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Original article

GENES CONFERRING ANTIMICROBIAL RESISTANCE IN CATTLE WITH SUBCLINICAL MASTITIS

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Summary

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This study was carried out to evaluate the antimicrobial resistance (AMR) as a risk factor associated with some microorganisms isolated from subclinical mastitis (SCM) milk samples from Holstein Friesian dairy animals in Fayoum area, Egypt. The percentage of the SCM in the farm was found to be 41.18% and 63.88% at quarter and cows level respectively, with mean somatic cell count (SCC) of $8.8\times10^5 \pm 9.2\times10^3$ cells/mL and electrical conductivity (EC) 6.27 ± 0.066 mS/cm for SCM quarter milk samples. Out of the total 444 SCM cow milk samples, the most often isolated microorganisms were *Staphylococcus aureus:* 296 (66.6%), *Enterococcus* spp.: 230 (51.80%), *Escherichia coli:* 210 (47.29%) and *Streptococcus agalactiae:* 106 (23.87%). AMR was determined by disc diffusion test and the corresponding resistance genes were detected by PCR. Results of the *in vitro* susceptibility tests performed and the phenotypes indicated that the highest resistance to antibiotics for isolated microorganisms was against penicillin followed by amoxicillin + clavulanic acid, oxacillin and tetracycline, whereas moderate resistance was exhibited to oxytetracycline, ampicillin, sulfamethazole/trimethoprim, cefotaxime and erythromycin. However the most effective antibiotics against most isolates were nitrofurantoin and gentamicin followed by enrofloxacin, norfloxacin and cefoxitin. It was shown that the resistance to tetracyclines was due to the *tetK* or *tetA(A)* genes, the resistance to β-lactams (penicillins) – to *blaZ* and *blaTEM* genes, to macrolides (erythromycin): to *ermB* and *ermC* genes. Methicillin resistance genes were *mecA, mec1* and *mecC*, glycopeptides (vancomycin) resistance gene was *vanA*, and norfloxacin resistance was attributed to *norA* gene.

Key words: antimicrobial resistance (AMR) gene, risk factor, subclinical mastitis (SCM)

INTRODUCTION

Mastitis is defined as inflammatory disease condition of the udder affecting milk production and having a real effect on the dairy farm economy. It is considered to be the foremost costly disease of dairy animals through discarded milk, reduction in milk yield, premature culling of animals and replacements. If the subclinical mastitis (SCM) infection persists for longer periods, it may lead to formation of fi-

brous tissue barrier between the organisms and the antibiotic preparations limiting their efficacy (Pleguezuelos *et al.,* 2015).

Most of the time, the treatment of mastitis is applied before knowing the causative microorganism or without appropriate anti-microbial testing that leads to the use of antibiotics which are not effective to the pathogens (Suleiman *et al.,* 2018).

To approach suitable treatment and control degree, it is imperative to perform antibiotic susceptibility test of relevant antimicrobials because the regular utilisation of commonly used antibiotics for the treatment of cows or the overuse and abuse of antimicrobial agents have led to the evolution of resistant forms of previously harmless bacteria (Seyoum *et al.,* 2018). Antibiotic resistance is carried on plasmids and transposons that can pass from one species to another (Padol *et al.,* 2015).

Therefore this study was carried out for evaluation of the prevalence of subclinical mastitis, isolation and identification of microbial pathogens from SCM milk samples in a private dairy farm located in Fayoum district, Egypt. Another goal was to determine antibiotic sensitivity for most bacterial isolates and to detect the genes of drug resistance using PCR.

MATERIALS AND METHODS

Study area and animals

A farm (located in Fayoum district, Egypt) with a herd with 2,300 dairy Holstein Friesian cows was chosen for this study. For prevalence of SCM, 2780 quarter milk samples from 695 lactating cows were examined. The farm used milking machine supported by A fiMilk MPC (an Afimilk system) – a milk meter measuring milk yield and milk conductivity for monitoring cow health and milk production. The milking machine detected the SCM automatically through increase of electrical conductivity accompanied with decrease in milk yield production and alarmed so accurate detection allowed the operator to focus on the specific cows that require attention.

Aseptic milk samples collection

Quarter milk samples (n=1,145) from 444 apparently healthy animals suspected to harbour SCM based on increase of electrical conductivity (EC) accompanied with decrease in milk yield production and absence of visible abnormalities of milk secretions or any sign of clinical mastitis, were collected according to Radostitis *et al.* (2007) and subjected to further examination by a California Mastitis Test (CMT) according to Schalm *et al.* (1971). EC was measured according to Linzell & Peaker (1971) and somatic cell counts (SCC): according to Gonzalo *et al.* (2006).

The collected milk samples were prepared for bacterial investigation according to Carter & Cole (2012). The isolated suspected colonies were identified by conventional methods (appearance on incubated plates, colony morphology, Gram staining and different biochemical tests) and by the Vitek 2 compact system (BioMérieux, France).

Antimicrobial sensitivity testing

Antimicrobial sensitivity testing for isolated bacteria was performed using the disk diffusion method (CLSI, 2016). Antibiotic discs and their concentration (μg/mL) used in this study are shown in Table 1.

PCR detection of drug resistance genes

Extraction of DNA was performed with The QIAamp DNA Mini Kit (Qiagen,

Table 1. The concentration of used antibiotic discs (µg/mL)

Antimicrobial agent	Disc concentration	Antimicrobial agent	Disc concentration
Penicillins - Beta-lactams		*Cephalosporins - Beta-lactams*	
Ampicillin – Sulbactam	20 µg	Cephazolin	30 µg
Penicillin	10 IU	Cefepime	30 µg
Ampicillin	10 µg	Cefoperazone	75 µg
Methicillin	5 µg	Cefoxitin	30 µg
Oxacillin	1 µg	Cefotaxime	30 µg
Amoxacillin+clavulanic acid	30 µg	*Macrolides, lincosamides and streptogramins*	
Aminoglycosides		Clindamycin	2 µg
Gentamicin	10 µg	Erythromycin	15 µg
Amikacin	30 µg	*Quinolones*	
Kanamycin	30 µg	Epicoflosin	5 µg
Neomycin	30 µg	Ciprofloxacin	5 µg
Glycopeptides		Norfloxacin	10 µg
Vancomycin	30 µg	Enrofloxacin	5 µg
Tetracyclines		*Sulfa drugs*	
Oxytetracycline	30 µg	Sulfamethoxazole/trimethoprim	25 µg
Doxycycline	30 µg	*Aminocoumarin*	
Tetracycline	30 µg	Novobiocin	5 µg
Miscellaneous antibiotics		*Rifamycins*	
Chloramphenicol	30 µg	Rifampicin	5 µg
Nitrofurantoin	300 µg		

Germany, catalogue no. 51304) according to the manufacturer instructions. Ethanol 96% (Applichem, Darmstadt, Germany) was used for the first washing step. PCR Master Mix used for PCR was Emerald Amp GT PCR Master Mix (Takara, BIO INC., Japan, Code No. RR310A). The mixture also contained a vivid green dye that separated dye fronts into blue and yellow when run on an agarose gel. After PCR, the reaction mixture was applied directly to a gel for analysis. Nine pairs of primers were supplied from Metabion (Germany) or Biobasic (Canada). Their specific sequences and amplified specific products are shown in Table 2.

RESULTS

In this study, subclinical mastitis was found to affect 444 dairy cows (1,145 SCM quarters) out of a total of 695 dairy cow based on CMT, electrical conductivity and SCC data.

The mean SCC value of the examined subclinical mastitic quarter milk samples was $8.8\times10^5 \pm 9.2\times10^3$ cells/mL, with maximum value of 2.7×10^6 cells/mL and minimum value 2.5×10^5 cells/mL. The mean electrical conductivity of examined SCM quarter milk samples was 6.27 ± 0.066 mS/cm (range 5.50–10.83 mS/cm).

The most predominant microorganisms isolated from the samples (Table 3) were *Staphylococcus aureus* (n=296; 66.6%), *Enterococcus* spp. (n=230; 51.80%), *Escherichia coli* (n=210; 47.29%) and *Streptococcus agalactiae* (n=106; 23.87%). Other different pathogens were detected: coagulase-negative *Staphylococcus*, *Klebsiella oxytoca*, *Bacillus subtilis*, *Pseudomonas aeruginosa*

Table 2. Oligonucleotide primers sequences and PCR conditions

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
MecC	F: GCTCCTAATGCTAATGCA R: TAAGCAATAATGACTACC	304 bp	Cuny *et al.,* 2011
MecA	F: GTAGAAATGACTGAACGTCCGATAA R: CCAATTCCACATTGTTTCGGTCTAA	310 bp	McClure *et al.,* 2006
MecI	F: GACACGTGAAGGCTATGATATAT R: ATTCTTCAATATCATCTTCGGAC	344 bp	Stegger *et al.,* 2012
BlaZ	F: ACTTCAACACCTGCTGCTTTC R: TGACCACTTTTATCAGCAACC	173 bp	Duran *et al.,* 2012
TetK	F: GTAGCGACAATAGGTAATAGT R: GTAGTGACAATAAACCTCCTA	360 bp	Duran *et al.,* 2012
NorA	F: TTCACCAAGCCATCAAAAAG R: CTTGCCTTTCTCCAGCAATA	620 bp	Pourmand *et al.,* 2014
Primary denaturation: 94 ˚C/5 min; secondary denaturation: 94 ˚C/30 sec.; annealing: 50 ˚C/30 sec.; extension: 72 ˚C/30 sec.; No. of cycles: 35; final extension: 72 ˚C/7 min.			
ErmC	F: ATCTTTGAAATCGGCTCAGG R: CAAACCCGTATTCCACGATT	295 bp	Schlegelova *et al.,* 2008
ErmB	F: CATTTAACGACGAAACTGGC R: GGAACATCTGTGGTATGGCG	425 bp	Schlegelova *et al.,* 2008
VanA	F: CATGAATAGAATAAAAGTTGCAATA R: CCCCTTTAACGCTAATACGATCAA	1030 bp	Kariyama *et al.,* 2000
tetA(A)	F: GGTTCACTCGAACGACGTCA R: CTGTCCGACAAGTTGCATGA	576 bp	Randall *et al.,* 2004
BlaTEM	F: ATCAGCAATAAACCAGC R: CCCCGAAGAACGTTTTC	516 bp	Colom *et al.,* 2003
Primary denaturation: 94 ˚C/5 min; secondary denaturation: 94 ˚C/30 sec.; annealing: 53 ˚C/30 sec.; extension: 72 ˚C/30 sec.; No. of cycles: 35; final extension: 72 ˚C/7 min.			

F: forward primer; R: reverse primer.

and *Bacillus cereus* with prevalence of 17.34%, 13.73%, 10.13%, 7.20% and 6.08% respectively. The lowest isolation rate in this study was for *Sphingomonas paucimobilis, Enterobacter aerogenes, Raoultella ornithinolytica*, *Pantoea* species, *Bacillus* other species, *Citrobacter amalanaticus* and *Enterobacter cloaca* complex with percentages of 3.82%, 2.92%, 2.70%, 2.25%, 2.02%, 0.67% and 1.12% respectively.

The results of the performed *in vitro* antibiotic sensitivity tests (Tables 4 and 5) indicated that the highest resistance for most isolated microorganisms was against penicillin followed by amoxicillin + clavulanic acid, oxacillin and tetracycline, whereas moderate resistance was exhibited to oxytetracycline, ampicillin, sulfamethazole/trimethoprim, cefotaxime and erythromycin. However the most effective antibiotics against most isolated microorganisms were nitrofurantoin and gentamicin followed by enrofloxacin, norfloxacin and cefoxitin.

The results of the examination of antibiotic resistance genes (Table 6; Fig 1–7) showed that the resistance to tetracyclines was attributed to the *tetK* or *tetA(A)* genes which were expressed in most examined microorganisms, while *blaZ* and *blaTEM* genes related to β-lactams resistance were expressed in all examined isolates. Macrolides resistance genes (*ermB* and *ermC)* showed variable positive reactions in examined microorganisms, while the examined *Staphylococcus aureus* strain was positive for the methicillin-resistant gene *mecA*; however *vanA* and *norA* genes were detected in the examined *E. gallinarum* isolate.

DISCUSSION

Bovine mastitis is considered a remarkable disease that affects dairy cows. It not only causes changes in glandular tissues but also affects the quality and quantity of milk, moreover the health risk to consumers that can be associated with milk due to the presence of zoonotic pathogens and antimicrobial drug residues (Mia *et al.,* 2017). Subclinical mastitis is considered more difficult to be detected because of a lack of clinical signs that can be easily identified by visual inspection and palpation of the udder compared with clinical mastitis. So reliable diagnostic methods are needed to detect subclinical mastitis such as CMT, SCC and electrical conductivity.

The presented data showed that the percentage of the SCM at the farm at quarter and cow level was 41.18% and 63.88% respectively based on CMT and electrical conductivity data. Inspection of our data revealed that the mean value of SCC of subclinical mastitic quarter milk samples was high. Somatic cell count in milk has been accepted as the world standard for mastitis diagnosis and the milk from healthy udders usually has a SCC less than 200,000 cells/mL (Fernandes *et al.,* 2004).

The results presented in this study showed that the high mean EC value of examined SCM milk samples was in agreement with Yoshida (2005).

Table 3. Bacterial species isolated from the positive SCM cow's milk samples (n=444)

Microorganism	Number	%
Staphylococcus aureus	296	66.66
Enterococcus species *(faecalis, faecium, avium, gallinarum)*	230	51.80
Escherichia coli	210	47.29
Streptococcus agalactiae	106	23.87
Coagulase negative *Staphylococcus (Staphylococcus chromogenes)*	77	17.34
Klebsiella oxytoca	61	13.73
Bacillus subtilis	45	10.13
Pseudomonas aeruginosa	32	7.20
Bacillus cereus	27	6.08
Sphingomonas paucimobilis	17	3.82
Enterobacter aerogenes	13	2.92
Raoultella ornithinolytica	12	2.70
Pantoea species	10	2.25
Other *Bacillus* species	9	2.02
Enterobacter cloaca complex	5	1.12
Citrobacter amalanaticus	3	0.67

Table 4. Antibiotic sensitivity test results of *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* isolates

Antimicrobial agent	*Staphylococcus aureus* (n=24)			*Streptococcus agalactiae* (n=30)			*Escherichia coli* (n=30)		
	S	R	I	S	R	I	S	R	I
Cefoxitin	62.5%	37.5%	–	–	–	–	50%	–	50%
Ampicillin	25%	62.5%	50%	40%	60%	–	10%	60%	30%
Oxacillin	50%	25%	25%	50%	50%	–	–	100%	–
Gentamicin	100%	–	–	60%	–	40%	66.6%	10%	23.3%
Clindamycin	50%	50%	–	–	–	–	–	100%	–
Erythromycin	–	100%	–	60%	40%	–	–	70%	30%
Tetracycline	25%	25%	50%	57%	33%	10%	20%	80%	–
Nitrofurantoin	100%	–	–	100%	–	–	70%	–	30%
Trimethoprim/ sulfamethazole	37.5%	62.5%	–	100%	–	–	30%	50%	20%
Amikacin	37.5%	62.5%		40%	60%		20%	80%	
Penicillin	25%	50%	25%		100%			100%	
Enrofloxacin	75%		25%	100%			70%	10%	20%
Amoxacillin+ clavulanic acid	50%	25%	25%	–	50%	50%	30%	50%	20%
Norfloxacin	100%	–	–	100%	–	–	70%	30%	–
Epicoflosin	–	–	–	100%	–	–	100%	–	–
Oxytetracycline	–	100%	–	–	–	100%	–	100%	–
Cefotaxime	–	100%	–	–	–	–	50%	50%	–
Doxycycline	25%	75%	–	–	–	–	–	100%	–
Kanamycin	–	100%	–	–	–	–	50%	50%	–
Cephradine	33%	33%	33%	–	–	–	–	–	–
Novobiocin	–	100%	–	–	–	–	–	–	–
Cefobid	50%	–	50%	–	–	–	–	100%	–
Cephazoline	–	100%	–	–	–	–	–	100%	–
Cefepime	–	50%	50%	–	–	–	–	–	100%
Chloramphe-nicol	–	–	–	–	–	–	70%	30%	–
Rifampicin	–	–	–	–	–	–	–	100%	–
Neomycin	–	–	–	–	–	–	–	100%	–

S=sensitive; R=resistant; I=intermediate

The commonest microorganisms isolated from the samples in our study was *Staphylococcus aureus.* This high prevalence may be due to poor hygienic practices and lack of effective udder washing, post milking teat dipping & drying, this result is nearly similar to results that obtained by Abdel-Rady & Sayed (2009); Alemu *et al.,* (2014); In other studies (Gao *et al.,* 2017; Vakkamäki *et al.,* 2017; Seyoum *et al.,* 2018; Suleiman *et al.,* 2018), this organism showed lower frequency 10.2%, 21%, 47.2% and 36.8% respectively.

The *Enterococcus* spp. was isolated at a high percentage comparable to what was reported by others (Giraffa, 2002; Scheidegger *et al.,* 2009); on the other hand, lower frequencies of 16.4%, 1.3% and 8.02 % were obtained by Kateete *et al.*

Table 5. Antibiotic sensitivity tests results of *Enterococcus* species isolates

Antimicrobial agent	*Enterococcus faecalis* n=10			*Enterococcus faecium* n=10			*Enterococcus gallinarum* n=6			*Enterococcus avium* n=6		
	S	R	I	S	R	I	S	R	I	S	R	I
Cefoxitin	–	–	–	–	–	–	100%	–	–	–	–	100%
Ampicillin	70%	30%	–	–	–	100%		100%	–	–	–	–
Oxacillin	100%	–	–	100%	–	–		100%	–	–	–	–
Gentamicin	100%	–	–	–	–	100%	100%	–	–	–	50%	50%
Clindamycin	100%	–	–	–	–	–	50%	50%	–	–	–	–
Erythromycin	–	100%	–	–	–	–	100%	–	–	–	–	–
Tetracycline	–	100%	–	–	100%	–		100%	–	100%	–	–
Nitrofurantoin	100%	–	–	–	100%	–		–	–	–	–	–
Trimethoprim/sulfamethazole	50%	50%	–	–	100%	–		–	–	–	–	–
Amikacin	70%	30%	–	–	100%	–	100%	–	–	–	–	–
Penicillin	–	100%	–	–	–	–	–	100%	–	–	–	100%
Enrofloxacin	100%	–	–	–	–	100%	–	100%	–	100%	–	–
Amoxacillin/clavulanic acid	–	100%	–	100%	–		–	100%	–	100%	–	–
Norfloxacin	100%	–	–	–	–	100%	–	–	–	–	–	–
Epicoflosin	100%	–	–	–	–	100%	–	–	–	–	–	–
Oxytetracycline	–	100%	–	100%	–	–	–	–	–	–	–	–
Cefotaxime	–	100%	–	–	–	–	–	–	–	–	–	–
Doxycycline	–	–	–	100%	–	–	–	–	–	–	–	–
Kanamycin	100%	–	–	100%	–	–	–	–	–	–	–	–
Cephradine	–	–	–	–	100%	–		–	–	–	–	–
Novobiocin	100%	–	–	–	–	–		–	–	–		–
Vancomycin	100%	–	–	100%	–	–		–	100%	–		–

S=sensitive ; R=resistant; I=intermediate

Table 6. Percentage of positive examined antimicrobial resistance genes for the most dominant isolated microorganisms

Isolated microorganisms	Number	*tet*K	*tet*A(A)	*bla*Z	*bla*TEM	*van*(A)	*nor*A
Staphylococcus aureus	1	100%	–	100%	–	–	–
Enterococcus faecalis	2	100%	–	100%	–	–	–
Enterococcus faecium	2	100%	–	100%	–	–	–
Enterococcus gallinarum	1	100%	–	100%	–	100%	100%
Enterococcus avium	1	100%	–	100%	–	–	–
Escherichia coli	2	–	100%	-	100%	–	–
Streptococcus agalactiae	3	–	–	100%	–	–	–

Isolated microorganisms	Number	*erm*C	*erm*B	*mec*1	*mec*C	*mec*A
Staphylococcus aureus	1	–	–	0%	0%	100%
Enterococcus faecalis	2	50%	–	–	–	–
Enterococcus faecium	2	–	–	–	–	–
Enterococcus gallinarum	1	–	–	–	–	–
Enterococcus avium	1	100%	–	–	–	–
Escherichia coli	2	–	50%	–	–	–
Streptococcus agalactiae	3	–	100%	–	–	–

(–) not examined.

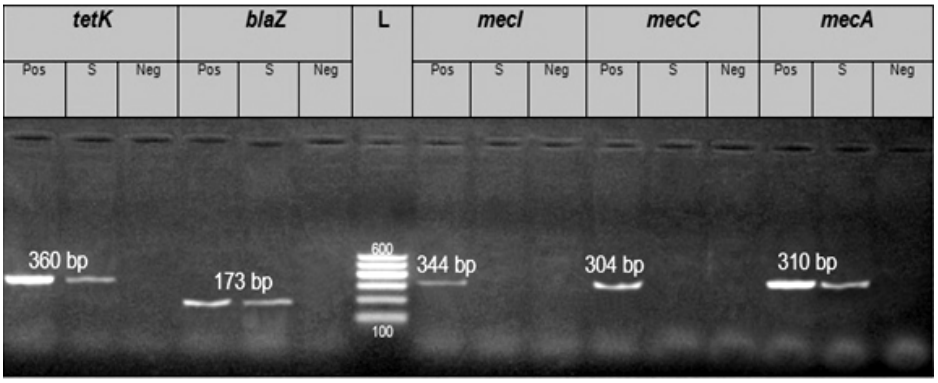

Fig. 1. Agarose gel electrophoresis of detected antibiotic resistance gene DNA fragments of *S. aureus* amplified by PCR from selected isolates (*tetK* – 360 bp), (*blaZ* – 173 bp), (*mecA* – 310 bp), (*mec1* – 344 bp), (*mecC* – 304 bp).

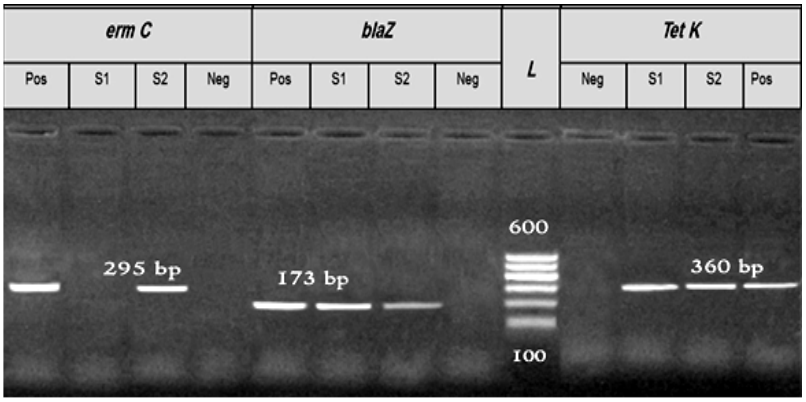

Fig. 2. Agarose gel electrophoresis of detected antibiotic resistance gene DNA fragments of *E. faecalis* amplified by PCR from selected isolates (*tetK* – 360 bp), (*blaZ* – 173bp), (*ermC* – 295bp).

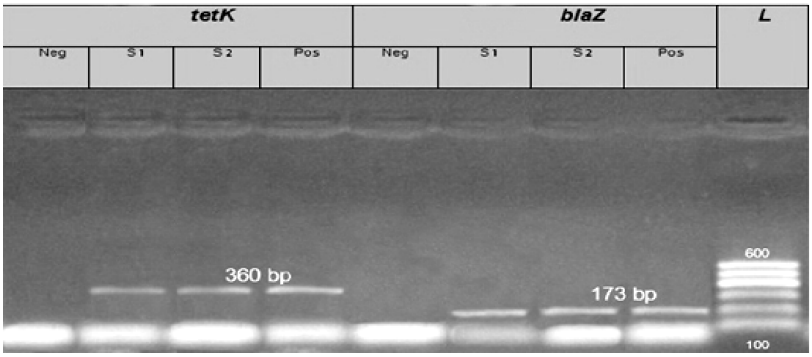

Fig. 3. Agarose gel electrophoresis of detected antibiotic resistance gene DNA fragments of *E. faecium* amplified by PCR from selected isolates (*tetK* – 360 bp), (*blaZ* – 173 bp).

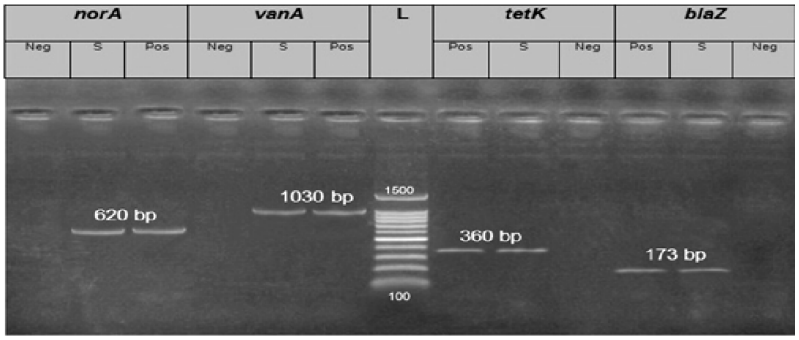

Fig. 4. Agarose gel electrophoresis of detected antibiotic resistance gene DNA fragments of *E. gallinarum* amplified by PCR from selected isolates (*tetK* – 360 bp), (*blaZ* – 173bp), (*norA* – 620bp), (*vanA* – 1030 bp).

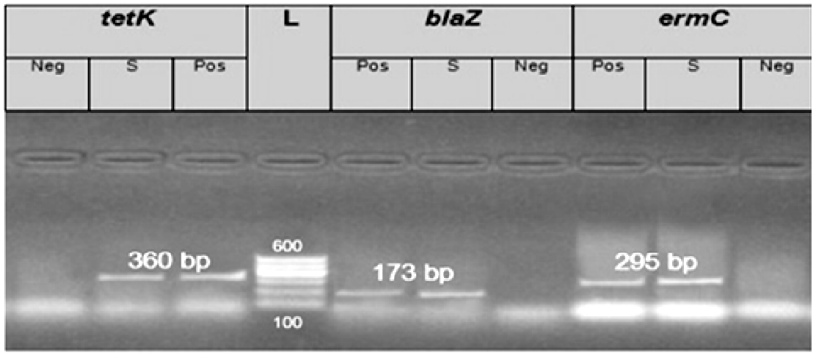

Fig. 5. Agarose gel electrophoresis of detected antibiotic resistance gene DNA fragments of *E. avium* amplified by PCR from selected isolates (*tetk* – 360 bp), *(blaZ* – 173 bp), (*ermC* – 295 bp).

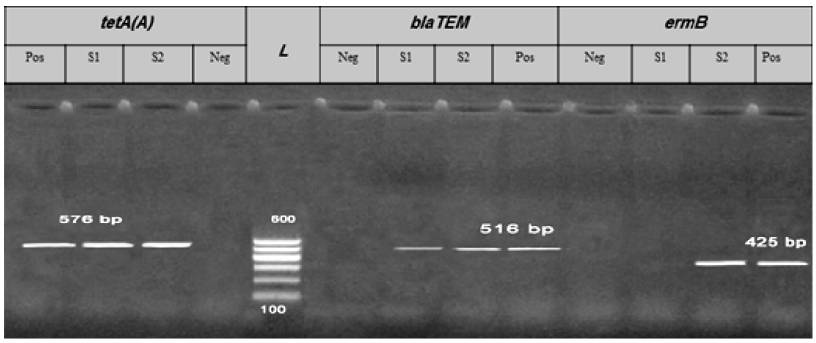

Fig. 6. Agarose gel electrophoresis of detected antibiotic resistance gene DNA fragments of *E.coli* amplified by PCR from selected isolates (*tetA(A)* – 576 bp), (*blaTEM* – 516 bp), (*ermB* – 425 bp).

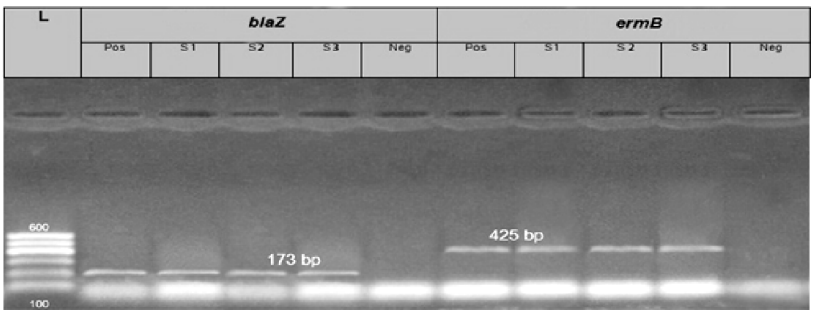

Fig. 7. Agarose gel electrophoresis of detected antibiotic resistance gene DNA fragments of *S. agalactiae* amplified by PCR from selected isolates (*blaZ* – 173 bp), (*ermB* – 425 bp).

(2013), Ganda *et al.* (2016), Trajchev *et al.,* (2017) respectively, while Hamzah & Kadim (2018) reported a higher frequency (67.4%). The differences in the microbial community on the teat surface varied from one farm to another due to many different factors as microbial load and type in the bedding material and milking machines which can contaminate the surface of teat and can potentially enter to the milk.

The prevalence of *Escherichia coli* was similar to that obtained by Barbour *et al.,* (2015), while lower incidences was reported by Bhat *et al.* (2017) and Darbaz *et al.* (2018): 13% and 7.4% respectively. Nevertheless Khan *et al.* (2017) recorded a higher incidence of 54.5%. The high percentage of SCM caused by coliform bacteria indicates unsanitary milking process or faulty sterilisation of utensils, improper preparation of dairy animals and using contaminated water supplies or contamination from soil and faecal matter.

The prevalence of *Streptococcus agalactiae* was in accordance with previously reported results (Elhaig *et al.,* 2014; Trajchev *et al.,* 2017). However other studies reported higher prevalence of 31% and 34.4% (Abdel-Rady & Sayed, 2009; Ramirez & Tolmasky, 2014) or lower prevalence between 5.8% and 15.6% (Leelahapongsathon *et al.,* 2014; Oliveira *et al.,* 2015; Sztachańska *et al.,* 2016). Although *S. agalactiae* can live outside the udder for short periods of time in the right conditions, it is considered to be an obligate udder pathogen. A high percentage of cows may be affected where control procedures are not implemented effectively or due to the unsanitary conditions of strip cups, towels, milkers' hands, cross suckling calves, milking machines that are considered as the most potential sources of infection in cows.

Other different pathogens detected in this study were coagulase negative *Staphylococcus*, *Klebsiella oxytoca*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Bacillus cereus* in accordance with the data of Gao *et al.* (2017), Darbaz *et al.* (2018); Suleiman *et al.* (2018). The lowest isolation rate in this study for *Sphingomonas paucimobilis, Enterobacter aerogenes, Raoultella ornithinolytica*, *Pantoea* species, other *Bacillus* species, *Citrobacter amalanaticus* and *Enterobacter cloaca* complex were in line with data reported by Memon *et al.* (2012), Kateete *et al.* (2013), Oluchi (2016) and Ali & Ali (2017). The origin of SCM due to these microorganisms may be related to contaminated water sources, hoses and nozzles in milking parlors, the pipes and tanks in cattle sheds. Other potential causes include intramammary antibiotic infusions under unhygienic conditions.

Antibiotics are key components of the treatment regimen for common diseases including mastitis and there are prophylactic uses of antimicrobials in the dairy industry such as dry cow therapy and foot bath disinfection programs (Schewe & Brock, 2018). Recently, there are great concerns by the World Health Organization related to milk and associated antimicrobial residues that lead to development of resistance genes and transmission between human and animal pathogens, hence the recommendation that use of antibiotics must be limited for treatment of infected animals only (Ismail *et al.,* 2018).

Our data revealed that the randomly chosen 24 *Staphylococcus aureus* isolates were 100% resistant to cefazolin, novobiocin, erythromycin, kanamycin, oxytetracycline and cefotaxime in accordance with other data (Elango *et al.,* 2010; Girma *et al.,* 2012; Memon *et al.,* 2012; Bhat *et al.,* 2017) while the examined

isolates were less resistant to doxycycline (75%), ampicillin (62.5%), amikacin and sulfamethazole/trimethoprim comparable to the results of Abera *et al.* (2010), Haftu *et al.* (2012), Nahed *et al.* (2013), Abrahmsén *et al.* (2014) and Prabhu *et al.* (2015). Half of isolates showed resistance to clindamycin, penicillin and cefepime in line with Bhat *et al.* (2017).

The lowest resistance was observed against cefoxitin (37.5%), cephradine (33%), tetracycline, oxacillin and amoxacillin+clavulanic acid (each with 25%) in agreement with previously reported data (Mekuria *et al.,* 2013; Chaturvedi *et al.,* 2017; Ssajjakambwe *et al.,* 2017; Seyoum *et al.,* 2018). However, gentamicin, enrofloxacin and nitrofurantoin were found to be the most effective antibiotics on most isolated strains in line with Vásquez-García *et al.* (2017) and indicating that most of the strains tested did not acquire resistance determinants for these antibiotics.

Multiple-drug resistant staphylococci that are resistant mostly to beta-lactam antibiotics and the methicillin-resistant strains generally occur following routine use of these drugs by the veterinarians (penicillin, erythromycin and tetracycline) either for prophylaxis or for growth promotion as well as imprecise dosage to sick or healthy animals. So *S. aureus* is considered as a major cause of mastitis in dairy cows causing huge financial losses worldwide (Wang *et al.,* 2015) due to its wide range of resistance to antibiotics

Enterococci are considered one of the most important farm pathogens causing high mortality rate of up to 61%. *Enterococci* can colonise the genitourinary tract, oral cavity and skin but the gastrointestinal tract, delicate tissue, wounds and ulcers are the major colonisation sites (Hamzah & Kadim, 2018). The examined isolates of different *Enterococcus* species showed different pattern of resistance against tested antibiotics. Ten examined *E. faecalis* isolates were 100% resistant to amoxacillin+clavulanic acid, oxytetracycline, cefotaxime, penicillin, tetracycline and erythromycin; 50% of isolates showed resistance to sulfamethazole/trimethoprim and 30% of isolates were resistant to ampicillin and amikacin.

All of the examined ten isolates of *E. faecium* were resistant against sulfamethazole/trimethoprim, tetracycline, amikacin, cephradine and nitrofurantoin, as also shown by Hamzah & Kadim (2018). All six examined isolates of *E. gallinarum* were resistant to ampicillin, oxacillin, penicillin, tetracycline, enrofloxacin and amoxacillin+clavulanic acid, while 3 isolates were resistant to clindamycin. Half of the examined 6 *E. avium* isolates were resistant to gentamicin and the examined strains showed no resistance against tetracycline, enrofloxacin and amoxicillin+ clavulanic acid.

Enterococci have been known to be resistant to most antibiotics used in clinical practice. They are known naturally resistant to cephalosporins, aminoglycosides and clindamycin. The vancomycin resistant enterococci (VRE) are a global biological hazard to public health (Linden, 2007). This study demonstrates that enterococci isolated from dairy cows with SCM were most frequently resistant to tetracyclines, beta-lactams, fluoroquinolones and macrolides. These antibiotics are the most intensively used for dairy cows treatment during the last years. The lack of restrictions to the antimicrobial agents generally led to critically uncontrolled usage of most antimicrobial agents leading to a high prevalence of resistant *Enterococcal* isolates (Eputiene *et al.,* 2012).

The thirty *E. coli* isolates examined were 100% resistant to penicillin, clindamycin, oxacillin, oxytetracycline, doxycycline, cefobid, rifampicin, neomycin and cephazoline. Lower resistance – 80% was exhibited to tetracycline and amikacin. However 70% of isolates were resistant to erythromycin, 60% to ampicillin and 50% to kanamycin, cefotaxime, amoxicillin + clavulanic acid, sulfamethazole/trimethoprim). Thirty percent showed resistance to chloramphenicol and norfloxacin the lowest resistance of 10% was against enrofloxacin and gentamicin. These results are in accordance with those reported by Ceniti *et al.* (2017); Hinthong *et al.* (2017); Verma *et al.* (2018).

The high resistance rates of the *E. coli* isolates observed in this study suggested that the emergence of resistant strains in diseased animals could be increased by the misuse of antibiotics as aminoglycosides, tetracyclines and fluoroquinolones used for animal treatment and metaphylaxis and the improper use of antimicrobial agents can lead to the failure of treatment (Ssajjakambwe *et al.,* 2017). Also the extended-spectrum β-Lactamase (ESBL) producing *Enterobacteriaceae* can be transferred between human and livestock (Gonggrijp *et al.,* 2016).

The results demonstrated that all of the examined 30 isolates of *S. agalactiae* were resistant to penicillin, while a lower resistance of isolates was observed against ampicillin, amikacin, oxacillin, amoxacillin+ clavulanic acid, erythromycin and tetracycline in line with other data (Chaturvedi *et al.,* 2017; Ssajjakambwe *et al.,* 2017; Verma *et al.,* 2018). It is known that the beta-lactams especially penicillin remain the antibiotic of choice in the treatment of streptococcal infection for several decades, the macrolide rythromycin is considered the most important used alternative for treatment of streptococcal infection, so a significant increase in the frequency of resistance to beta-lactams and erythromycin was observed.

Streptococcus agalactiae is considered one of the major causes of economic losses in dairy farms. The unsanitary conditions and some milking equipment such as strip cups, towels, milkers' hands, cross suckling calves; milking machines are all considered potential sources of infection in cows (Merz *et al.,* 2016). It breaks the natural barriers of the udder, enters the teat canal, and ascends in the milk through the quarter, so in later stages it increases SCC of the infected quarter.

The examined *S. aureus*, *S. agalactiae*, *E. faecalis*, *E. gallinarum*, *E. faecium* and *E.avium* isolates showed high resistance to penicillins and they expressed the *blaZ* gene encoding resistance to beta-lactams in support to data of Xu *et al.* (2015), and Mello *et al.* (2017), while the *mec*A gene which encodes penicillin binding protein responsible for encoding resistance against oxacillin was detected in the examined *S. aureus* (Xu *et al.,* 2015). In addition the presence of *tetK* gene that encodes resistance to tetracycline was detected in each *S. aureus*, *E. faecalis*, *E. gallinarum*, *E. faecium* and *E. avium.* However both the *ermC* gene in *E. faecalis* and *E. avium* and the *ermB* gene in the examined three *S. agalactiae* isolates were detected by PCR as a phenotypically resistant to macrolide-class antimicrobials (erythromycin). The *van A* and *norA* genes was detected in *E. gallinarum* as genes encoding resistance against vancomycin and norfloxacin respectively.

E. coli expressed *tetA (A)* gene and *blaTEM* gene genetically responsible for resistance against tetracyclines and β-lactams respectively in agreement with the

data by Das *et al.* (2017). In addition the *ermB* gene was detected in one *E. coli* strain as one of the genes encoding resistance against macrolides.

β-lactams are antimicrobials, most commonly utilised for treatment of mastitis. This explains that wide use or misuse of broad spectrum antimicrobials is one of several risk factors that can lead to the rise of multidrug resistance (Tassew *et al.,* 2016). *Staphylococcus* isolates of are characterised as methicillin resistant (MRSA for *S. aureus* and MRCNS for coagulase negative staphylococci) if they show the presence of the *mecA* gene and display phenotypic resistance to oxacillin/methicillin (Kulangara *et al.,* 2017). The mechanism of action of the macrolides, such as erythromycin, is the inhibition of bacterial protein synthesis by binding reversibly to the subunit 50S of the bacterial ribosome, there by inhibiting translocation of peptidyl-tRNA (Stevens *et al.,* 2018).

Mechanisms via which the bacteria became resistant to tetracycline are cytoplasmic exocytosis channels, ribosomal conservation and deactivation of enzymatic system. Efflux protein genes *tetA* is associated with tetracycline resistance and contribute to the active elimination of the antibiotic from a cell (Arab *et al.,* 2018).

CONCLUSION

The prevalence of the subclinical mastitis in the examined farm in Fayoum, Egypt not only affected the economy of the farm but also is of great concern because of the antibiotic resistance patterns showing widespread emerging resistance among mastitis pathogens to antibacterial drugs. Therefore, it is recommended that training and guidance should be given to farmers and animal handlers. The determination of the particular antibacterial should be based on lab investigations and the choice of the satisfactory dose, to avoid/reduce the chance of inducing microbial resistance and to diminish their side impacts for people and animals alike.

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