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ORIGINAL ARTICLE

Antimicrobial profile of multidrug-resistant *Streptococcus* spp. isolated from dairy cows with clinical mastitis

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ABSTRACT

Objective: The current investigation was designed to point out the prevalence of multidrug-resistant *Streptococcus* spp. causing acute clinical mastitis and their pattern of antibiotic resistance in dairy cows.

Materials and methods: Milk was sampled from 128 dairy cows with 191 infected quarters during the period from August 2017 to December 2018. Bacterial species were isolated from the milk samples and identified based on colony morphology and biochemical tests. Multiplex PCR was done for confirmatory detection of the *Streptococcus* spp. isolates.

Results: The chief isolation percentages, from the sampled milk, were *Escherichia coli* (26%), then *Staphylococcus aureus* (23%), and *Streptococcus dysagalactiae* (23%), then *Streptococcus agalactiae* (20.1%), and finally coagulase-negative *Staphylococci* (7.7%). In confirmed PCR streptococci isolates, the antibiotic resistance genes have been detected, including macrolides antibiotic resistance genes (*ermB* and *mefA* genes), lincosamides antibiotic resistance genes (*linB* gene), and tetracycline resistance genes (*tetM* and *tetO* genes). Age, parity number, cleaning of bedding materials, cleaning of milking facilities, and utensils and udder cleaning practice were significant risk factors for multidrug-resistant streptococcal mastitis in dairy cows.

Conclusion: The results of this study explored the phenotypic and genotypic traits of *Streptococcus* spp. which constitute a usual cause of acute clinical mastitis in dairy cows. The *ermB*, *mefA*, *tetM*, and *tetO* antibiotic-resistant genes were identified in streptococci isolates from dairy cows' milk with acute clinical mastitis, indicating a public health hazard. Thus, veterinary clinical breakpoints are needed to improve surveillance data, improve the hygiene regimen on the farms, and promote the wise use of antimicrobials.

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Introduction

Clinical mastitis is perceived to be one of the leading illnesses that influence dairy farms' productivity and dramatically affects the welfare of dairy cows [1]. The hallmarks of clinical mastitis in dairy cows include alterations in the physicochemical and microbiological characters of the milk, along with pathological changes in the glandular tissue, which may be accompanied by noticeable clinical signs on the animal [2].

Bovine mastitis, a complex multi-factorial disease, occurs depending on variables related to the animals, environment, and pathogens [2]. Among these pathogens, bacterial agents are the most common and widely distributed in the dairy cows' environment. Hence, they represent a common threat to the mammary glands in dairy cows [3]. These pathogenic agents invade the udder, multiply, often produce toxins that have a significant detrimental effect on the mammary tissue itself and the general health status of the animal [4]. Furthermore, infection by one microorganism will pave the way for the entry of other bacterial pathogens, especially during disturbances of the immune system of the animals as a whole and the mammary defense system as well [2].

Streptococcus agalactiae (S. agalactiae), Streptococcus dysgalactiae (S. dysgalactiae), and *Streptococcus uberis (S.*

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uberis) are potential causes for both subclinical and clinical mastitis [3,5]. Penicillin (PEN), amoxicillin (AML)/clavulanic acid, ampicillin (AMP), erythromycin (ERY), and clindamycin (CLI) are the most common antibiotics used to treat bovine mastitis with ideal cure rates [6]. Unfortunately, resistance to these antibiotics has been increased in Gram-positive bacteria, including streptococci. The uncontrolled use of antibiotics usually affects the microbial system of sensitive bacteria, which causes mutations, and thus allows bacteria to survive and further proliferate as antibiotic-resistant bacteria. Therefore, the antibiotic resistance rises to dangerously high levels worldwide, which is a usual threat to the ability to treat the common infectious diseases [7].

Macrolides, lincosamides, and streptogramin B bind to the 50S subunit of the bacterial ribosome, thereby blocking the synthesis of protein [8]. The resistance to these antibiotics is referred to as macrolides-lincosamides-streptogramin B (MLSB) resistant phenotypes, which are widely seen in Gram-positive bacteria [9], including *Streptococcus* spp. isolated from dairy cows with acute clinical mastitis [10]. Likewise, the widespread use of tetracycline (TET) made it included in the resistance list of many bacteria, including streptococci [11].

In cows with clinical mastitis, the pattern of antimicrobial resistance in isolated streptococci revealed that these bacteria remain susceptible to PEN, with rare exceptions [12]. The resistance to ERY appeared in the USA and Europe, with a prevalence rate between 20% and 50% [13,14], while in Brazil, it did not exceed 10% [15]. However, it is known that their resistance rate to TET reached up to 70% -80%, where the resistance genes that have been detected more frequently were *ermB*, *tetL,* and, *tetM* [16].

The data regarding streptococcal genetic resistance to antibiotics are not as extensive as other contagious pathogens [17]. Therefore, it is a fascinating organism to study, which need periodical monitoring for both its antibiogram profile and its genetic resistance pattern. The current study was planned to investigate the prevalence of multidrug-resistant *Streptococcus* spp. causing clinical mastitis and their pattern of antibiotic resistance in dairy cattle.

Material and Methods

Animals

A total number of 128 Holstein dairy cows, with 191 infected quarters, aged between 3 and 10 years old expressing the clinical signs of acute clinical mastitis were included to complete the current study. The studied dairy cows were selected from 20 farms located in Dakahlia and Damietta governorates, Egypt, during the period between August 2017 and December 2018. The Research Ethics Committee, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt, approved this study with code No R/11. The studied lactating cows were kept under semi-open sheds and were maintained under the same system of feeding at the farm. All the selected dairy cows were milked twice daily by hand or machine at 6.00 AM and 3.00 PM throughout the lactation period. The diseased cows were thoroughly examined, including examination of their mammary glands, and all clinical findings were recorded. Furthermore, their milk secretion was examined using a strip cup test to detect any milk abnormalities and identify the affected udder quarters [2]. All the selected dairy cows showed the signs of acute clinical mastitis in the form of a systemic illness along with abnormalities in the udder as well as milk secretion.

Milk sampling

Following the method of milk sampling described by the national mastitis council, the udder of each of the studied cow was washed using fresh running water, and then it was wiped with tissue papers. The teat surface in the affected quarter was then sterilized using swabs containing 75% ethanol. The first stream from the infected quarter was discarded then about 30 ml was collected in a separate sterile cup and kept on ice for immediate examination upon delivery to the Diagnostic and Animal Research Laboratory, Department of Internal Medicine, Infectious and Fish Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt. All milk samples were collected before treating the cows with any antibiotic. The analysis of somatic cell count (SCC) was performed on the same day of milk sampling using a cell counter (Fossomatic™ FC SCC for raw milk testing, Foss, Foss Allé 1, DK-3400 Hilleroed, Denmark). The dairy cow with SCC in milk ≥ 500,000 cells/ml was considered to have clinical mastitis.

Isolation and identification of Streptococcus spp.

The collected milk samples were centrifuged (3,000 rpm for 15 min) to concentrate the bacterial cells at the sediment. Using an automatic pipette, 1 µl was taken from the sediment, mixed with 5 ml of tryptone soya broth (Oxoid) in a sterile test tube, and incubated at 37°C for 18 h. A loopful was taken from the enriched samples and streaked on three different selective media. Edward's media (Oxoid) supplemented with 6% defibrinated sheep blood was used for the selective isolation of *Streptococcus* spp., including *S. agalactiae, S. dysgalactiae*, and *S. uberis*. Eosin methylene blue media (Oxoid) was used for the selective isolation of *Esherichia coli (E. coli).* Baird parker media (Oxoid) supplemented with 5% egg yolk-tellurite emulsion was used for the selective isolation of *Staphylococcus* spp., including *Staphylococcus aureus (S. aureus),* and coagulase-negative *Staphylococci*. Bacteriological identification of the isolated

bacterial species was performed based on the colony morphology on the plates of specific media and other biochemical tests [18].

Confirmatory detection of Streptococcus spp. using multiplex PCR

To extract the DNA of streptococci isolates, a mixture of a bacterial culture grown overnight (200 µl) and distilled water (800 µl) was boiled for 10 min. The mixture was then centrifuged, and the supernatant was taken as a DNA template in the polymerase chain reaction (PCR). *Streptococci* isolates were confirmed by multiplex PCR as previously described [19] using primers and conditions compiled in Table 1. The amplification reaction was performed using an aliquot of the supernatant (10 µl), deoxynucleoside triphosphate (250 µm), $MgCl_2$ (2.5 mm), primer (50 pmol), and AmpliTaq Gold™ DNA Polymerase (1 U) (Applied Biosystems, Fisher Scientific Company, Ottawa, ON, Canada). After that, the final mixture volume was completed using distilled water until the final quantity reached 50 µl. The initial denaturation was conducted at 95°C for 15 min, and then denaturation was performed at 94°C for 60 sec. The annealing temperature was 54.8°C for 60 sec. The extension was performed at 72°C for 60 sec, and the final extension was done at 72°C for 10 min. After PCR reactions, the amplified products were stained with ethidium bromide dye, then were run on agarose gel (1.5%) (Biotechnology grade AGA001.100—Bioshop Life Science Products, Bioshop Canada Inc., Ontario, Canada), and finally visualized under UV light to detect the amplicon size.

Antimicrobial susceptibility testing

Out of 73 isolates of *Streptococcus* spp., 50 confirmed PCR isolates were randomly selected to perform the antibiotic sensitivity test using the Kirby–Bauer disk diffusion assay [20], where Muller–Hinton agar medium plates (Oxoid) were used. The isolates were tested for sensitivity against 13 different antibiotic discs (Oxoid) to determine the efficacy of them as well as their sensitivity and / or resistance pattern. The used antibiotics were PEN, 10 µg; AML, 25 µg; ceftriaxone (CRO), 30 µg; cefoxitin (FOX), 30 µg; cefepime (FEP), 30 µg; TET, 10 µg; ERY, 15 µg; lincomycin (LCM), 10 µg; CLI, 2 µg; streptomycin (STR), 10 µg; gentamycin (GEN), 10 µg; enrofloxacin (ENR), 5 µg; and imipenem (IPM), 10 µg According to the diameter of the inhibition zone around each antibiotic disc and the interpretative chart supplied by the manufacturer, the antimicrobial susceptibility was classified as susceptible, intermediate, and resistant.

Detection of macrolides-lincosamides-resistant phenotypes

Macrolides resistant phenotype (M) and lincosamides resistant phenotype (L) were assessed by a double disk diffusion test using ERY (15 µg), LCM (10µg), and CLI (2 µg) disks (Oxoid) [21]. The M phenotype was recorded when the isolates were resistant to ERY only. The L phenotype was proved when the isolates were non-susceptible to LCM and/ or CLI. The resistance to both ERY (15 µg) and CLI (2 µg) was designated as MLSB phenotype. Both the inducible macrolides lincosamides streptogramin B (iMLSB) resistant phenotype and the constitutive macrolides lincosamides streptogramin B (cMLSB) resistant phenotype were studied [21]. The iMLSB phenotype was assigned if a D-shaped inhibition zone was observed around the DA disk, but the absence of an inhibition zone around the two discs marked the cMLSB phenotype.

Screening of antimicrobial resistant genes in streptococci isolates

Isolates of *S. agalactiae* and *S. dysgalactiae* were tested for the presence of macrolides, lincosamides, and TET drug classes resistant genes using conventional PCR based on their phenotypic resistance on Muller–Hinton agar plates. The *ermB* and *mefA* genes were screened using the primer pairs and under conditions previously stated [22,23]. The *linB* gene was identified using primers and under conditions previously described [24]. Meanwhile, *tetM* and *tetO* gene were detected using primers and under conditions previously reported [25,26] (Table 2).

Table 1. Multiplex PCR primer sequences used for identification of *Streptococcus* spp. isolated from milk samples from dairy cows with acute clinical mastitis.

Bacteria	Primers	Sequence (5'-3')	Target gene (Protein)	Reference
Streptococcus agalactiae	GSag-S	ATTGATAACGACGGTGTTACTGT	*sklA3* (fibrinogen binding protein)	[19]
	GSag-AS	CATAGTAGCGTTCTGTAATGATGTC		
Streptococcus dysgalactiae	GSdys-S	GTGCAACTGCATCACTATGAG	*16S rRNA*	[19]
	GSdys-AS	CGTCACATGGTGGATTTTC		
Streptococcus uberis	GSub-S	TGATTCCGACTACTACGCTAGAT	*pauA* (plasminogen activator A)	[19]
	GSub-AS	ATACTTTGAGTTTCACCGAGTTC		

Risk factors associated with multi-drug resistant streptococcal mastitis

All the required data were collected using questionnaires directed to the herd managers on every visit and also by direct observation of the milking and farming practices. All data related to the potential risk factors, including age, parity number, stage of lactation, the season of the year, cleaning of the bedding materials, cleaning of milking facilities and utensils, udder health monitoring, udder cleaning, and milking practice were obtained and scored (Table 3).

Statistical analysis

The analysis of data was performed using a statistical software program (SPSS for Windows, Version 21.0, SPSS Inc., USA). The results of the categorical variables were expressed as number (percentage). Association between the prevalence of multidrug-resistant genes in *Streptococci* spp. isolated from dairy cows' milk with clinical mastitis and the potential risk factors were studied using a univariate logistic regression analysis model. In this method, the dependent variable was the presence of resistant genes (*Streptococcus* spp. with identified antibiotic resistance genes or *Streptococcus* spp. without identified antibiotic resistance genes). Risk factors with a significant association at $p < 0.05$ (two-sided) were selected for further analysis using a multivariate logistic regression model. Regression coefficient (B), standard error (S.E.), wald, *p*-value, odds ratio (OR), and a 95% confidence interval (95% CI) were documented for each assessed risk factor. In all statistical analyses, the results were statistically significant at $p < 0.05$.

Results

The isolates of *S. agalactiae* appeared as colorless colonies with a bluish hue and surrounded with the complete zone of hemolysis on Edward's media (β-hemolysis). Meanwhile, *S. dysgalactiae* subspecies *dysgalactiae* appeared as

Table 2. PCR primer sequences used for identification of *ermB*, *mefA*, *linB*, *tetM*, and *tetO* antibiotic resistance genes in *Streptococcus* spp. isolated from milk samples from dairy cows with acute clinical mastitis.

Primer	Strand	Primer sequence 5'-3'	Target genes	Annealing temperature	Reference
ermB	F	GAAAAGGTACTCAACCAAATA	*ermB*	50°C for 30 sec	[22]
	R	AGTAACGGTACTTAAATTGTTTAC			
mefA	F	CTGTATGGAGCTACCTGTCTGG	*mefA*	52°C for 20 sec	[23]
	R	CCCAGCTTAGGTATACGTAC			
linB	F	CCTACCTATTGTTTGTGGAA	*linB*	54°C for 45 sec	[24]
	R	ATAACGTTACTCTCCTATTC			
tetM	F	TGGAATTGATTTATCAACGG	*tetM*	49°C for 60 sec	[25]
	R	TTCCAACCATACAATCCTTG			
tetO	F	AGCGTCAAAGGGGAATCACTATCC	*tetO*	55°C for 1 min	[26]
	R	CGGCGGGGTTGGCAAATA			

Table 3. Scoring protocol for the potential risk factors related to dairy cows with acute clinical mastitis.

Risk factor	Score
Age	2–4 years old = 1; 5–7 years old = 2; 8-year old or more = 3.
Parity	Parity number 1–3 = 1; parity number 4–7 = 2; parity number >7= 3.
Stage of lactation	Early stage (1–3 months of lactation period) = 1; mid stage (4–6 months of lactation period) = 2; late stage (>6 months of lactation period and till onset of the dry period) = 3.
Season of the year	Summer months =1; autumn months =2; winter months = 3.
Cleaning of the bedding material	Regular cleaning and removal of dirt underneath the animals: Yes =1; No =2.
Cleaning of milking facilities and utensils	Careful cleaning of teat cups of milking machine, hands of milking staff, and other milking utensils: Yes = 1; No = 2.
Udder health monitoring	Periodical application of California Mastitis Test on the farm: Yes = 1; No =2
Udder cleaning	Washing udder with clean water stream before milking and teat dipping in povidone-iodine (Betadine antiseptic solution) after milking process (Good) = 1; just removal of dirt if present or washing the udder with water before milking process with a sponge and then dry it with a towel (Bad) = 2.
Milking practice	Automatic milking machine =1; hand milking by farm staff = 2.

bluish hue colored colonies surrounded with brownish and greenish zones of hemolysis (α- hemolysis). The isolates of *E. coli* appeared as green metallic sheen colonies on the Eosin Methylene Blue. The *Staphylococcus* spp. appeared as black shiny colonies on Baird parker. *Streptococcus* subspecies *dysgalactiae* was detected at 279 base pairs (bp), while *S. agalactiae* was detected at 487 bp. *S. uberis* was not detected in any of the milk samples from the studied dairy cows neither by using of conventional bacteriological methods nor multiplex PCR.

In the present study, 169 bacterial isolates were obtained from 128 cultured milk samples collected from the studied dairy cows with acute clinical mastitis. Of these, 39 isolates (23%) were *S. dysgalactiae*, 34 isolates (20.1%) were *S. agalactiae*, 44 isolates (26%) were *E. coli*, 39 isolates (23%) were *S. aureus*, and 13 isolates (7.7%) were coagulase-neg *Staphylococci*.

On the animal level (128 dairy cows with clinical mastitis), Streptococcus spp. were isolated from 73 cows (57.03%), while both *E. coli* and *Staphylococcus* spp. were isolated from 55 cows (42.97%). As for the quarter level (191 infected quarters), *Streptococcus* infection was recorded in 139 quarters (72.77 %), while *E. coli* and *Staphylococcus* spp. infection was recorded in 52 quarters (27.23%). The *Streptococcus* infection rate of one quarter was 37/139 (26.62 %), two quarters was 30/139 (21.58 %), three quarters was 36/139 (25.90 %), and four quarters was 36/139 (25.90 %).

In studied cows with streptococcal mastitis (73/73), the recorded systemic illness were fever (more than 40°C) lasting for a day or two days, depression, congested mucosa, tachycardia, decreased appetite, decreased milk production to agalactia, ruminal stasis, and reduced mobility, due to either the pain of a swollen udder or feeling unwell. Furthermore, local udder abnormalities and changes in the physical characters of the milk secretion were recorded. The affected quarter of the udder was abnormal and appeared to be red, swollen, hot, painful, and firm compared to other healthy quarters with inflamed supra-mammary lymph nodes. The milk secretion seemed to be visibly abnormal (i.e., is not "drinkable") varied between increased viscosity, white to yellow clots, flakes, greenish pus, yellowish serous fluid, and sometimes tinged with blood. The presence of milk clots at quarter level was the most prevalent detectable

Table 4. Phenotypic susceptibility pattern of *Streptococcus* spp. isolated from milk samples from dairy cow with acute clinical mastitis.

Antibiotic discs	Phenotypic isolates (*n* = 50)		
	Susceptible No. (%)	Intermediate No. (%)	Resistant No. (%)
Penicillins			
Penicillin (PEN)	6 (12 %)	18 (36 %)	26 (52 %)
Amoxicillin (AML)	8 (16 %)	12 (24 %)	30 (60 %)
Cephalosporins			
Ceftriaxone (CRO)	38 (76 %)	10 (20 %)	2 (4 %)
Cefoxitin (FOX)	18 (36 %)	28 (56 %)	4 (8 %)
Cefepime (FEP)	38 (76 %)	12 (24 %)	0 (0 %)
Tetracycline			
Tetracycline (TET)	6 (12 %)	10 (20 %)	34 (68 %)
Macrolides			
Erythromycin (ERY)	2 (4 %)	14 (28 %)	34 (68 %)
Lincosamides			
Lincomycin (LCM)	0 (0 %)	10 (20 %)	40 (80 %)
Clindamycin (CLI)	2 (4 %)	8 (16 %)	40 (80 %)
Aminoglycosides			
Streptomycin (STR)	14 (28 %)	28 (56 %)	8 (16 %)
Gentamycin (GEN)	32 (64 %)	18 (36 %)	0 (0 %)
Fluoroquinolones			
Enrofloxacin (ENR)	42 (84 %)	8 (16 %)	0 (0 %)
Carbapenems			
Imipenem (IPM)	20 (40 %)	28 (56 %)	2 (4 %)

abnormality as compared with other recorded changes (85/139 quarters; 61.15% vs. 54/139 quarters; 38.85%).

Antibiotic susceptibility and resistance testing for streptococci isolates (n = 50) revealed that the prevalence of lincosamide phenotypic resistance, including LCM and CLI was 80% for each. The prevalence of phenotypic resistance to both macrolides, ERY, and TET was 68% for each. The prevalence of AML, PEN, STR, and FOX phenotypic resistance was 60%, 52%, 16%, and 8%, respectively. The prevalence of phenotypic resistance to CRO and IPM was 4% for each of them. However, *Streptococci* isolates demonstrated complete susceptibility to GEN, FEP, and ENR (Table 4).

The M resistance phenotype and L resistance phenotype were identified in 2/50 (4%) and 14/50 (28%) streptococci isolates, respectively. However, the iMLSB resistance phenotype and the cMLSB resistance phenotype were detected in 10/50 (20%) and 22/50 (44%) streptococci isolates, respectively. In contrast, 2/50 (4%) of selected streptococci isolates did not show any of these resistance phenotypes (Table 5).

With respect to macrolides resistance genes (*ermB* and *mefA*) identified in the selected streptococci isolates, the *ermB* gene was detected in 32/50 (64 %) isolates and yielded amplification product at 635 bp. Of these, six isolates were susceptible to *in vitro* sensitivity to ERY, but were resistant to both LCM and CLI, representing the L resistance phenotype, two isolates expressed intermediate resistant phenotype to these antibiotic classes, and 24 isolates were completely resistant to ERY and exhibited variable degree of resistance to both LCM and CLI in the form of either cMLSB resistant phenotype (6 isolate) or the iMLSB resistance phenotype (18 isolates) (Table 5). Furthermore, the *mefA* gene was identified in 10/50 (20 %) of streptococci isolates and yielded amplification products at 294 bp. From which two isolates exhibited intermediate phenotypic resistance to ERY, and eight isolates exhibited complete phenotypic resistance to the same class of antibiotic (Table 5).

With regard to TET resistance genes (*tetM* and *tetO*) detected in the selected streptococci isolates, 22/50 (44 %) isolates contained the *tetM* gene with a target amplicon size 1,060 bp. Of these, 20 isolates showed complete resistance, one isolate exhibited intermediate resistance, and one isolate was susceptible to TET. However, *tetO* gene was not detected in all of the screened isolates (Table 5).

In the selected streptococci isolates, 8/50 (16 %) isolates (four cMLSB resistant phenotype and four iMLSB resistant phenotype) harbored both *mefA* and *ermB* genes. 20/50 (40 %) isolates harbored both *ermB* and *tetM* genes. 2/50 (4 %) isolates, representing the M phenotype, harbored both *mefA* and *tetM* genes (Table 5). Although antibiotic phenotypic resistance demonstrated by the streptococci isolates to LCM and CLI was detected at large scale (80%), it was not expected that none of the selected isolates contained the *linB* gene.

Multivariate logistic regression model of the studied risk factors for the prevalence of multidrug-resistant *Streptococcus* spp. pointed out that the age (p = 0.031, OR: 0.300, 95% CI: 0.084–1.073), parity number (p = 0.001, OR: 12.500, 95% CI: 2.992–53.478), cleaning of bedding materials (p = 0.008, OR: 6.000, 95% CI: 1.596–22.551), cleaning of milking facilities and utensils (p = 0.040, OR: 3.857, 95% CI: 1.067–13.943) and udder cleaning practice (p = 0.001, OR: 9.873, 95% CI: 2.934–33.220) are significant effectors for the prevalence of multidrug-resistant streptococcal mastitis in dairy cows. In contrast, there was no significant association between the stage of lactation, the season of the year, udder health monitoring, milking practice, and the occurrence of multidrug-resistant streptococcal mastitis in dairy cattle (Tables 6 and 7).

Discussion

There is no doubt that the investigation of clinical mastitis in cattle has attracted the attention of researchers all over the world. It has been proven to be the most complex and disturbing disease among dairy cattle production system, which has both economic and zoonotic importance [27,28]. Accurate and periodical diagnosis of mastitis in cattle is a mast that helps to combat the occurrence or at least to reduce the costs of the disease outcome that may be worsened with any delay [29]. In the examined cows with streptococcal mastitis, systemic signs were recorded with udder abnormalities, as previously reported [2,30]. The occurrence of streptococcal mastitis is the process of invasion and inflammation of mammary tissue lobules' in a series of crises. Initially, in the lactiferous ducts, the organism multiplies rapidly with the shedding of its lining epithelium and appearance of milk clots. The bacteria then pass to the lymphatic vessels and supra-mammary lymph nodes with the flow of neutrophils to the milk ducts. During the initial invasion of tissues, a short-term systemic reaction occurs with a sharp decrease in milk production due to damage of the acinar and duct epithelium.

Studies of antibiogram for mastitis pathogens are essential; as they provide a rational antibiotic therapy, limit the antimicrobial resistance, and the potential health hazard for the public [31]. In this study, the antibiotic susceptibility and resistance testing of streptococci isolates were almost similar to the previously recorded results [26,32]. It is assumed that the high prevalence of macrolides, lincosamides, and TET resistance is a therapeutic problem originating from the extensive use of these drugs in the veterinary field without control or supervision either in treatment or even prophylaxis [33]. The problem of penicillin resistance is currently outstanding as it is the first

Table 5. Distribution of different antibiotic resistance, detected antibiotic resistant genes, and resistance phenotypes in *Streptococcus* spp. isolated from milk samples from dairy cows with acute clinical mastitis.

Isolate no.	Different antibiotic resistance	Detected antibiotic resistant genes	Resistance phenotypes
1	CLI, LCM, AML, PEN	*ermB, tetM*	L
2	ERY, TET, CLI, IPM, LCM	*ermB, tetM*	cMLSB
3	ERY, TET, CLI, LCM, AML, PEN	*ermB,tetM*	cMLSB
4	CLI, LCM, AML	*ermB*	L
5	ERY, TET, CLI, LCM, AML, PEN	*ermB, tetM*	iMLSB
6	ERY, TET, CLI, LCM, FOX, PEN	*ermB*	cMLSB
7	None	*ermB*	None
8	ERY, AML	*mefA, tetM*	M
9	ERY, TET, CLI, LCM, FOX, PEN	*ermB,mefA, tetM*	cMLSB
10	ERY, TET, CLI, LCM, AML, PEN	*ermB, mefA*	cMLSB
11	ERY, TET, CLI, LCM, AML, STR, PEN	*ermB, mefA, tetM*	iMLSB
12	ERY, TET, CLI, LCM	*ermB, tetM*	cMLSB
13	ERY, TET, CLI, AML, CRO, STR	*ermB, tetM*	cMLSB
14	LCM, AML, PEN	*ermB*	L
15	ERY, TET, CLI, PEN	*ermB, tetM*	cMLSB
16	ERY, TET, CLI, LCM,AML, STR, PEN	*ermB, tetM*	cMLSB
17	ERY, TET, CLI, LCM, AML, PEN	*ermB, mefA*	iMLSB
18	LCI, LCM	No	L
19	ERY, LCM	No	cMLSB
20	TET, CLI, LCM, STR	No	L
21	ERY, TET, LCM, AML	No	cMLSB
22	TET, CLI, AML	No	L
23	ERY, TET, CLI, LCM	No	iMLSB
24	CLI, LCM, AML, PEN	No	L
25	ERY, TET, CLI, LCM, AML, PEN	No	iMLSB
26	CLI, LCM, AML, PEN	*ermB, tetM*	L
27	ERY, TET, CLI, IPM, LCM	*ermB, tetM*	cMLSB
28	ERY, TET, CLI, LCM, AML, PEN	*ermB,tetM*	cMLSB
29	CLI, LCM, AML	*ermB*	L
30	ERY, TET, CLI, LCM, AML, PEN	*ermB, tetM*	iMLSB
31	ERY, TET, CLI, LCM, FOX, PEN	*ermB*	cMLSB
32	None	*ermB*	None
33	ERY, AML	*mefA, tetM*	M
34	ERY, TET, CLI, LCM, FOX, PEN	*ermB,mefA, tetM*	cMLSB
35	ERY, TET, CLI, LCM, AML, PEN	*ermB, mefA*	cMLSB
36	ERY, TET, CLI, LCM, AML, STR, PEN	*ermB, mefA, tetM*	iMLSB
37	ERY, TET, CLI, LCM	*ermB, tetM*	cMLSB
38	ERY, TET, CLI, AML, CRO, STR	*ermB, tetM*	cMLSB
39	LCM, AML, PEN	*ermB*	L
40	ERY, TET, CLI, PEN	*ermB, tetM*	cMLSB

Continued

Isolate no.	Different antibiotic resistance	Detected antibiotic resistant genes	Resistance phenotypes
41	ERY, TET, CLI, LCM,AML, STR, PEN	*ermB, tetM*	cMLSB
42	ERY, TET, CLI, LCM, AML, PEN	*ermB, mefA*	iMLSB
43	CLI, LCM	No	L
44	ERY, LCM	No	cMLSB
45	TET, CLI, LCM, STR	No	L
46	ERY, TET, LCM, AML	No	cMLSB
47	TET, CLI, AML	No	L
48	ERY, TET, CLI, LCM	No	iMLSB
49	CLI, LCM, AML, PEN	No	L
50	ERY, TET, CLI, LCM, AML, PEN	No	iMLSB

PEN = Penicillin; AML = Amoxicillin; CRO = Ceftriaxone; FOX = Cefoxitin; FEP = Cefepime; TET = Tetracycline; ERY = Erythromycin; LCM = Lincomycin; CLI = Clindamycin; STR = Streptomycin; GEN = Gentamycin; ENR = Enrofloxacin; IPM = Imipenem; L = Lincosamides resistant phenotype; cMLSB = Constitutive macrolides lincosamides streptogramin B resistant phenotype; iMLSB = Inducible macrolides lincosamides streptogramin B resistant phenotype; M = Macrolides resistant phenotype.

line that deals with *Streptococcus* infection in the veterinary field with massive doses [34].

Macrolides and lincosamides phenotypic resistance are increasingly reported, at different geographic locations worldwide, in Gram-positive bacterial isolates, including streptococci. The ribosomal modification, the antibiotic efflux, and drug inactivation constitute the various mechanisms responsible for a variety of phenotypes of their resistance. In our study, the ERY and LCM resistant phenotypes belonged to MLSB resistant phenotypes of either constitutive or inducible rather than M phenotype and/or L phenotype alone as previously recorded [35–39]. The majority of the selected streptococci isolates harbored *ermB* gene in accordance with the ones previously reported in New York, Brazil, and Italy [40–43]. The identified *ermB* gene in both intermediate resistant phenotypes (six isolates) and susceptible phenotypes (two isolates) indicated that this gene was already present but was not expressed.

The *mefA* gene was detected in 10 isolates where two isolates exhibited intermediate phenotypic resistance to ERY, and eight isolates showed complete phenotypic resistance to such antibiotic class in agreement with those previously reported in Italy [43] and both Asia and Australasia [44]. The obtained results revealed that all positive *mefA* gene isolates were also positive for the *ermB* gene except for two isolates which were found to be belonged to M phenotype exhibiting resistance to ERY only and not to LCM nor CLI as previously reported in France, Spain, and Italy [24,36,43]. The isolates that conferred phenotypic resistance to ERY without detected macrolides resistance genes were assumed to harbor resistance genes other than *ermB* or *mefA* genes, such as the *ermA* subclass TR, *ermC* and *mefC* genes [45].

In the current study, *tetM* gene was frequently detected and widely distributed among streptococci isolates due to the widespread and random use of such antibiotics worldwide. However, *tetO* gene was not detected in any of these isolates in harmony with those previously stated [36,43]. However, TET resistance shown by streptococci isolates that were negative for *tetM* and *tetO* genes may be attributed to other TET resistance determinants such as *tetQ*, *tetS*, *tetK*, *tetL*, and other resistant genes [46]. Interestingly, the majority of isolates carrying *tetM* gene were also positive for *ermB* gene. Such a link supports the theory that *ermB* gene is frequently linked with the *tetM* gene on the same mobile element suggesting evidence of a horizontal gene transfer [36,47].

In the selected streptococci isolates, the unexpected negative results for *linB* gene detection were a controversial issue due to the high phenotypic resistance with which these isolates behave against lincosamides. This could be explained by the presence of *ermB* gene in the majority of these isolates, which might be responsible for the cMLSB resistant phenotype and iMLSB resistant phenotype mediating both lincosamides and macrolides resistance. Likewise, other genes mediating antibiotic inactivation enzymes for such antibiotic class might be present such as *mphC* and *lnuD* among this collection of isolates [48] but were not investigated.

The highest prevalence of multidrug-resistant streptococcal mastitis in dairy cows was recorded in the 5–7 years old age group with parity number 4–7 times in agreement with those previously reported [49]. Older cows with multiple parities have large teats with more relaxed sphincter muscles, increasing the possibility of entrance of the infectious agent to the cows' udder through their large teat orifice [2]. Besides, a competent innate host defense mechanism for younger age animals with less parity number is one of the possibilities that make them less susceptible to infection [50].

Table 6. Distribution of potential risk factors associated with multi-drug resistant *Streptococcus* spp. isolated from milk samples from dairy cows with acute clinical mastitis.

Variable and category	Streptococci isolates		*p*-value	95% Confidence Interval (95% CI)
	Without antibiotic resistant genes (*n* = 16)	With antibiotic resistant genes (*n* = 34)		
Age				
2–4 years	0 (0%)	6 (17.6%)	0.031	0.08–1.07
5–7 years	12 (75%)	24 (70.6%)		
8 years or more	4 (25 %)	4 (11.8%)		
Parity				
1–3 times	10 (62.5%)	4 (11.8%)	0.001	2.99–53.47
4–7 times	6 (37.5%)	30 (88.2%)		
More than 7	0 (0%)	0 (0%)		
Stage of lactation				
Early lactation	10 (62.5%)	16 (47.1%)	0.425	0.47–6.01
Mid lactation	4 (25%)	12 (35.3%)		
Late lactation	2 (12.5%)	6 (17.6%)		
Season of the year				
Summer months	0 (0 %)	0 (0%)	0.945	0. 28–3.24
Autumn months	1 (12.5%)	10 (29.4%)		
Winter months	14 (87.5%)	24 (70.6%)		
Cleaning of bedding materials				
Yes	9 (56.3%)	6 (17.6%)	0.008	1.59–22.55
No	7 (43.7%)	28 (82.4%)		
Cleaning of milking facilities and utensils				
Yes	8 (50%)	7 (20.6%)	0.040	1.06–13.94
No	8 (50%)	27 (79.4%)		
Udder health monitoring				
Yes	4 (25%)	14 (41.2%)	0.945	0.05–24.06
No	12 (75%)	20 (58.8%)		
Udder cleaning				
Good	6 (37.5%)	3 (8.8%)	0.001	2.93–33.22
Bad	10 (62.5%)	31 (91.2%)		
Milking practice				
Milking machine	4 (25%)	8 (23.5%)	0.204	0.34–140.79
Hand milking	12 (75%)	26 (76.5%)		

Inadequate cleaning of the house and bedding materials underneath the cows significantly affected the prevalence of multidrug-resistant streptococcal mastitis in harmony with those previously recorded [28]. Environmental pathogens can survive and multiply in unhygienic organic bedding materials. Therefore, poor housing conditions, including faulty drainage of manure, slapdash care in barn cleaning, presence of muddy bedding, and poor infection control, were the major contributors that are involved in the contamination of the udder as well as the teat ends with dirt harboring the microorganisms [51,52]. Furthermore, the presence of mud on the teat is indicative of unhealthy teat conditions as when the mud dries on the teat, and it pulls moisture from the skin making it less elastic and prone to cracks and fissures thus facilitating the bacterial invasion of the teat canal [53].

Cleaning of milking facilities and utensils significantly affected the prevalence of multidrug-resistant streptococcal mastitis. They act as a fundamental risk factor for the

Table 7. Multivariate logistic regression model for risk factors associated with multi-drug resistant *Streptococcus* spp. isolated from milk samples from dairy cows with acute clinical mastitis.

Variable	B	S.E.	Wald	*p*-value	OR	95% CI
Age						
	−1.205	0.651	3.427	0.031	0.300	0.084–1.073
Parity						
	2.526	0.742	11.599	0.001	12.500	2.992–53.478
Cleanliness of bedding materials						
	1.792	0.676	7.035	0.008	6.000	1.596–22.551
Cleanliness of milking facilities and utensils						
	1.350	0.656	4.239	0.040	3.857	1.067–13.943
Udder cleaning						
	2.290	0.619	13.681	0.001	9.873	2.934–33.220
Constant						
	−2.400	3.761	0.407	0.523	0.091	–

B = Regression coefficient; S.E. = Standard error; OR = Odds ratio; 95% CI = Confidence interval at 95%.

spread of contagious microorganisms, including multi-drug-resistant *Streptococcus* spp. from one cow to another. Failure to maintain adequate sanitation and disinfection of milkers' hands, teat cups of the milking machine, and other tools used in the milking process was associated with a higher incidence of spread of resistant bacteria [54]. The cleaning of the udder during the milking time significantly influenced the prevalence of multi-drug resistant streptococcal mastitis as well. In the present study, the bad cleaning protocol of the udder was superior to other good protocols in the occurrence and spread of multidrug-resistant streptococcal mastitis in dairy cows. Moreover, the use of the common sponge, washing rags, or towels between cows help in the spread of such contagious pathogens, especially multidrug-resistant streptococci with a resultant intra-mammary infection in agreement with those previously stated [55]. The current study emphasizes the need for veterinary clinical breakpoints to improve surveillance data, improve the cleanliness of the udder during the milking process as well as the milking facilities and utensils, improve treatment of various animal diseases, and promote the wise use of antimicrobials.

Conclusion

The results of the current study explored the phenotypic and genotypic traits of multidrug-resistant *Streptococcus* spp., which are a common cause of acute clinical mastitis in dairy cows on the species level. The isolated streptococci appeared to be highly resistant to lincosamides, macrolides, and TET classes of antibiotics with complete susceptibility to ENR, GEN, and FEP. The *ermB*, *mefA*, *tetM* gene, and *tetO* antibiotic-resistant genes were identified in multidrug-resistant *Streptococcus* spp. isolated from dairy cows' milk with clinical mastitis, indicating a public health hazard. Cows' age, parity number, cleaning of the bedding materials, cleaning of milking facilities and utensils, and udder cleaning protocol are considered as potential risk factors, playing a significant role in the occurrence of multidrug-resistant streptococcal mastitis in Egypt. Further investigations for the detection of other antibiotic resistance genes in multidrug-resistant *Streptococcus* spp. are required to offer detailed data for their content of antibiotic resistance genes in such species genome.

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Conflict of interest

The authors declared that they have no conflict of interest related to this research.

Authors' contributions

Both the authors contributed equally in designing, experimentation, analysis, and manuscript preparation and finalization. All the authors finally approved for publication of the article.

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