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FREQUENTLY ISOLATED BACTERIA FROM LACTATING COWS WITH CLINICAL AND SUBCLINICAL MASTITIS IN GHARBIA GOVERNORATE, EGYPT

Fatma M. Abo Zaid[1]*, Hassan Y. Abd ElHamed[2]*, El-Sagher O. Ahmed[2]*, Amal M. Eid[1]*, Adel E. A. Mohamed[2]

[1]Animal Health Research Institute, Tanta, Egypt, [2]Department of Animal Medicine, Faculty of veterinary Medicine, South Valley University, Qena, Egypt.

ABSTRACT

This study was carried out on a total number of 122 mixed breed cows (46 clinical and 76 subclinical mastitic cows) aged between 2-8 years old and collected from 17 dairy farms and also single cases from farmers located in different localities in Gharbia, Egypt. Bacteriological culturing revealed CNS isolated from clinical and subclinical cases 28 (60.9%), 19 (25%) respectively, CPS isolated from clinical cases only 9 (19.6%) and E. coli isolated from clinical and subclinical cases 9 (19.6%), 30 (39.5%) respectively. Serological identification of 10 E. coli isolates revealed following serotypes (O26, O111, O114, O125, O146 and O166). Sensitivity test for (CPS) showed that the highest resistance was to amoxicillin 66.7% and the lowest resistance was to Gentamycin and Sulphatrimethoprime 11%, (CNS) showed that the highest resistance was to Oxacillin 46.8% then Kanamycin 8.5% and E. Coli showed that the highest resistance was to Oxacillin 59%, the lowest resistance was to Gentamycin 5.1%, complete susceptibility to Enrofloxacine, Ciprofloxacine and high susceptibility to Gentamycin and Sulphatrimethoprime. Multiplex PCR of (CPS) isolates resulted in all isolates give a positive reaction at 279bp of nuc genes (Staphylococcus aureus) (S. aureus) meanwhile 2 isolates 1.6% (2/122) give a positive reaction at 147bp of mecA genes (methicillin resistance S. aureus) (MRSA). The objective of this study is to isolate and identify the common bacterial isolates from mixed breed mastitic cows, detection the antibiotics sensitivity of the studied bacteria and molecular detection of nuc genes (S. aureus specific primers) and mecA genes (methicillin resistance S. aureus specific primers) in (CPS).

Key words: ***Bovine mastitis; E. Coli; CPS; CNS; S. aureus; MRSA; nuc genes; mecA genes.***

INTRODUCTION:

Mastitis can be defined as an inflammation of the mammary gland which cause physical, chemical, bacteriological and cytological changes in milk, pathological changes in the udder glandular tissues and can notice effects on the quality and quantity of milk (Amir, 2013).

Its severity can be categorized into clinical, sub-clinical and chronic forms, while its degree is relying on the kind of causative organism, breed, age, immunological health and lactation stage of the infected animal (Yalcin, 2000).

It is mainly caused by microorganisms which usually including gram-negative and gram-positive bacteria, yeasts, algae and mycoplasma (Zadoks et al., 2011). S. aureus is the most widespread and economically important pathogen which causing inflammatory infections in dairy ruminants (Katsuda at al., 2005). S. aureus can reach milk either by direct excretion from udders

with clinical or subclinical Staphylococcal mastitis or by environmental contamination during handling of raw milk, so approximately 30-40% of all mastitis cases are associated with S. aureus (Jorgensen et al.,2005).

Coagulase Negative Staphylococcai (CNS) are constantly existing on the udder skin and in teat canals, under a suitable condition they penetrate the galactogenic pathway to the quarter, the pathogenic mechanisms of them can be represented by two parameters which are invasiveness (ability to penetrate the protective barriers, adhere to host cells and to form a biofilm) and toxicity (can produce toxins and enzymes which including proteases and hemolysis) (Bochniarz and Wawron, 2012). E. coli is the prevalent coliform species leading to mastitis infection in dairy animals during parturition and lactation stages by prominent local or systemic clinical symptoms.

It is widely known that animal, environmental and bacterial factors are interdependently and affecting mastitis susceptibility (Quesnell et al., 2012).

Mastitis is the most common frequent reason for using of antimicrobial drugs in dairy herds as antibiotic therapy is a primary instrument in its controlling in lactating and dry cows (Sawant et al., 2005).

The most widely used antibiotics in treatments of bovine mastitis are β-lactams which working by inhibiting cell wall synthesis by the bacterial organism (Kwon et al., 2006).

Mechanism of β-lactams resistance in Staphylococci include forming of β-lactamases and/or production of low affinity Penicillin binding protein (PBP), identified as methicillin resistance (MR) which prevent therapy of any currently available β-lactam antibiotics and may cause resistance to other classes of antibiotics beside β-lactams among all Staphylococci (Olsen et al., 2006).

Production of a modified form of penicillin binding protein 2A (PBP2) is encoded by the mecA gene (Kilic et al., 2006).

MATERIAL AND METHODS:

This study carried on 122 mixed breed cows aged between 2-8 years old and including 76 cases of subclinical mastitis and 46 cases of clinical mastitis. These cows selected from Gharbia governorate, Egypt.

The data involved including age, parity, stage of lactation, udder health monitoring, milking practice and cleanliness of the bedding materials.

Clinical examinations of selected cows were performed according to (Constable et al., 2014).

The California Mastitis Test (CMT) was performed for detection of subclinical mastitis according to the manufacturer's instruction (Mellenberger and Carol, 2000).

Sampling of milk using standard methods described by (National Mastitis Council ,1999).

Bacteriological examination of milk samples according to (Qunin et al., 2002).

While Identification of Staphylococcus and E-Coli spp. according to (Macfaddin., 2000) and (Harley and Prescott, 2007).

Serological identification of E. coli isolates according to (Kok et al., 1996) at Animal health research institute, El Dokky, Egypt. Using rapid diagnostic E. coli antisera sets (DENKA SEIKEN Co., Japan).Virulence factors for E. coli isolates were detected by hemolytic activity according to (Beutin et al., 1989) and Congo Red (CR) binding test according to (Panigrahy and Yushen, 1990).

Antibiotic sensitivity test for bacterial isolates according to (Jorgensen and Ferraro,2009).

Penicillin G; P (10 units/disk), Amoxicillin; AML (10µg/disk) and Oxacillin; Ox (1µg/disk) were used to test phenotypic expression of nuc and mecA genes. The following antibiotics were also tested including Enrofloxacin; ENR (5µg /disk), Ciprofloxacin; Cip (5µg/disk), Kanamycin; k (30µg/disk), Gentamycin; CN (10µg/disk), Sulpha-Methoxazole Trimethoprime; SXT (25µg/disk), Oxytetracycline; OT(30µg/disk).

Multiplex Polymerase Chain Reaction (PCR) for (CPS) isolates were performed according to (Reischl et al., 1994).

Primers used for PCR amplification were synthesized in Bio Basic Inc. (Canada). Details of primer sequences, their specific targets, amplicon sizes and references are summarized in Table 1.

Table 1: Primer sequences, references, their specific targets and amplicon sizes

Primer name	Primer sequence 5′-3′	Product size	Specificity	References
16SrRNAf 16SrRNAr	5′-GTA GGT GGC AAG CGTTAT CC 3′ 5′- CGC ACA TCA GCG TCA G 3′	228bp	Staphylococcus genus specific primers	Monday and Bohach, 1999
nuc1 nuc2	5′-GCGATTGATGGTGATACGGTT- 5′AGCCAAGCCTTGACGAACTAAAGC-3′	279bp	Staphylococcus aureus specific primers	Brakstad et al., 1992
mecA f mecA r	5′-GTG AAG ATA TAC CAA GTG ATT3′ 5′-ATG CGC TAT AGA TTG AAA GGAT 3′	147bp	Methicillin resistant staphylococci specific primers	Zhang et al., 2005

RESULTS

Based on the history, results of clinical examination, clinical signs, isolation and identification of causative pathogens, clinical and subclinical mastitis were diagnosed in the examined cases.

Table 2 showed bacteriological isolation of 122 milk samples (clinical 46 and subclinical 76) which resulted in CNS isolated from clinical and subclinical 28 (60.9%), 19 (25%) respectively, CPS isolated from clinical cases only 9 (19.6%) and E. coli isolated from clinical and subclinical 9 (19.6%), 30 (39.5%) respectively.

It was essential to mention that 27 milk samples obtained from clinically healthy cows (subclinical mastitis) were negative for the tested bacteria.

Table 3 showed serological identification of 10 E. coli isolates.

Table 4 showed virulence factors of all E. Coli isolates including hemolytic and CR activity.

Table 5 showed sensitivity test of (CPS) isolates in which the highest resistance to amoxicillin (66.7%), lowest resistance to gentamycin and sulphatrimethoprim (11.11%) and complete susceptibility to enrofloxacin and ciprofloxacin antibiotic discs.

Table 6 showed sensitivity test of (CNS) isolates in which the highest resistance to oxacillin (46.8%) then kanamycin (8.5%) and complete susceptibility to all other tested antibiotic discs.

Table 7 showed that E. Coli isolates had the highest resistance to oxacillin (59%), lowest resistance to gentamycin (5.1%) and complete susceptibility to enrofloxacin, ciprofloxacin and kanamycin antibiotics.

Table 8 showed molecular typing of (CPS) isolates.

Table 2: Bacteriological isolation of pathogenic bacteria from mastitic milk samples.

Isolated bacteria / Type of mastitis	CNS		CPS		E. Coli	
	No.	%	No.	%	No.	%
Clinical (N=46)	28	60.9	9	19.6	9	19.6
Subclinical (N=76)	19	25	0	0	30	39.5

Table 3: Serological identification of E-Coli isolates. *

Serotypes	Strain	N.	%
O26	Shiga Toxin Producing (STEC)	1	10%
O111	Enterohaemorrhagic	1	10%
O114	Enteropathogenic	1	10%
O125	Enteropathogenic	3	30%
O146	Enteropathogenic	3	30%
O 166	Enterotoxogenic	1	10%
Total		10	100%

*(N=10)

Table 4: Virulence factors of E. Coli isolates*

Hemolytic Activity test				Congo Red Binding test	
Alpha hemolytic		Beta hemolytic		Positive	%
N.	%	N.	%		
3	7.7%	1	2.6%	8	20.5%

*(N=39)

Table 5: Sensitivity test of(CPS) isolates*

Antibiotic tested	Number of phenotypic isolates*			% of resistant phenotypes
	Susceptible	Intermediate	Resistant	
Penicillin	3	1	5	55.6%
Amoxicillin	3	0	6	66.7%
Oxacillin	2	3	4	44.4%
Gentamycin	7	1	1	11%
Enrofloxacin	9	0	0	0
Kanamycin	6	1	2	22.2%
Sulphatrymethoprim	7	1	1	11%
Ciprofloxacin	9	0	0	0

*(N=9)

Table 6: Sensitivity test of(CNS) isolates*

Antibiotic tested	Number of phenotypic isolates *			% of resistant phenotypes
	Susceptible	Intermediate	Resistant	
Penicillin	29	18	0	0
Amoxicillin	38	9	0	0
Oxacillin	14	11	22	46.8%
Gentamycin	36	11	0	0
Enrofloxacin	38	9	0	0
Kanamycin	34	9	4	8.5%
Sulphatrymethoprim	47	0	0	0
Ciprofloxacin	38	9	0	0

*(N=47)

Table 7: Sensitivity test of E-Coli isolates*

Antibiotic tested	Number of phenotypic isolates*			%of resistant phenotypes
	Susceptible	Intermediate	Resistant	
Penicillin	28	0	11	28.2%
Amoxicillin	26	0	13	33.3%
Oxacillin	16	0	23	59%
Gentamycin	32	5	2	5.1%
Enrofloxacin	35	4	0	0
Kanamycin	26	13	0	0
Sulphatrymethoprim	32	2	5	12.8%
Ciprofloxacin	38	1	0	0

***(N=39)**

Table 8: Molecular typing of (CPS)isolated from examined samples*

Genes	*Coagulase positive Staphylococcus*	
	N.	%
nuc genes*(Staphylococus aureus)*	9	100%
mecA genes *(MRSA)*	2	22.2%

***(N=9)**

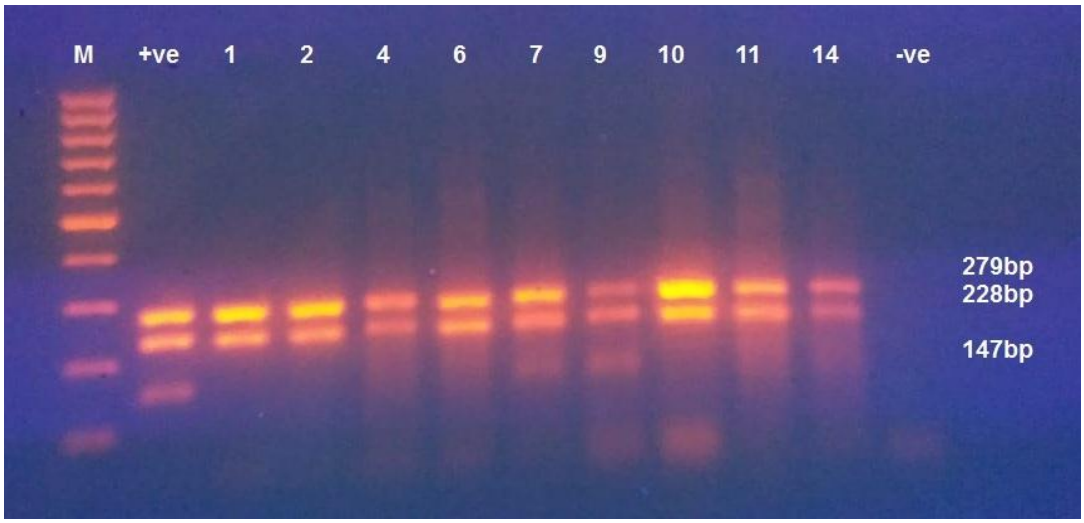

Figure 1: Multiplex PCR assay of 9 (CPS) isolates for detecting the nuc genes at (279 bp) S. aureus specific primers, 16SrRNA genes at (228bp) Staphylococcus genus specific primers and mecA genes at (147 bp) Methicillin Resistant Staphylococcus specific primers.

M: 100 bp ladder DNA marker, +ve.: mecA positive S. aureus positive control,

-ve.: negative control.

Lanes 1, 2, 4, 6, 7, 9, 10, 11, 14 positives to 16SrRNA and nuc (S. aureus). Lanes 1, 2, 4, 6, 10, 11, 14 are mecA negative S. aureus isolates. lanes 7&9: mecA positive S. aureus isolates

DISCUSSION

Bovine mastitis is the most complicated disease affecting production systems of dairy farms and having both zoonotic and economic significance (Zeryehun et al., 2013).

Precise and periodical diagnosis of bovine mastitis assist us in decreasing its incidence rate so we can reduce its economic costs which would be aggravated with any delay (Viguier et al., 2009).

Bacteriological isolations revealed CNS which isolated from clinical and subclinical 28 (60.9%), 19 (25%) respectively, CPS isolated from clinical cases only 9 (19.6%) and E. coli isolated from clinical and subclinical 9 (19.6%), 30 (39.5%) respectively. These results are closely related to (Jeykumar et al., 2013) and (Nadeem et al., 2014) and disagree with (Mekibib et al., 2010).

The high incidence rate of (CNS) meaning increasing of their importance as mastitis pathogen which reported by (Smith. 2001). The present findings revealed high proportion of (CNS) as an important cause of clinical and subclinical mastitis and this agree with (Oliver et al., 2005).

The variations in the results of the bacterial isolates due to several factors such as the age, parity, breed, stage of lactation, season of the year, udder health monitoring, cleanliness of milking facilities and utensils, udder cleanliness, the milking practice and the body condition score (Constable et al., 2014).

E. coli isolates serotyping was done to give information about the most important and prevalent serotypes helping in mastitis occurrence. Our results showed serotypes (O26, O111, O114, O125, O146 and O166). These results showed that amongst the serotyped strains of E. coli there was no dominant serotype. These results are agreeing with (Sayed, 2014).

Previous results showed that E. coli mastitis is not caused by a fixed number of particular pathogenic strains, but it is associated with environmental fecal contamination and be multi factorial causes (Rangel and Marin, 2009).

We can use Congo red (CR) agar test as a detective method for E. coli virulence and it also help in differentiation between virulent and a virulent E. coli strains (Sharma et al., 2006).

CR binding ability showed that 8/39 isolates (20.5%) had CR binding activity. These results are partially related to (Sharma et al., 2006) and (Amira et al., 2013) and disagree with (Zaki et al., 2004).

E. Coli hemolytic activity revealed Alpha hemolytic 7.7% and beta hemolytic 2.6%. These results are closely agreeing with (Zaki et al., 2004) and (Amira et al., 2013) .

The antimicrobial studies for mastitis causative pathogens are important because they detect the most suitable antibiotic therapy, decreasing their resistance and reduce the public potential health hazards (Akram et al., 2013).

The highest resistance of (CPS) isolates was to amoxicillin (66.7%) then oxacillin (44.4%). These results are closely agreeing with (Jagadeeswari et al., 2013).

The highest resistance of (CNS) was to oxacillin (42.3%) then kanamycin (9.9%). These results are agreeing with (Basappa et al., 2011).

The highest resistance of E. Coli was to oxacillin (56%) then amoxicillin (36%). These results are agree with (Sumathi et al., 2008) and disagree with (Singh et al., 2018).

The variability of different pathogens in their response to different antibiotics with the random use of these drugs without testing is the vital reason for treatment crash. Agar gel diffusion test for antimicrobial drugs must be used to choose the correct therapy, avoiding time waste and heavy costs on owners (Grag, 2001).

Multiplex PCR revealed that all (CPS) positive for nuc genes (S. aureus) and 22.2% isolates were methicillin resistance S. aureus (MRSA). These results are partially related to (Vanderhaeghen et al., 2010).

Results showed that incidence of methicillin resistance among (CPS) and (CNS)were higher than among S. aureus isolates and that declared the importance of both (CPS) and (CNS)as mastitis pathogens. These results are agree with (Luthje et al., 2006).

Conclusion: The findings of this study showed prevalence of the common bacteria causing bovine mastitis in Gharbia governorate, Egypt which included (CPS), (CNS) and E. Coli. Multiplex PCR assay showed that all tested (CPS) were positive to nuc genes (S. aureus) meanwhile mecA genes observed in two isolates only(MRSA). Results of sensitivity test for all bacterial isolates showed that enrofloxacin, ciprofloxacin and gentamycin were the most suitable antibiotics used for bovine mastitis control in the studied area so the antibiotic sensitivity test for mastitis causing bacteria is important to get maximum efficacy of antibiotics which will reduce the development of resistance to antibiotics.

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البكتيريا المعزولة فى كثير من حالات التهاب الضرع السريرى وتحت السريرى من الابقار المرضعة بمحافظة الغربية ، مصر.

د. فاطمة مرغنى طه أبوزيد ١*، د. حسن يوسف عبد الحميد٢* ، د. الصغير عمران احمد على ٢*

د. أمل مصطفى عيد ١* ، ا.د. عادل السيد احمد محمد ٢* .

١* معهد بحوث الصحة الحيوانية ، طنطا ، مصر .

٢* قسم طب الحيوان ، كلية الطب البيطرى ، جامعة جنوب الوادى، قنا، مصر

الملخص العربي :

أجريت هذه الدراسة على ١٢٢ بقرة من سلالة مختلطة (٤٦ بقرة مصابة بالتهاب الضرع السريرى و٧٦ بقرة مصابة بالتهاب الضرع تحت ألسريري) وتتراوح أعمارهم بين ٢–٨ سنوات وتم الحصول عليها من ١٧ مزرعة ألبان وكذلك من حالات مفردة من المزارعين في مناطق مختلفة في محافظة الغربية بمصر.

كشفت الدراسات البكتيريولوجية عن وجود معزولات المكورات العنقودية (CNS) من الابقار السريرية وتحت السريرية بنسبة ٢٨ (٦٠.٨%) و ١٩ (٢٥%) على التوالى ، معزولات المكورات العنقودية (CPS) من الابقار السريرية فقط بنسبة ٩ (٢٥%) ثم معزولات الاشريكية القولونية (E. Coli) من الابقار السريرية وتحت السريرية بنسبة ٩ (١٩.٦%) و ٣٠ (٣٩.٥%) على التوالى.

كشف التعريف السيرولوجى لعشرة معزولات من البكتيريا الاشريكية القولونية عن وجود . (O146,O125, O114,O166 ,O111, O26)

أظهر اختبار حساسية المضادات الحيوية لمعزولات البكتيريا العنقودية (CPS) أن أعلى مقاومة كانت للاموكسيسيلين ٦٦.٧% واقل مقاومة للجنتاميسين والسلفاترايميثوبريم ١١% ثم المعزولات البكتيرية العنقودية (CNS) أظهرت أعلى مقاومة للاوكسيسيلين ٤٦.٨% و اقل مقاومة للكاناميسين ٨.٥% بينما أظهرت الاشريكية القولونية (E.coli)أعلى مقاومة للاوكساسيلين ٥٩% واقل مقاومة للجنتاميسين ٥.١% وكذلك سجلت نتائج اختبار الحساسية القابلية الكاملة للانروفلوكساسين والسيبروفلوكساسين و حساسية عالية للجنتاميسين والسلفاترايميثوبريم .

أظهر تفاعل البلمرة المتسلسل المتعدد لتسعة معزولات من البكتيريا العنقودية الموجبة لاختبار الكواجيوليز أن جميع المعزولات تعطى تفاعلا ايجابيا عند جينات البكتيريا العنقودية اوريوس بينما ١.٦ % (١٢٢/٢) فقط موجبة عند جينات البكتيريا العنقودية اوريوس المقاومة للمسيسيللين .

الهدف من هذه الدراسة هو عزل وتحديد المعزولات البكتيرية الشائعة من الأبقار المصابة بعدوى التهاب الضرع، الكشف عن حساسية المضادات الحيوية للبكتيريا المدروسة والكشف الجزيئي للجينات الخاص بالبكتيريا العنقودية (CPS).