene was positive in six out of eight isolates of oxacillin resistance group. Results of SAA4 and Cp levels were differed in both pathogen groups. For *E. coli* group, the two levels were significantly increased in serum and only Cp elevated in milk comparing with control group. Conversely, in case of *S. aureus*, both parameters elevated significantly in milk and only SAA4 level was significant elevated in serum.

In conclusion, resistance to β -Lactam group, methicillin resistant group and tetracycline has reached a critical level in mastitic cattle with *E. coli* and *S. aureus*. But still the most effective and available antibiotics were enrofloxacin, amikacin and gentamicin. For diagnosis, SAA4 and Cp level has helped as a rapid tool in differentiation between both organisms.

Keywords: Mastitis; Cattle; Resistant gene; *Escherichia coli*; *Staphylococcus aureus*; SAA4; Ceruloplasmin

Introduction

Bovine mastitis is an inflammatory infectious disease of mammary glands and has difficulty to overcome. It affects normal milk flow and quality. Enterobacteriaceae (as *Escherichia coli*) and *Staphylococcus* bacteria species are one of the most problematic bacteria caused bovine mastitis [1]. They have a developing resistance for antibiotics especially for β -Lactam group (penicillin, cephalosporins, monobactams and carbactams) [2].

Prevalence of *E. coli* and multidrug resistance is of great concern in many countries especially in North Africa. Resistance to penicillin, tetracycline and sulphonamides and extension to β -lactamase spectrum in bovine mastitis are recorded in many reports [3-5]. Antimicrobial resistance genes extend to β -lactam group and tetracycline in *E. coli*. The blaTEM is an important gene of *E. coli* livestock [3]. Furthermore, *mecA* gene is encoded penicillin-binding protein 2A and cause methicillin resistance in *Staphylococcus aureus* [4].

Acute APPs are hepatic non-structurally related proteins in different body fluid and are diagnostic for inflammation in dairy cattle [6]. However, not all diseases change the APPs level. Mammary SAA-3 had antibacterial activity [7]. Serum amyloid A is one of the APPs. It had many highly linked genes (SAA1, SAA2, SAA3 and SAA4) [8]. Mammary SAA-3 had antibacterial activity [7] and activates the involution via increasing cytokines related to innate immunity in cattle infected with *S. aureus* [9,10]. Also, ceruloplasmin is one of the APPs protect the cell from danger of oxygen species. However, the relation between pathogen and APPs in mastitic cattle needs more studies [11].

Present study investigated prevalence of mastitic cattle with *E. coli* and *S. aureus* including resistance genes and susceptibility to some antibiotics with diagnostic reference of SAA4 and Cp levels in serum and milk.

Materials and Methods

Samples collection

The study was done on milk and corresponding blood samples (from the jugular vein) of 120 mastitic Holstein cows (3-7 years of age) diagnosed in the veterinary hospital, faculty of veterinary medicine, Mansoura University, Dakahlia Governorate, Egypt in two years (2014 and 2015). Each sample was taken from one animal and one quarter (most inflamed), changed milk (watery or viscous and yellowish or tinged with blood). Hand milking samples (10 mL of each) were collected in sterile vials after teat disinfection (70% ethanol) and discarding the first milk jets and directly conveyed to the laboratory. Blood samples

*Corresponding author: Verginia Mohamed El-Metwally Farag, Department of Clinical Pathology, Faculty of Veterinary Medicine, Mansoura University, Egypt, Tel: 02201126210224; E-mail: faragverginia@gmail.com

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tubes were put in an inclined position (20 min at room temperature) then in the refrigerator (one h) for improving the clot. All samples were centrifuged at 5000 and 3000 rpm for 30 and 10 min for milk and serum respectively. Milk samples precipitates were used for bacteriological examination. The clear blood serum and milk supernatant were kept at -20°C till used. California Mastitis test was negative for control group samples (from clinically healthy cows). After bacteriological identification, random ten samples of serum and corresponding milk were taken from each *S. aureus* and *E. coli* positive groups rather than control one for SAA4 and Cp analysis.

Bacteriological analysis and isolates identification

Milk samples precipitate was used for microbial enrichment procedure [12]. MacConkey agar (Biomark, Pune) and Eosin Methylene Blue (EMB) agar (Oxoid) for obtaining pure *E. coli* colonies (appeared as metallic sheen green colonies on EMB). For obtaining *S. aureus* isolates, inoculum were streaked on Baird Parker agar media (Oxoid) containing Egg yolk tellurite emulsion (5%) (Oxoid). The colony was seen as grey black and Gram stain film showed gram-positive cocci in bunched grape like irregular clusters. While *E. coli* appeared as gram-negative, large, pink, rod shaped. Then all suspected colonies were conducted to rapid identification by VITEK 2 compact system (Biomérieux) at the institute of animal health research, Dokki, Cairo, Egypt.

Antibiotic susceptibility assay

All *E. coli* and *S. aureus* isolates were subjected to antibiotic susceptibility tests using disk diffusion method [13] on Muller Hinton agar (Oxoid). The visible clear inhibition zone was determined (mm) and interpreted according guidelines chart. The isolates were graded for resistance to three or more antibiotics were recorded as multiple antibiotic resistant [14]. The antimicrobial discs (Oxoid) used were penicillin, enrofloxacin, amikacin, cefotaxime, cefoperazone, meropenem, imipenem, gentamicin, tetracycline and oxacillin.

DNA extraction and PCR amplification

Extraction of DNA from isolates was done using Mini kit of QIAamp DNA (Qiagen, Germany, GmbH) followed the manufactured instructions. Amplification of the PCR was done using oligonucleotide primers (supplied from Metabion, Germany) listed in Table 1. The primers were utilized in a reaction containing Emerald Amp Max PCR Master Mix (Takara, Japan). A functional thermal cycler biosystem 2720 was used.

Analysis of the Polymerase Chain Reaction (PCR) products

By electrophoresis separation, PCR products analysis was prepared

on agarose gel (Applichem, Germany, GmbH) in 1X TBE buffer (at room temperature). The DNA Ladders (Qiagen, Germany, GmbH) have been used. Photographing the gel was done via gel documentation system (Alpha Innotech, Biometra) computer software analyzed.

Bovine SAA4 and Cp analysis

Serum and milk SAA4 and Cp concentrations were determined in faculty of medicine, Cairo University, Egypt, using commercial Enzyme-Linked Immunosorbent Assay (ELISA) Kits (for serum). Quantitative immunoassay techniques were used for determination of bovine SAA4 (Nori bovine SAA4 ELISA Kit, Genorise Scientific, INC., USA, Cat. GRC112079) and bovine Cp (MyBioSource, Cat. No. MBS 703493). Samples were analyzed in duplicate following the manufacturer's instructions.

Statistical analysis

The biochemical parameters were statistically analyzed by using SPSS 17.0 program for windows, analysis of variance (ANOVA), LSD test for defining the statistical significance. Data were presented as means ± Standard Deviations (SD).

Results

Prevalence and bacteriological identification

Single bacterial isolates of *Escherichia coli* and *Staphylococcus aureus* were constituted 23 (19.2%) and 17 (14.2%) of 120 mastitic milk samples respectively. Meanwhile the mixed infection was 35 (29.16%) isolates.

Antibiotic susceptibility assay

Multidrug resistance of *E. coli* were 9 out of 23 isolates; three (for penicillin, cefotaxime, and tetracycline), four (for cefotaxime, penicillin, cefoprazone and gentamicin) and two (for penicillin, cefotaxime and amikacin). Table 2 demonstrates antibiotic susceptibility for *E. coli*.

Regarding to *S. aureus*, multidrug resistance was eight out of 17 (penicillin, oxacillin and cefotaxime) isolates. Antibiotic susceptibility of *S. aureus* isolates were tabulated (Table 3).

Gene detection

Two out of four *E. coli* isolates carried bla_{TEM} (516 bp) gene. These isolates resisted cefotaxime, penicillin, cefoprazone and gentamicin. TetA (A) (576 bp) gene was present in two out of four isolates resisted to tetracycline (Figure 1).

Concerning to *S. aureus* isolates, positive amplification of bla_Z (173) gene was investigated in all eight isolates resisted to penicillin group.

Target gene	Primers sequences	Ampl. segment (bp)	Prim. Denat.	Amplification (35 cycles)			Final exten.	Ref.
				Sec. Denat.	Ann.	Exten.		
<i>Staphylococcus aureus</i> bla _Z	ACTTCAACACCTGCTGCTTTC	173	94°C 5min	94°C 30 s	54°C 30 s	72°C 30 s	72°C 7min	[37]
	TGACCACTTTTATCAGCAACC							
<i>Staphylococcus aureus</i> mecA	GTAGAAATGACTGAACGTCGGATAA	310	94°C 5 min	94°C 45 s	50°C 45 s	72°C 45 s	72°C 10 min	[38]
	CCAATTCCACATTGTTTCGGTCTAA							
<i>Escherichia coli</i> bla _{TEM}	ATCAGCAATAAACCAGC	516	94°C 10 min	94°C 45 s	54°C 45 s	72°C 45 s	72°C 10 min	[39]
	CCCCGAAGAACGTTTTTC							
<i>Escherichia coli</i> TetA	GGTTCACCTCGAACGACGTC	576 bp	94°C 5 min	94°C 45 s	50°C 45 s	72°C 45 s	72°C 7 min	[40]
	CTGTCCGACAAGTTGCATGA							

Ampl: Amplified, Prim, Primary; sec: Secondary; denat: Denaturation; Ann: Annealing; exten: Extension; Ref: References

Table 1: The target genes, sequences of primers, sizes of amplification and cycling conditions in *Staphylococcus aureus* and *Escherichia coli* bacteria isolated from mastitic Holstein cattle (3-7 years old) milk in 2014-2015.

Antibiotic	Susceptible		Intermediate		Resistant	
	Number	%	Number	%	Number	%
Penicillin	-	-	3	13.04	20	86.9
Enrofloxacin	18	78.26	5	21.73	-	-
Amikacin	19	82.60	2	8.69	2	8.69
Cefotaxime	-	-	7	30.43	15	65.21
Cefoprazone	15	65.21	5	21.73	5	21.73
Imipenem	23	100	-	-	-	-
Meropenem	20	86.95	3	13.04	-	-
Gentamicin	17	73.91	4	17.39	2	8.69
Tetracycline	5	21.73	4	17.39	14	60.86
Oxacillin	5	21.73	8	34.78	10	43.47

Table 2: Results of antibiotic sensitivity test of *Escherichia coli* milk samples (n=23) of mastitic Holstein cattle (3–7 years of age) in 2014-2015.

Antibiotic	Susceptible		Intermediate		Resistant	
	Number	%	Number	%	Number	%
Penicillin	-	-	-	-	17	100
Enrofloxacin	16	94.11	1	5.88	-	-
Amikacin	10	58.82	2	11.76	5	29.41
Cefotaxime	7	41.17	3	17.64	8	47.58
Cefoprazone	12	70.58	5	29.41	-	-
Imipenem	16	94.11	1	5.88	-	-
Meropenem	14	82.35	3	17.64	-	-
Gentamicin	13	76.47	4	23.52	-	-
Tetracycline	10	58.82	2	11.76	7	41.17
Oxacillin	5	29.41	3	17.64	9	52.94

Table 3: Results of antibiotic sensitivity test of *Staphylococcus aureus* milk samples (n=17) of mastitic Holstein cattle (3–7 years of age) in 2014-2015.

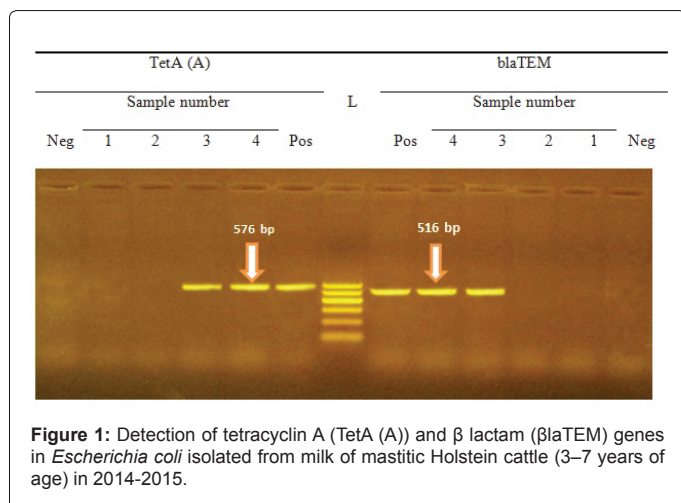


Figure 1: Detection of tetracyclin A (TetA (A)) and β lactam (β laTEM) genes in *Escherichia coli* isolated from milk of mastitic Holstein cattle (3–7 years of age) in 2014-2015.

Also, there were six out of eight (75%) isolates resisted to oxacillin group were positive for *mecA* (310 bp) gene (Figure 2).

Bovine SAA4 and Cp analysis

As shown in Table 4, in *E. coli* group, both SAA and Cp levels increased significantly in serum meanwhile Cp increased significantly in milk only as compared to control group. While, in *S. aureus* group, each parameter level elevated significantly in milk while only SAA level elevated in serum in comparative with control one.

Discussion

Escherichia coli and *Staphylococcus aureus* constitute important

cause of contagious and environmental mastitis in dairy cattle respectively [15]. Regarding to *E. coli* isolates, the present results were closely like to results (17.82%) of clinical mastitis study in Egypt [16] but higher than others in Slovakia [17] and in Uganda [18].

Prevalence percent of *S. aureus* isolates was nearly similar as a study in Egypt (11 isolates of 86) [19], contraries, another study [17] establish moderately low percent (9.74%). The differences are due to variations in management or season, as well immunity and closed houses in winter are of a great concern [15]. Also, current study selected only single isolates of both microorganisms.

Haphazard usage of antimicrobial agents in treatment of mastitis affected the resistance of *E. coli* and *S. aureus* isolates [17]. Susceptibility results were agreed with Chandrasekaran et al. [20] for *E. coli* isolates. For *S. aureus*, results were closely agreed with Beyene [21] and nearly (57.14%) with others [22]. *Staphylococcus* spp. resisted methicillin was attributed to β -lactamase and penicillin binding protein production [23] and *blaZ* gene was responsible for β -lactamase production [24].

In serum, present elevation level of SAA did not differ from previous reports [7,25] except in percentage which may differed according to the severity or chronicity of disease or isoforms nature with strong correlation with mammary cells [26]. But, its elevation in milk was confused. With serum ELISA kits, it did not change in serum and milk, while it varied with milk ELISA kits [27].

In general, inflamed mammary tissue raise the permeability of blood vessels and leakage of APPs to the milk flow [28]. The SAA improved chemotaxis (movement in response to chemical substance) of monocyte and neutrophil and opsonization (adsorption to facilitate adhesion to bacteria) for further phagocytosis as well as getting rid

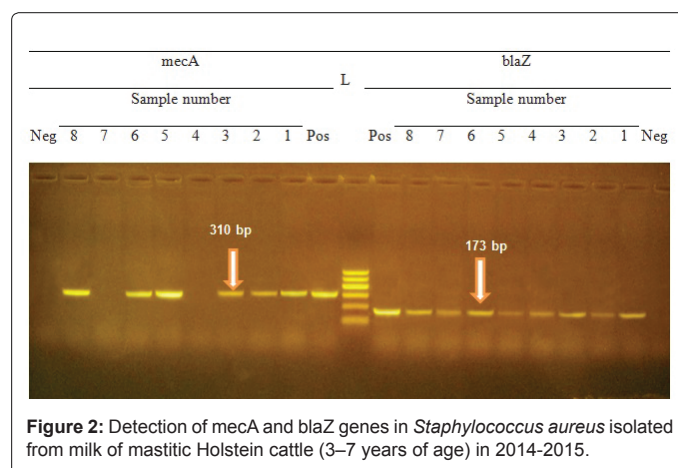


Figure 2: Detection of *mecA* and *blaZ* genes in *Staphylococcus aureus* isolated from milk of mastitic Holstein cattle (3–7 years of age) in 2014-2015.

Parameters	Groups		
	Control	<i>Escherichia coli</i>	<i>Staphylococcus aureus</i>
Blood			
SAA4 (mg/dL)	17.56 ± 4.33 ^b	23.61 ± 7.29 ^a	22.23 ± 2.27 ^a
Cp (mg/dL)	24.44 ± 3.40 ^b	33.61 ± 13.17 ^a	25.63 ± 8.44 ^{ab}
Milk			
SAA4 (mg/dL)	2.15 ± 0.13 ^b	2.53 ± 0.63 ^{ab}	2.88 ± 0.40 ^a
Cp (mg/dL)	6.06 ± 0.47 ^b	7.18 ± 1.57 ^a	7.34 ± 0.60 ^a

SAA4: Serum Amyloid A4; Cp: Ceruloplasmin. Means with the same letter (a, b) are not significantly different at P<0.05 in the same row

Table 4: Serum Amyloid A4 (SAA4) and Ceruloplasmin (Cp) concentration (mean ± SD) in blood serum and milk of mastitic Holstein cattle (3–7 years of age) infected with *Escherichia coli* and *Staphylococcus aureus* in 2014-2015.

of the endotoxins [29]. Further, not only type of pathogen (as *E. coli* and *S. aureus*) affects SAA level [25] but different strains of *S. aureus* are also inducing variable response in vitro in bovine mammary cells [26]. Consequently, SAA may be included for screening cows' mastitis [30,31].

Ceruloplasmin level elevated with the two studied mastitic groups. It is a major source of copper which has excellent anti-oxidative properties against inflammation and infection [32]. All aerobic microorganisms need copper. Meanwhile, when copper exceed the normal limit, it possesses two cycling ability in two oxidation process makes it very toxic for bacteria in assistance with the host macrophage which is copper dependent [33]. Moreover, Cp activates pathogen recognition in dairy cows that intramammary challenged with *E. coli* lipopolysaccharide [34]. Similarly, when treating mammary epithelial cells of murine with *E. coli* endotoxin or lipopolysaccharide *S. aureus*, up-regulation and transportation of some inflammatory marker genes are affected [35]. Both milk Cp and SAA levels depend on the method of measurement to be diagnostic tools for subclinical mastitis and many inflammatory diseases in dairy cattle [7,36].

In conclusion, prevalence of *E. coli* and *S. aureus* isolates was high in mastitic cattle of the studied samples and affect human life. High percent of the isolates resistance genes confirmed antibiotics problem particularly against methicillin resistance groups and multidrug resistance that was dramatically growing. Consequently the chance of treatment was decreased by excluding some important drugs such as penicillin then cefotaxime and oxacillin. On the other hand the diagnosis appeared to be helpful via determination of SAA and Cp in serum and milk. They may be included to quick differentiation between the two microbes and save time for correct treatment.

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