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PREVALENCE, ANTIMICROBIAL RESISTANCE PROFILES OF CLINICAL AND SUBCLIN-ICAL MASTITIS IN LACTATING COW WITH ASSESSMENT OF TREATMENT TRIAL

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Abstract: This study looked at the prevalence of bovine mastitis in a dairy farm in Egypt's Ismalia Governorate, as well as the phenotypic and genotypic characteristics of the causative bacteria and their antimicrobial susceptibility. Also, a treatment trial with a combination of Cefalexin and Kanamycin was evaluated. The total prevalence of mastitis was 31.82% (119/374) at cow level and 17.01% (247/1452) at quarter level. 261 isolates were detected. As major microorganisms, 74.33% are Staphylococcus spp. and 25.67% are Streptococcus spp. The isolates were tested against 15 antimicrobial agents, with Gentamycin (CN), Ciprofloxacin (CIP), Cefalexin (CL), and Kanamycin (K) having the lowest percentage of resistant bacteria. Molecular characterization of isolated pathogens and antimicrobial resistance genes was performed by PCR on 15 isolates. *blaTEM-1*was the most frequently detected gene, followed by *aadA1, dfrA1, cmlA, sul1,* and *tetA*. 110 infected udder quarters were enrolled for 21 days to evaluate the treatment with Terrexine LC intramammary suspension 10g (Cefalexin ph. Eur 200mg, Kanamycin monosulfateph.Eur 100,000 I.U.) on six occasions at 12-hour intervals and Gentamycin intramuscular injection (1 cm/20 kg BW for 3-5 days) in cows with systemic reactions. A highly significant reduction was recorded for the log10 SSC, log10 TBC, and the level of LDH in milk after treatment compared to their level before treatment (P-value 0.0001***). The milking season, severity of mastitis, or type of microorganism isolated prior to treatment have no effect on the recovery rate (P-value 0.05). In conclusion, bacterial isolates in the present study revealed multidrug resistance to the majority of commonly used antimicrobial agents, so antibiotic usage must be restricted. PCR is a helpful technique for the detection of resistant bacteria. Treatment of bovine mastitis with a combination of antibiotics significantly improves the bacteriological cure, SSC, TBC, and the level of LDH in milk.

Key words: bovine mastitis; drug resistance; resistance gens; treatment; Cafalexin; Kanamycin

Introduction

Bovine mastitis is a devastating and great challenge to dairy production, disturbing animal health and welfare in addition to causing considerable economic losses and a public health hazard. It lowers milk yield and quality, raises treatment and labour costs, a causes milk to be held after treatment, resulting in premature culling or death of the infected cow (1, 2).

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Mastitis is caused by a wide range of pathogens that vary in their virulence, abilities to stimulate an immune response, and structures of their cell walls, resulting in differing susceptibilities to antimicrobials, so accurate determination of the cause is important to prescribe the appropriate treatment (3).

Mastitis pathogens are classified into 1-Contagious bacteria (*S. aurus, S. agalactia*, and *mycoplasma*) that spread from an infected quarter during milking.*2.* Environmental bacteria (*S. uberis*, S. dysagalctia, E. coli*, and other coliforms*) that are commonly present in cows' environments. (4).

The diagnosis of clinical mastitis depends on udder examination and changes in the physical and chemical properties of milk, as well as an increase in somatic cell count, while the diagnosis of subclinical mastitis is more difficult and depends on indirect techniques such as the California mastitis test, an ,electrical conductivity test, and the detection of body enzymes released due to tissue damage, such as the lactate dehydrogenase enzyme. (5,6)

The lactate dehydrogenase enzyme is present in the cytoplasm of all animal cells. During udder infection, the activity of this enzyme increases as a result of udder tissue damage, making it a responsive indicator for early detection of mastitis and a reliable outcome to evaluate treatment success (6, 7).

Microbiological examination of milk is considered to be the standard method for diagnosing bovine mastitis, despite its limitations, such as the time needed for culturing and the costs (8).

PCR is a unique molecular technique that was developed in order to provide higher sensitivity and specificity while minimising costs. (9)

Mastitis is the most common disease that leads to the use of antimicrobials on dairy farms. Imprudent use of antimicrobials can lead to a rise in antimicrobial resistance, and there is a risk of the subsequent spread of resistant genes to other microbial populations. (10)

Combining two or more antibiotics may reduce the emergence and spread of resistant pathogens by increasing bactericidal activity against specific pathogens due to a synergistic effect, or it may allow antimicrobial action against more pathogens that are not affected by a single antibiotic (11, 12).Also, this combination may extend the period between dose administrations or decrease the number of administrations required, or both, so it can achieve similar or enhanced bactericidal activity using a lower total amount of antibiotics (13).

Our study aimed to investigate the prevalence of bovine mastitis in dairy cows, study antimicrobial resistance, and assess the recovery rate through intramammary infusions of cephalexin and kanamycin.

Material and methods

The protocol for this study was reviewed and approved by Zagazig University's Ethical Committee on Animal Care and Use Experimentation (ZU-IACUC Committee), with approval number ZU-IACUC/2 /F/165/2021.

Study design

This research was carried out in a dairy farm in Ismailia Governorate, Egypt, between 2020 and 2021.The farm was purposefully selected based on the high population of cows (374 black and white Holstein lactating cows) and the diversity of cattle age and lactation stage.

The farm was raised using an open system, with machinery milking three times per day.The udder was washed with water before and after milking teat dipping. The soil was muddy and scraped every six months.The farm was screened with the California mastitis test (CMT) every 2 months. Dry cows were treated with long-acting udder infusions. Prior to our study, mastitis was treated in the farm without the regular application of antimicrobial sensitivity to the causative pathogen.

Clinical examination

Cows were generally examined for systemic illness and for clinical mastitis with a special physical examination of each udder, teat, and secreted milk.

Sample collection

Aseptic milk samples were collected from each quarter separately. Udder was carefully cleaned and disinfected with 70% alcohol. After discarding the early jets of milk, 10 ml of milk from each quarter was collected in a screw-capped bottle. The samples were then labelled and transported to Zagazig University,Faculty of Veterinary Medicine, infectious diseases laboratory for examination.Each milk sample was physically checked for any abnormalities in colour, consistency, and the presence of blood, clots, and flak.

California mastitis test

Apparently healthy udder quarters with apparently normal milk were tested using the California mastitis test(CMT) to detect the subclinical mastitis. The test procedures were

performed according to the prior study (14), and the reading of the test was scored as 0, trace, 1, 2, and 3. A milk sample was counted as positive if it had a reading of 1-3.

Isolation and identification of the causative agent

Ten microliters of each milk sample were streaked onto blood agar (Oxoid, Hampshire, UK; CM0271 with 5% sheep blood) and incubated at 37 C for 24-48 h. The colony morphology and type of hemolysis were used to identify the growth on blood agar at first.Aloopful of pure culture was smeared and stainedwith Gram´s stain to differentiate the shape of bacteria. Separate colonies from blood agar were subcultured onto mannitol salt agar (Oxoid Ltd., Hampshire, UK) for primary identification of the genus Staphylococcus. And to Edward Media (Oxoid, Hampshire, UK) for the identification of the genus Streptococcus spp. The colonies were morphologically and biochemically identified (15).

Antimicrobial Susceptibility Testing (16)

The sensitivity of the isolated strains was determined using the disc diffusion technique against 15 antimicrobial agents commonly used in Egypt for the treatment of mastitis and of public health importance (Gentamicin CN (10 g/ml), Streptomycin S (10 g/ml), Kanamycin K (30 g/ml), Ciprofloxacin CIP (5 g/ml), Vancomycin VA (30 g/ml), Cefalexin CL (30 g/ml) Amoxicillin+ Clavulanic acid AMC(30 µg/ml), Ceftriaxone CRO(30 µg/ml), Penicillin G P(10 µg/ml), Doxycycline DO(30 µg/ml), Chloramphenicol C(30 µg/ml), Erythromycin E(15 µg/ml), Oxacillin OX(1µg/ml), Sulfamethoxazole + Trimethoprim SXT (25 µg/ml)and Clindamycin DA(2 µg/ml)).

A few colonies of similar morphology were mixed in a tube with 5 ml of sterile 0.85% physiological saline, and the turbidity was adjusted to match a 0.5 McFarland standard tube (1.5 x 10^8 CFU) and streaked onto Mueller-Hinton agar plates. The antimicrobial discs were applied and incubated at 37 C for 24–48 h. The diameter of the zone of growth inhibition was measured to determine the degree of sensitivity.It was interpreted according to the national committee for the Clinical Laboratory Standards Institute (17). Isolates were considered multidrug-resistant (MDR) if they were resistant to at least three different classes of antimicrobial agents (18).

Determination of the minimum inhibitory concentration (MIC)

MIC values of antibiotics were determined by the microdilution method following the recommendations of Papich(19).

Molecular Identification of Isolates and Resistance Genes

Extraction of DNA

DNA was extracted from the from biochemically identified bacterial isolate according to QIAamp DNA mini kit instructions (Catalogue no.51304). QIAGEN protease, 200 µl of the sample, 200 µl buffer were mixed in a 1.5 ml microcentrifuge tube. The tube was incubated at 56˚C for 10 min. 200 µl ethanol (96%) were added. The mixture was carefully applied to the QIAamp mini spin column (in a 2ml collecting tube). 500 ml buffer AW1, 500 ml buffer AW2 and 100 µl buffer AE were added separately.

Preparation of the PCR Master Mix

According to Emerald Amp GT PCR mastermix (Takara) Code No.RR310Akit:PCR reactions were of 25 µlvolum (Emerald Amp GT PCR mastermix (2x premix) 12.5*µl*, PCR grade water 4.5*µl,* Forward primer (20 pmol), Reverse primer (20 pmol) 1 *µl*for each andTemplate DNA 6 *µl).* Eight pairs of oligonucleotide primers were used in conventional PCR assays for amplification of *16S RNA* gene of genus *staphylococcus*, *tuf*gene of genus *Streptococcus spp*and six antimicrobial resistance genes (Table 1).

Agarose gel electrophoreses

20 µl of each PCR product samples, negative control and positive control were loaded to the gel. The power supply was 1-5 volts/cm of the tank length. The run was stopped after about 30 min and the gel was transferred to UV cabinet.

The gel was photographed by a gel documentation system and the data was analyzed through computer software.

Treatment

One hundred and ten udder quarters with intramammary infection (16 showed sub clinical mastitis and 94 with clinical mastitis) form total 247 infected quarters (146 with sub clinical mastitis and 101 with clinical mastitis) were enrolled in this study for 21 days after treatment with Terrexine LC intramammary suspension 10g (Cefalexin ph. Eur 200mg, Kanamycin monosulfateph.Eur 100,000 I.U.; Univet Limited) on six occasions at 12 h intervals. A gentamycin intramuscular injection was administered to 32 cows (1 cm/20 kg BW for 3-5 days) and showed a systemic reaction (Table 2).

Each quarter was sampled at 0, 7, 15,21days after initiation of treatment to evaluate the bacteriological cure, level of somatic cell count(SCC),total bacterial count(TBC) and lactate dehydrogenase (LDH) activity.

Bacteriological cure

Bacteriological cure was assessed by sample taken from affected quarter on(7,15and21)days after treatment were free of the bacterial species isolates in the pre-treatment.

Somatic cell count and total bacterial count

Somatic cells as well as total bacterial count were measured at quarter level. Milk samples were collected from each quarter just before treatment and on 7, 15, and 21 days aftertreatment. These parameters were enumerated in raw cow's milk by The FOSS BacSomatic™ analyser. The samples were tested within 2h after collection. Udder quarters enrolled in treatment were with SCC ≥ 500,000 cells/ml pre-treatment.

Lactate dehydrogenase activity

LDH activity was measured using the Health Mate™ milk LDH mastitis test DFI-car. It is a dipstick semi-quantitative colorimetric test to determine elevated levels of LDH in milk as early indicator of mastitis. The test strip is dipped in 5mL of fresh cow milk for 2minutes then read the change in its colour.

Analysis of the data

The analysis of the datawas carried out in the R environment for statistical computing and visualization (28), which is an open-source dialect of the S statistical computinglanguage, and were expressed the mean ± standard deviation (SD) or percentage (%). For normality investigation, the Shapiro–Wilks test was used for numerical data, and the Chi-square test ($\chi 2$) was used for categorical data. The means of samples with normal distribution and of sufficient size were compared by Student's t test and one-way ANOVA. A p value < 0.05 was considered significant.

Table 1: Oligonuclotide primers for molecular identification of *Staphylococcus spp.*, *Streptococcus spp.* and antimicrobial re-sistance genes)

Target gene	Primers sequences	Amplified segment (bp)	Reference
16S RNA	AACTCTGTTATTAGGGAAGAACA CCACCTTCCTCCGGTTTGTCACC	756	**(20)**
tuf	CCACCTTCCTCCGGTTTGTCACC TGG GTT GAT TG AACC TGG TTT A	110	**(21)**
blaTEM-1	AGGAAGAGTATGATTCAACA CTCGTCGTTTGGTATGGC	535	**(22)**
aadA1	TATCAGAGGTAGTTGGCGTCAT GTTCCATAGCGTTAAGGTTTCATT	484	**(23)**
dfrA1	TGGTAGCTATATCGAAGAATGGAGT TATGTTAGAGGCGAAGTCTTGGGTA	425	**(24)**
TetA	'GGCGGTCTTCTTCATCATGC 'CGGCAGGCAGAGCAAGTAGA	502	**(25)**
Sul 1	CC GATATTGCTGAGGCGG CCAACGCCGACTTCAGCT	435	**(26)**
cmlA	CCGCCACGGTGTTGTTGTTATC CACCTTGCCTGCCCATCATTAG	698	**(27)**

Table 2: 110 udder quarters enrolled for treatment with Cefalexin and Kanamycin monosulfate

No. of treated quarters	Clinical	Subclinical
	94	16
Treatment	- Terrexine LC intramammary suspension 10g (Cefalexin ph. Eur 200mg, Kanamycin monosulfateph.Eur 100,000 I.U.; UnivetLimited,UK) on six occasions at 12 h intervals. - Gentamycin intramuscular injection was administrated to 32 cows (1cm/20kg Bw for 3-5 days) showed systemic reaction.	Cases of subclinical mastitis were isolated and milked six times daily for one month, then retested with CMT and the positive cases were treated with an antibiotic.

Results

Prevalence of mastitis on the farm before treatment

At cow level:

A total of 374 lactating cows were tested, and 119 were found to have clinical or subclinical mastitis, for a total prevalence of 31.82%.

At the quarter-udder level:

44 udder quarters (2.9%) were completely dried out of a total of 1496 examined.From the remaining 1452 examined quarters, 101 (6.96%) revealed different degrees of inflammation (asymmetry, redness, hotness, and pain during palpation). Also, changes in the secreted milk from these quarters varied from slight deformity in milk quantity and milk colour to clot formation, bloody milk, and the appearance of yellow fluid or pus secreted from the affected quarters. 146 quarters (10.06%) were diagnosed as subclinical mastitis using the California mastitis test, with the total prevalence of mastitis at 17.01% (247/1452) (Table 3).

Microbiological results

261 bacterial isolates were yielded from a total of 247 milk samples. The isolates were characterised by phenotypic characteristics and biochemical reactions as two major microbial species. 194 (74.33%) *Staphylococcus spp.* and 67 (25.67%) Streptococcus *spp.* isolates (Table 4).

Antimicrobial sensitivity

All of the bacteria isolated were multidrug resistant**.**The highest proportion of antimicrobial-resistant bacteria was recorded for AMC, followed by E, S, C, and P, and the lowest for K, CIP, CL, and OX. No resistance was recorded for VA.

Regarding the Staphylococcus spp. isolates, the highest percentage of resistance was recorded for AMC (60.82%), and the lowest resistance rates were recorded for K, CIP, and CL (3.61% for each); no resistance was recorded for VA. For Streptococcus spp. isolates, the highest resistance rate was recorded for AMC (100%) and the lowest rate was recorded for CL (11.94%). No resistance was recorded for CN, K, CIP, OX, CRO, and VA (Table 5).

Table 3: Prevalence of mastitisat quarter level

		Clinical					Subclinical					Total (1452*)
At quarter level	N	Q1	Q2	Q3	Q4	Total	Q1	Q2	Q3	Q4	Total	247
		23	28	17	33	101	24	49	44	29	146	
	P(%)	1.58	1.93	1.17	2.27	6.96	1.65	3.37	3.03	1.99	10.06	17.01

Q: quarter; N : number; p: percentage ; *Other quarters (N= 44) were either dried or blind& excluded from the total examined number.

Table 4: Prevalence of isolated bacteria

Isolates	*Staphylococcus spp*			*Streptococcus spp*			
No. of isolates	CNS	*S. aurus*	Total	*S. uberis*	*S. agalactia*	*S. dysagalacti*	Total
	111	83	194	32	23	12	67
Prevalence	42.53%	31.80%	74.33%	12.26%	8.81%	4.60%	25.67%

Table 5: Antimicrobial resistance of *Staphylococcus spp.* and *Streptococcus spp.* isolated from lactating cows with mastitis

Antimicrobial Disc (Concentration **µg/ml**)	Microrganism	Resistance No (%)	Total (%)
CN (10)	Staph Strept	21 (10.8) 0 (zero)	21(8.05)
S (10)	Staph Strept	76 (39.18) 48 (63.16)	124 (47.51)
K (30)	Staph Strept	7(3.61) 0 (zero)	7 (2.68)
CIP (5)	Staph Strept	7(3.61) 0 (zero)	7 (2.68)
VA(30)	Staph Strept	0(zero) 0 (zero)	0 (0.00)
CL(30)	Staph Strept	7(3.61) 8 (11.94)	15 (5.75)
AMC(30)	Staph Strept	118(60.82) 67 (100)	185 (70.88)
CRO (30)	Staph Strept	35 (18.04) 0 (zero)	35 (13.41)
P (10)	Staph Strept	55 (28.35) 42 (62.69)	97 (37.16)
DO(30)	Staph Strept	49 (25.26) 34 (50.75)	83(31.80)
C (30)	Staph Strept	83 (42.78) 34 (50.75)	117 (44.83)
E (15)	Staph Strept	83 (42.78) 50 (74.63)	133(50.96)
OX (1)	Staph Strept	21(10.82) 0 (zero)	21 (8.05)
SXT (25)	Staph Strept	62 (31.96) 34 (50.75)	96(36.78)
DA (2)	Staph Strept	49 (25.26) 17 (25.37)	66 (25.29)

PCR and resistance gens

Eight *Staphylococcus spp.* and seven *Streptococcus* isolates, with phenotypic resistance to a large number of antimicrobial agents, were confirmed by PCR in addition to the detection of six resistance genes. β-lactamase gene (*blaTEM-1)* was the most detected resistance gene (86.67%), followed by aminoglycoside adenylyl-transferase gene (*aadA1)*, dihydrofolate reductase gene (*dfrA1),* chloramphenicol resistance gene *(cmlA),*Sulfonamide resistance gene *(sul1),* and finally tetracycline resistance gene (*tetA)*, with a percentage of 60.00%, 60.00%, 47.67%, 40.00%, and 26.67%, respectively (Table 6).

Treatment evaluation

Clinical and Bacterial cure

By the 21st day after treatment, 92.55 % of clinical cases (87/94) were cured where the appearance of milk and mammary glands returned to normal**.** In addition, the bacteriological cure was achieved in 88.18% of enrolled quarters(97/110).

Milk yield

No significant change in the level of milk production per cow after treatment (18.39± 4.75) comparing its level before treatment (17.87± 4.51; P-value 0.76) (Figure 1).

Table 6: Antimicrobialresistantprofileof 15 isolates(8 *Staphylococcus spp* and 7 *Streptococcus spp*)

NO	M.O	PHENOTYPE	RESISTANTGENES
1	*S.aurus*	CN, S, CL, AMC, CRO, P, DO, C, E, DA, TE, AX, CX	*cmlA, aadA1, bla*$_{TEM-1}$*, dfrA1, sul1, tetA*
2	*S.aurus*	S, AMC, C, DO, E, OX, SXT, DA, TE, CX	*aadA1, bla*$_{TEM-1}$*, sul1*
3	*S.aurus*	CN, S, AMC, CRO, P, DO, C, E, OX, SXT, DA, TE, AX, CX	*aadA1, bla*$_{TEM-1}$*, tetA*
4	CNS	S, AMC, CRO, P, DO, C, DA, TE	*cmlA, aadA1, bla*$_{TEM-1}$*, tetA*
5	CNS	CN, S, CIP, AMC, CRO, P, DO, C, E, OX, SXT, DA, IP, M, AX	*cmlA, aadA1, bla*$_{TEM-1}$*, dfrA1*
6	CNS	CN, S, K, AMC, CRO, C, SXT, CX	*cmlA, aadA1, bla*$_{TEM-1}$*, dfrA1*
7	CNS	CN, S, AMC, CRO, C, SXT, CX	*aadA1, dfrA1, sul1*
8	CNS	S, AMC, CRO, P, DO, E, CX	*cmlA, aadA1, bla*$_{TEM-1}$*, sul1*
9	Strept	S, AMC, P, E, SXT	*dfrA1, sul1, aadA1, bla*$_{TEM-1}$
10	strept	S, AMC, E, SXT	*dfrA1, sul1,*
11	strept	S, AMC, P, DO, C, E, DA	*cmlA, dfrA1, bla*$_{TEM-1}$
12	Strept	S, AMC, P, DO, C, SXT	*cmlA, bla*$_{TEM-1}$
13	Strept	S, AMC, C, E, SXT	*bla*$_{TEM-1}$
14	Strept	S, AMC, CRO, P, C, E	*bla*$_{TEM-1}$
15	Strept	S, AMC, CRO, P, C, E	*bla*$_{TEM-1}$

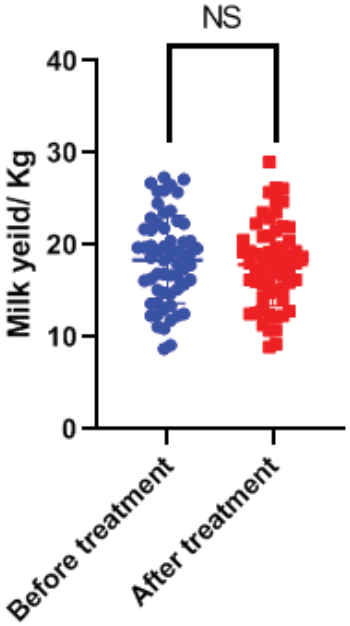

Figure 1: Change in milk yield at cow level before and after treatment with combination of Kanamycin and Ce-falexin

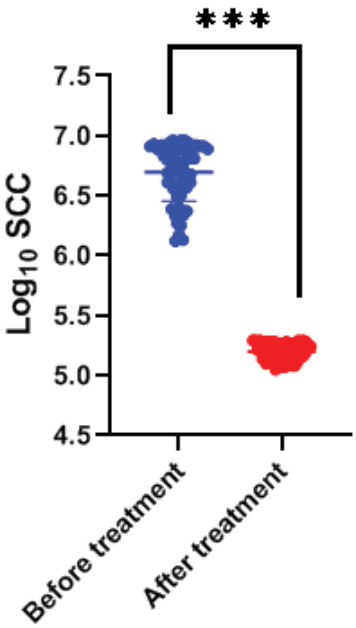

Figure 2: Change in $\log_{10}$ SCC at quarter level before and after treatment with combination of Kanamycin and Ce-falexin

SCC

Quarter-level SCC gradually declined in milk samples during the posttreatment follow-up. A high significance was recorded for the log10 SSC in milk at 21stday after treatment (5.20± 0.07) compared the level before treatment (6.69 ±0.24 P-value <0.0001***) (Figure 2).

Total bacterial count

The TBC was increased before treatment to 7.40 ±0.21 which was then significantly decreased to 3.67 ±0.16 after treatment (Figure 3).

Milk samples revealed significant increase LDH Activity as high as 497.33 ±14.36 U/L before treatment that restored its normal level after treatment(Figure 4).

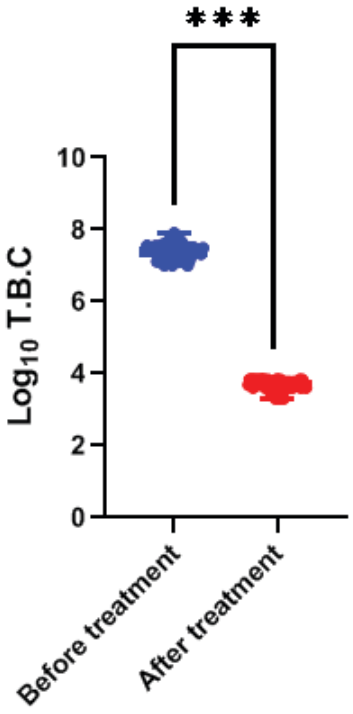

Figure 3: Change in log10 TBC at quarter level before and after treatment with combination of Kanamycin and Ce-falexin

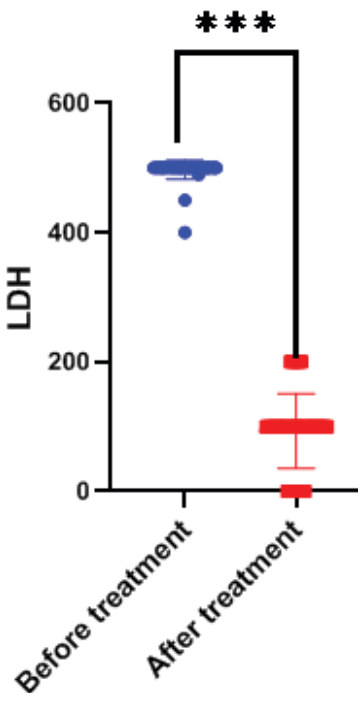

Figure 4: Change in LDH activity at quarter level before and after treatment with combination of Kanamycin and Cefalexin

Lactat dehydrogenase activity

Table 7: The development of the 4 outcomes (milk yield, Log_{10} SCC, Log_{10} TBC and LDH)in relation to day of treatment

parameters	Before treatment	After treatment			P-value (Anova test)	Significance
	0 day	7days	15 days	21 days		
Milk yield	17.87± 4.51	17.50± 4.52	17.49± 4.77	18.39± 4.75	0.76	Not significance
Log_{10} TBC	7.40 ±0.21	5.13± 0.18	4.34 ±0.20	3.67 ±0.16	<0.0001***	Highly significance
Log_{10} SCC	6.69 ±0.24	5.51 ±0.09	5.35 ±0.13	5.20± 0.07	<0.0001***	Highly significance
LDH	497.33 ±14.36	93.33 ±57.83	10.00 ±30.25	-	<0.0001***	Highly significance

Discussion

Bovine mastitis is the most common production disease in dairy herds worldwide, not just in developed countries.It is responsible for several production effects (29).

The obtained results on 374 lactating cows revealed that the prevalence of mastitis was 31.82% (119/374) at the cow level and 17.01% (247/1452) at the udder quarter level, out of which 6.96% (101/1452) was clinical mastitis and 10.06% (146/1452) was subclinical mastitis. This prevalence of bovine mastitis was similar to the results obtained in other previous studies in Egypt (30), A higher prevalence of 52.1% and 49.9% for subclinical mastitis was recorded by Algammal et al. (31) in cattle in the same governorate. Mbindyo et al. (32) in Kenya reported a higher percentage for total mastitis (80%; 316/395) at cow level and (51.6%; 815/1580) at quarter level.

In the present study, CNS was the most prevalent pathogen (42.53%), followed by *S. aurus* (31.8%), *S.uberis*(12.26%), *S. agalactia (8.81%),* and *S. dysagalactia (4.59%).* This is in line with previous findings (33-34) that CNS were the dominant isolated bacteria from clinical mastitis

in cattle. Also, CNS was recorded as the major pathogen implicated in udders with subclinical mastitis in different countries (35–36).

Previously, CNS was proven to cause mild subclinical mastitis, so it was considered a minor mastitis pathogen compared with *Staphylococcus spp*aureus, streptococci, and coliforms (37).

Recently they associated with many economic losses due to significant increases in SCC and reductions in milk yield and quality (38, 39); moreover, it can cause intraalveolar fibrosis of the udder tissue and loss of secretory function of this tissue.

S. aurus had a prevalence of 31.80% in the current study.Nearly similar percentages were recorded by Mousa et al. (40) and Moustafa et al. (41) but a lower percentage of *S. aureus* (5.6%) was reported by El-Ashker et al. (42) and a higher percentage of 45.6% and 46.5% was recorded by Abdel-Tawab et al. (43) and El-Faramawy et al. (44) from clinical mastitis cases. The infection with *S. aurus* is transmitted from animal to animal through milker's hands, contaminated equipment, and a bad hygienic environment (45).

In this study, the prevalence of streptococcal mastitis was 25.67% (*S.uberis*(12.26%), *S. agalactia* (8.81%), and *S. dysagalactia* (4.59%). This related to the results recorded by Duse et al. (46) for *S.uberis* (11.4%), but they reported different prevalences for *S. agalactia (1.2*%) and *S. dysgalactia*(15.8%). A lower prevalence of streptococcal mastitis (5.0%) was found by Hasan et al. (47). Abd El-Aziz et al. (48) reported a higher percentage of *S.uberis* (20.59%) in lactating cows.

S.dysgalactiae detected in mastitis milk were 14 times higher than those detected in non-mastitis milk, suggesting that this species is a major pathogen in mastitis cases (49).

Successful treatment of bovine mastitis depends upon the selection of an appropriate antibiotic, so frequent surveillance of mastitis pathogens and updating information about their antibiotic resistance are very important. Here, we tested the isolated strains against 15 antimicrobials that have veterinary and public health importance. A high rate of resistance was observed. All of the isolates were drug resistant.This disagrees with Duse et al. (46), who reported low antibiotic resistance to many tested compounds among gramme-positive agents isolated from bovine mastitis.

A phenotypically high proportion of resistant isolates was found for amoxicillin + clavulanic acid, chloramphenicol, erythromycin, sulfa + trimethoprim, and penicillin. This partially agrees with Mbindyo et al. (32) in Kenya, who recorded the highest resistance for ampicillin followed by erythromycin in *Staphylococcus spp*. Abd El-Aziz et al. (48) in Egypt observed 100% resistance to cefalexin in *S.uberis* isolated from clinical bovine mastitis. In our study, cefalexin showed a low resistance rate in both *Staphylococcus spp.* and *Streptococcus spp.* isolates (3.61% and 11.94%, respectively). This difference in the antimicrobial resistance pattern reflects the need for regular monitoring of the antibiotic sensitivity of isolated pathogens and the strategic use of antibiotics.

Low proportions of resistant staphylococcal isolates were recorded for kanamycin and gentamicin, and no streptococcal resistant isolates were found for them. This strongly agrees with the results of Hasan et al. (47). This result indicated that optimum doses of kanamycin, cefalexin, gentamycin, and ciprofloxacin may be the drugs of choice to treat the most cases of bovine mastitis.

The molecular characterization of different resistance genes is an important measure to investigate the emergence and spreading of resistance. This study investigated six resistance genes in 15 isolates (8 *Staphylococcus spp. and 7Streptococcus spp.)* using PCR. (*blaTEM-1)* was the most detected resistance gene.It is the β-lactamase gene that mediates resistance to β-lactam antibiotics; it hydrolyzes the β-lactam ring and inactivates the antibiotic (50).

Molecular analysis of resistance genes provides a rapid screening method for investigating and controlling drug resistance.

In our study, treatment of bovine mastitis with a combination of antibiotics recovered significant improvements in the measured parameters (bacteriological cure, SSC, TBC, and the level of LDH), in addition to improvements in cow, udder, and milk states after treatment.

In our study, clinically and bacteriologically cured quarters were 92.55 percent and 88.18 percent, respectively.Clinical cure is the main goal on farms, but it is not a reliable outcome to assess antimicrobial therapy effectiveness as inflammations of the mammary gland and physical changes in milk are self-limiting and

usually return to their normal appearance within 7 days (51).

Bacterial cure in present study is near to that found by McDougall (52). Using acombination of lincomycin plus neomycin.

Cefalexin is a first generation cephalosporin. It inhibits the synthesis of bacterial cell wall with a time-dependent bactericidal activity, but kanamycin is an aminoglycoside that inhibit of bacterial protein synthesis. Its bactericidal activity is proportional to its concentration.This difference in the mechanisms of action allows a marked difference in the kinetics of killing (12).

Kanamycine's bactericidal activity is greatly reduced by the presence of milk, as is that of other aminoglycosides (53), but synergism observed between cefalexin and kanamycine overcomes this reduction (13).

Maneke et al. (54) proved a faster and greater bacterial killing rate for the combination of cefalexin and kanamycin at lower antibiotic concentrations than those observed with either drug alone.

In this study, post-treatment milk yield per cow did not obviously differ from that recorded pretreatment. The same result was revealed by Sérieys et al. (55), and Fuenzalida and Ruegg (56). Assessment of milk yield requires a prolonged follow-up period (57); this may be the reason why no significant difference in milk yield was recorded in the present study or other previous studies with similar results.

Our results proved a gradual decrease in SCC at the quarter level to near the normal value during the follow-up period after treatment. This is in line with that mentioned by Ruegg(3), who recommended that assessment of SCC responses be performed at the quarter level rather than the composite milk samples and should continue for at least 21 days post treatment, but a gradual (rather than immediate) decline should be expected and etiology influences the rate at which SCC returns to normal.

LDH is a sensitive mastitis indicator in cattle. The enzyme is primarily released by damaged udder epithelial cells, invading microorganisms, and leukocytes, which disintegrate as a response to inflammation by the animal's immune system (58)So the more severe the inflammatory response, the higher the level of this enzyme. LDH is a reliable, highly sensitive marker for early detection of udder infections and assessment of mastitis therapy (6).

Early detection of mastitis is of paramount importance in controlling the disease. The Health MateTM milk LDH mastitis test is a rapid, easily applied test. It does not require complicated steps, instruments, or the skimming of milk samples, making it an ideal mastitis screening test.

Conclusion

Regular updating of information about mastitis-causing pathogens and their antibiotic susceptibility is a very important measure. Molecular analysis of antibiotic resistance genes is a helpful screening method for investigating and controlling drug resistance. Treatment of bovine mastitis with a combination of antibiotics significantly improves the bacteriological cure, SSC, TBC, and the level of LDH in milk.

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