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Original Article *Bacteriology*

CylE and mig as virulence genes of streptococci isolated from mastitis in cows and buffalo in Egypt

Rehab El-Shafei[1], Azza Farag[2], Rasha M. Elkenany[3], Gamal Younis [3]

[1] Aga District Veterinary Hospital, Mansoura, Egypt.
[2]Animal Health Research Institute, Dokki, Giza, Egypt.
[3] Department of Bacteriology, Mycology, and Immunology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt.

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Address correspondences to
Rasha M. Elkenany, PhD; Tel: +2-01090226696; E-mail: dr_rashavet22@yahoo.com

ABSTRACT

Objective: A study was carried out to investigate the prevalence of mastitis caused by *Streptococcus agalactiae and Streptococcus dysgalactiae* in cows and buffalo reared in households and smallholder dairy farms, and to detect their antibiotics susceptibility, and molecular investigation of some virulence genes (*cylE* and *mig* genes).

Design: Observational study.

Samples: A total of 288 milk samples were gathered from 72 mastitic animals (48 cows and 24 buffalo) from households and smallholder dairy farms in Dakahlia and Domiatte Governorates, Egypt.

Procedures: Isolation and identification of *S. agalactiae* and *S. dysgalactiae* was performed usin conventional techniques. The identified isolates were examined for antimicrobial resistance by dis diffusion assay, Minimum inhibitory concentration MIC by broth microdilution method as well a virulence genes (*cylE* and *mig* genes) by polymerase chain reaction (PCR).

Results: Forty-five (62.5%) out of 72 animals showed the clinical signs of mastitis. Microbiological evaluation of 288 mastitis milk samples was displayed 190 (65.72%) *streptococci* strains composing of 114 (60%) *S. agalactiae* and 76 (40%) *S. dysgalactiae* strains. The antibiotic susceptibility tests revealed that *S. agalactiae* strains was resistant to trimethoprim (100%), followed by tetracycline and minocycline (37.7%), whereas intermediate resistant was observed to other tested antibiotics. Moreover, *S. dysgalactiae* strains were highly resistant to lincomycin, tetracycline (87.52 each), followed by trimethoprim (81.6) and minocycline (75%), while all strainswere susceptible to penicillin, amoxicillin, cephapirin, and cefquinome. Additionally, the highest MIC with the widest range (1 to ≥128 µg/mL) was observed to trimethoprim for *S. agalactiae* and to erythromycin and lincomycin for *S. dysgalactiae*. In contrast, the lowest MIC was detected to penicillin, amoxicillin, cefquinome, and erythromycin for *S. agalactiae* and to penicillin, amoxicillin, cephapirin, cefquinome, and rifaximin for *S. dysgalactiae*. The *cylE* gene was displayed in 6 (60%) *S. agalactiae* strains, whereas the *mig* gene was found in 4 (40%) *S. dysgalactiae* strains.

Conclusion and clinical relevance: Our data highlights the importance of awareness of antibiotic resistar strains of *S. agalactiae* and *S. dysgalactiae* in various mastitic animals (cows and buffalo) in Egypt.

Keywords: Antibiotic susceptibility; Cows and buffalo; Mastitis; *Streptococci*, Virulence genes (*cylE, mig*).

1. INTRODUCTION

Bovine mastitis is one of the furthermost challenging infections having a global economic influence on the dairy industry, numerous bacterial genera and species able to cause mastitis extensive in the environment of dairy cows [1]. Various pathogens have been pronounced as an etiology of bovine mastitis. Based on their epidemiology, mastitis microorganisms have been categorized into two groups contagious and environmental. The main reservoir of contagious pathogens is an infected udder while a contaminated environment is the most important reservoir of pathogens producing environmental mastitis [2]**.** *Streptococcus agalactiae (S. agalactiae), S. dysgalatiae,* and *S. uberis* have been stated as the furthermost three common causative agents of mastitis [3]**.** *S. agalactiae*, as well recognized as Group B *Streptococcus* (GBS) succeeding in the Lancefield category [4]**,** is extremely contagious agents and usually establish in the mammary gland of cattle. *S. agalactiae* is frequently related to acute clinical and persistent subclinical mastitis [5]**.**

There are scarce antibiotic investigation networks for animal bacterial strains meanwhile they are hard to set up, preserve, and progress. According to recent studies, the antibiotic resistance of udder pathogens is a global widespread. The local variances in resistance patterns of pathogens are predominant; even though considerable use and occasionally abuse some antibiotics remain efficient today however [6].

S. agalactiae has a diversity of virulence factors associated with pathogenicity. Several *s. agalactiae* virulence factors were identified in the mouse and rat models, main virulence factors of *S. agalactiae* comprise

fibrinogen binding protein (*fnb),* laminin-binding protein (*lmb)*, fibronectin-binding protein *(pavA*), β-C protein *(cba),* capsule, C5a peptidase (*scp)*, hyaluronate lyase (*hylB*), α-C protein, β- peptidase *(scp*), hyaluronate lyase (*hylB),* α-C protein, β- hemolysin/cytolysin (*cyl),* and CAMP factor *(cfb)* [*7]*. The *cyl* gene codes for β-hemolysin, a toxin that played a role in tissue injury and the systemic spread of the bacteria and contributes to meningitis [8].

S. dysgalactiae has been furthermost generally pronounced as a contagious pathogen nevertheless it also acts as environmental pathogens [2] *S. dysgalactiae* subsp. *dysgalactiae* have been considered exclusively as animal pathogens and are commonly associated with clinical and subclinical bovine mastitis.

Though the virulence factors of *S. dysgalactiae* are not so far completely unstated, a surface-expressed M-like protein, called (*mig)* can bind immunoglobulin G *(IgG*), K2 macroglobulin (K2-M) bovine immunoglobulin A (B-IgA), and acts a title role in anti-phagocytosis by bovine neutrophils in the attendance of bovine serum [9]. M-like protein and lipoteichoic acid (LTA) were present in strains of *S. dysgalactiae* isolated from bovine intramammary gland [10].

The main aim of this study was to determine the prevalence of mastitis produced by *S. agalactiae and S. dysgalactiae* in cows and buffalo that were tested from households and smallholder dairy farms in Dakahlia and Domiatte Governorates, Egypt and determine their susceptibility to different antibiotics. Besides, molecular investigation to detect some virulence genes (*cylE* and *mig* genes) in streptococcal isolates (*S. agalactiae* and *S. dysgalactiae*) by PCR was performed.

2. MATERIALS AND METHODS

2.1. Sampling

In total of 288 milk samples were gathered from 72 mastitic animals (48 cows and 24 buffalo) from households and smallholder dairy farms in Dakahlia and Domiatte Governorates, between October 2016 and July 2017. The age of affected animals ranged from 5 to 8 years and the milk yield per day ranged from 20 to 30 kilos. The udder of each animal was inspected before sample collection for the recognition of clinical signs of mastitis (inflammation, asymmetry, hotness, swelling, or any physical changes). Before sample collection for bacterial investigation, the first milk stream was rejected after that tedious teat cleaning and disinfection by 70% alcohol as recommended by the National Mastitis Council [11] was achieved. Samples were collected into sterile screw-capped McCartney bottle and immediately give in to the laboratory in an ice container for further bacteriological examination.

2.2. Isolation and identification of Streptococcus species

The collected milk samples were streaked onto the surface of 5% sheep blood agar and Edward's media *(Oxoid)* [12]. The incubation of such plates was aerobically done at 37°C for 24-48 h. All isolates were conventionally identified based on the following: colony morphology, Gram's stain, catalase, oxidase reactions, and esculin hydrolysis test [13]. Additionally, suspected streptococcal colonies giving gram-positive cocci with catalase and oxidase negative were biochemically identified by API 20 Strep (Biomerieux, Crappone France). Serological grouping of isolates was achieved with a commercial latex agglutination kits for the identification of streptococcal groups (A, B, C, D, F, and G) as recommended by the manufacturer [14].

2.3. 2.3. Antibiotic susceptibility test

The in vitro disk diffusion method was made as described by Bauer, Kirby [15] using Mueller-Hinton agar plates. The sensitivity of *Streptococcus* species to 15 chemotherapeutic agents belonged to seven classes Oxoid [14] was examined; penicillin G, amoxicillin, chloramphenicol, cloxacillin, cephalexin, cephazolin, cephapirin, cefquinome, erythromycin*,* lincomycin, tetracycline, minocycline, trimethoprim, rifampicin, and rifaximin. These tested antibiotics were commonly utilized for therapeutic uses in dairy Egyptian farms. The results were interpreted in accordance with Clinical Laboratory Standards [16].

2.4. Minimum inhibitory concentration (MIC)

MIC of 15 antibiotics against representative streptococcal species (8 *S. agalactiae* and 16 *S. dysgalactiae* strains) was determined using the broth microdilution method as outlined in Overesch, Stephan [17] The range of the concentration of the antibiotics was from 0.015 to 128 ug/ml for which only high-level resistance was mark off. Briefly, 3-5 colonies were transported into 5 mL of Mueller-Hinton broth (MHB*)* (Oxoid) and incubated for 16-20 h at 35°C. Subsequently, almost 0.5 mL of this pre-inoculum was transported to 5 mL of MHB and adjusted to a turbidity of McFarland standard 0.5 to get an inoculum with almost 1.5 × 10^8 CFU/mL. Next, 200 µL was transferred from the inoculum to 11 mL of MHB. Each well was completed to 100 µL of the adjusted inoculum. To verify the inoculum density of each isolate, 10 µL of the final inoculum was diluted in 10 mL of 0.9% NaCl, and 100 µL of this dilution was plated onto blood agar plates. The micro-titer plates were incubated aerobically for 16-20 h at 35°C and then scored by visual examination. The breakpoints used were those recommended by the CLSI [16] for streptococci. The lowest concentration of antibiotic required to inhibit bacterial growth is considered as MIC.

2.5. Molecular detection of Streptococcus virulence-associated genes

Bacterial DNA extraction was achieved by QIAamp DNA mini kit **(Metabion, Germany)** instructions for the detection of some virulence-associated genes (*cylE* and *mig* genes) in 20 randomly representative streptococcal isolates (10 ***S. agalactiae*** and 10 ***S. dysgalactiae***) was performed. The used primer pairs (**Metabion, Germany)** in the PCR protocols were illustrated in Table (2). The following cycling condition was performed; one cycle initial denaturation at 94 °C for 5 min, then 35 cycles comprising of denaturation at 94 °C for

30 sec, annealing at 55 °C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 7 min.

3. RESULTS

3.1. Prevalence of streptococcal species in mastitis milk

In practice of evaluation of the mastitis animals, a total of 72 animals encompassing of cows (48) and buffalo (24) were tested for clinical signs of mastitis. Milk samples of 45 (62.5%) animals were found mastitic. Among the 45 animals, 35 (72.29 %) were from cows and 10 (41.66 %) from buffalo. Regarding quarter, among 288 examined quarters, 190 (65.72%) quarters comprising of 147\192 (76.56%) from cows and 43\96 (44.79%) from buffalo were mastitic. Furthermore, microbiological assessment of samples showed 190\288 (65.72%) streptococci composing of 114 (60%) *S. agalactiae* and 76 (40%) *S. dysgalactiae* isolates from mastitis milk samples **(Table 2)**.

Table 1. Oligonucleotide primers sequences.

Target gene	Sequence	Amplified product	Reference
cylE	TGACATTTACAAGTGACGAAG TTGCCAGGAGGAGAATAGGA	248 bp	***Jain et al. (2012)***
Mig	CGTTTTTAGTTTCGGGAGCA TGCCTTCAATTGAGTCTGCTG	188 bp	***Krishnaveni et al. (2014)***

Table 2. Prevalence of mastitis and streptococcal species in milk samples (n=288).

Examined animals	No. of animals	Examined quarters	Animal		Quarter		Bacterial strains	
			Positive	%	Positive	%	*S. agalactiae*	*S. dysgalactiea*
Cows	48	192	35	72.29	147	76.56	114 (60%)	76 (40%)
Buffaloes	24	96	10	41.66	43	44.79		
Total	72	288	45	62.5	190	65.72		

Table 3: Antibiotic susceptibility *of S. agalactiae* (n=114) and *S. dysgalactiae* (n=76).

Antimicrobial agent	*S. agalactiae*						*S. dysgalactiae*					
	Resistant		Sensitive		Intermediate		Resistant		Sensitive		Intermediate	
	No	%	No	%	No	%	No	%	No	%	No	%
Pencillin (10 IU)	0	0	114	100	0	0	0	0	76	100	0	0
Amoxicillin|(10 ug)	0	0	114	100	0	0	0	0	76	100	0	0
Chloramphenicol (30 ug)	0	0	0	0	114	100	0	0	28	36.8	48	63.2
Cloxacillin (5 ug)	28	24.6	86	75.4	0	0	0	0	33	43.4	43	56.6
Cephalexin (30 ug)	28	24.6	0	0	86	75.4	0	0	0	0	76	100
Cefazolin (30 ug)	0	0	28	24.6	86	75.4	0	0	0	0	76	100
Cephapirin (30 ug)	0	0	43	37.7	71	62.3	0	0	76	100	0	0
Cefquinome (30 ug)	0	0	71	62.3	43	37.7	0	0	76	100	0	0
Erythromycin (30 ug)	0	0	43	37.7	71	62.3	0	0	0	0	76	100
Lincomycin (10 ug)	0	0	0	0	114	100	65	87.5	0	0	11	14.5
Tetracycline (30 ug)	43	37.7	0	0	71	62.3	65	87.5	0	0	11	14.5
Minocycline (30 ug)	43	37.7	0	0	71	62.3	57	75	0	0	19	25
Trimethoprim (5 ug)	114	100	0	0	0	0	62	81.6	0	0	14	18.4
Rifampicin (10 ug)	0	0	0	0	114	100	0	0	0	0	76	100
Rifaximin (10 ug)	0	0	0	0	114	100	0	0	0	0	76	100

3.2. Antibiotic susceptibility results and MIC

The results of antibiotic susceptibility tests performed on all isolates of *S. agalactiae* and *S. dysgalactiae* were illustrated in **Table (3)**. All *S. agalactiae* strains showed absolute resistant to trimethoprim, followed by tetracycline and minocycline (37.7%), while intermediate resistant was observed to chloramphenicol, lincomycin, rifampicin, rifaximin (100% each), followed by cephalexin, cefazolin (75.4% each), cephapirin, and erythromycin (62.3% each). All *S. agalactiae* strains were susceptible to penicillin and amoxicillin. Moreover, *S. dysgalactiae* strains showed high resistance to lincomycin, tetracycline (87.52 each), followed by trimethoprim (81.6) and minocycline (75%), while all strains were susceptible to penicillin, amoxicillin, cephapirin, and cefquinome.

Furthermore, **Table (4)** revealed that trimethoprim was the highest MIC and the widest range (1 to ≥128 µg/ml) for *S. agalactiae,* whereas penicillin, amoxicillin, cefquinome, and erythromycin were the lowest MIC detected (0.03 µg/ml). Furthermore, **Table (5)** showed that erythromycin and lincomycin was the highest MIC and the widest range (1 to ≥128 µg/ml) for *S. dysgalactiae*, whereas penicillin, amoxicillin, cephapirin, cefquinome, and rifaximin was the lowest MIC detected (≤0.6-0.015 µg/ml).

3.3. Molecular determination of streptococcal virulence-associated genes

In this study and by PCR detection, the *cylE* gene was detected in 6 (60%) strains of *S. agalactiae* **(Figure 1)**, while the *mig* gene was found in 4 (40%) strains of *S. dysgalactiae* **(Figure 2)**.

Table 4. Minimal inhibitory concentration (MIC) of *S. agalactiae* strains (n=8) and ranges of antibiotic solutions performed in the study.

Compound	0.6-0.015≤	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	≥128
Penicillin G	0	6	2	-	0	0	0	0	0	0	0	0	0	0	0
Amoxicillin	0	1	5	2	0	0	0	0	0	0	0	0	0	0	0
Cloxacillin	0	0	0	0	0	4	4	0	0	0	0	0	0	0	0
Cephalexin	0	0	0	0	0	0	0	3	5	0	0	0	0	0	0
Cefazolin	0	0	2	6	0	0	0	-	0	0	0	0	0	0	0
Cephapirin	0	0	3	5	0	0	0	0	0	0	0	0	0	0	0
Cephaquinome	0	5	3	0	0	0	0	-	0	0	0	0	0	0	0
Erythromycin	0	3	5	0	0	-	-	-	0	0	0	0	0	0	0
Lincomycin	0	0	0	0	8	0	0	0	0	-	0	0	0	0	0
Tetracycline	0	0	0	0	5	0	0	0	0	0	0	0	3	0	0
Minocycline	0	0	0	0	5	0	0	0	-	0	-	0	3	0	0
Chloramphenicol	0	0	0	0	0	0	0	8	-	0	-	0	0	0	0
Trimethoprim	0	0	0	0	0	0	0	0	-	0	-	0	0	0	8
Rifampicin	0	0	2	5	1	0	0	0	-	0	0	0	0	0	0
Rifaximin	0	0	0	0	5	3	0	0	0	0	0	0	0	0	0

Table 5. Minimal inhibitory concentration (MIC) distribution for *S. dysgalactiae* strains (n=16) and ranges of antibiotic solutions performed in the study.

Compound	≤0.6-0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	128≥
Penicillin	16	0	0	-	0	0	0	0	0	0	0	0	0	0	0
Amoxicillin	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cloxacillin	0	7	9	0	0	0	0	0	0	0	0	0	0	0	0
Cephalexin	0	0	0	0	10	6	0	0	0	0	0	0	0	0	0
Cefazolin	0	0	13	3	0	0	0	0	0	0	0	0	0	0	0
Cephapirin	9	7	0	0	0	0	0	0	0	0	0	0	0	0	0
Cephaquinome	14	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Erythromycin	0	0	13	0	0	0	0	0	0	0	0	0	0	0	3
Lincomycin	0	0	0	0	14	0	0	0	0	0	0	0	0	0	2
Tetracycline	0	0	0	0	0	0	0	1	1	6	2	0	2	4	0
Minocycline	0	0	0	8	3	0	0	0	0	0	2	3	0	0	0
Chloramphenicol	0	0	0	0	0	0	0	10	6	0	0	0	0	0	0
Trimethoprim	0	0	0	0	2	14	0	0	0	0	0	0	0	0	0
Rifampicin	0	0	15	1	0	0	0	0	0	0	0	0	0	0	0
Rifaximin	2	3	4	7	0	0	0	0	0	0	0	0	0	0	0

Zero (0) indicates no isolates with an MIC at that concentration

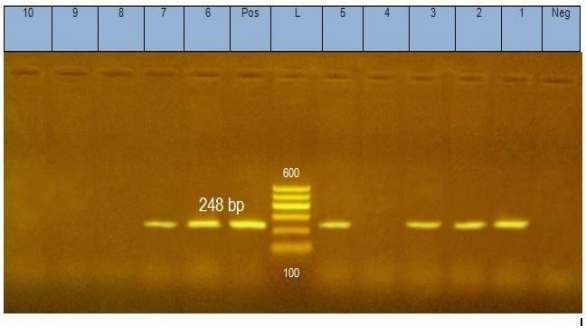

Figure 1. Agarose gel electrophoresis of *cylE* gene amplification (248 bp) for *S. agalactiae* strains isolated from milk samples. L: 100 bp ladder. Pos: control positive. Neg: control negative. Lanes (1-3,5-7): positive samples. Lanes (4, 8-10): negative samples.

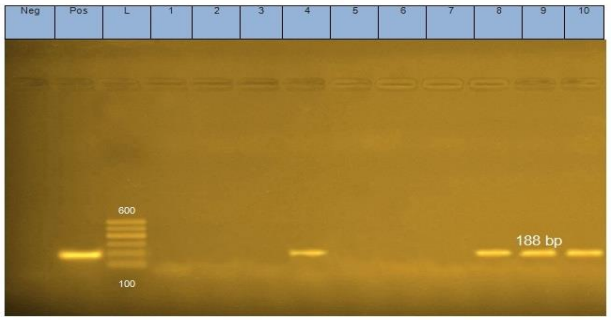

Figure 2. Agarose gel electrophoresis of *mig* gene amplification (188 bp) for *S. dysgalactiae* strains isolated from milk samples. L: 100 bp ladder. Pos: control positive. Neg: control negative. Lanes (4,8-10): positive samples. Lanes (1-3, 5-7): negative samples.

4. DISCUSSION

Currently, the commercial influence of clinical and subclinical mastitis is high in the dairy industry. The economic losses arise from diminished milk production, treatment and employment costs, veterinary fees, risk of culling or death of the cow, and decreased milk quality and milk value. Additionally, low-quality milk may hold pathogens and their toxins, that may harmful for human health [18].The previous data suggested that *S. agalactia* and *S. dysgalactiae* are the main causative agents of mastitis [6]. Biochemical and molecular analysis of isolates gotten from different mastitic animals (cows and buffalo) categorically revealed *S. agalactiae (60%)* and *S. dysgalactiae* (40%). Results agree with Kia [19] and Eldesouky, Refae [20] who reported a higher prevalence of *streptococci* from mastitic animals (cows and buffalo) with a percentage of 75% and 38%, respectively. In contrast, a lower prevalence of *streptococci* was shown by other investigators as Getahun, Kelay [21], Ranjan and Singh [22], Chen, Yang [23] and Jeykumar, Vinodkumar [24] with percentages of 22.8%, 5.7%, 15.5%, and 16.1%, respectively. The prevalence of mastitis was common in cows than buffalo because buffalo has been traditionally considered less susceptible to mastitis than cow. Buffalo have a long narrow teat canal, which may be expected to prevent the invasion of microorganism and give them higher absolute and relative resistance to pathogens [25]. The undiscriminating and ill-advised administration of antibiotics and the unreasonable treatment of bovine mastitis with diverse antibiotics were requested serious problems alike multiple drug resistance. Up to now, various kinds of antibiotics were used against the pathogens in bovine mastitis with or without identification and drug susceptibility test [26]**.** This study displayed high resistance of *S. agalactiae* strains to trimethoprim, tetracycline, and minocycline and intermediate resistance to the other examined antibiotics. On the other hand, the furthermost effective antibiotics against *S. agalactiae* were penicillin and amoxicillin. Therefore, β-lactams were persistence the primary choice of antibiotic recommended for the treatment of bovine mastitis. These results coincide with research performed by Erskine, Walker [27] and İKİZ, BaŞAran [18] The tetracycline resistance of *Streptococcus* was reported by Overesch, Stephan [17] and Tian, Zheng [28] This investigation was opposite to the results of Ebrahimi, Nikookhah [29] and Singh, Chandra [30] who displayed high resistance rates of *S. agalactiae* to amoxicillin in percentages of 76.92% and 93.03%, respectively. Furthermore, *S. dysgalactiae* demonstrated high resistance to lincomycin, tetracycline, followed by trimethoprim and minocycline, while all strains were susceptible to penicillin, amoxicillin, cephapirin and cefquinome. These results were contrary to Mosaferi, Mehrabani [31] who stated that *S. dysgalactiae was* the greatest sensitivity to tetracycline and the least sensitivity to penicillin.

Mig protein of *S. dysgalactiae* was previously proposed to be involved in resisting phagocytosis by bovine neutrophils in the existence of bovine serum [32]**.** Consequently, the mig protein, an M-like protein, is represented a potential virulence factor of *S. dysgalactiae*. Also, the *cyl* genes of *S. agalactiae* are needed to produce hemolysin [10]. Thus, some virulence-associated genes (*cylE* and *mig* genes) were determined in streptococcal isolates (*S. agalactiae* and *S. dysgalactiae*) by PCR in this study. The results revealed the attendance of *cylE* gene in 60% of tested *S. agalactiae* strains and *mig* gene in 40% examined *S. dysgalactiae* strains. Jain, Tewari [33] observed 22.2 % (6/27) strains had the *cylE* gene while [Spellerberg, Martin [34]] detected 23% strains positive for the *cyl*E gene.

Conclusion

The present investigation showed that a higher prevalence of *S. agalactiae* than *S. dysgalactiae* in various mastitic animals (cows and buffalo) in Egypt. Furthermore, the development of antibiotic-resistant and virulent strains of *S. agalactiae* and *S. dysgalactiae* occurred. Also, the slightly elevated MIC indicated the emergence of resistant streptococcal species. Consequently, continuous surveillance of antimicrobial-resistant mastitis pathogens is vital for the right decisions on bovine mastitis treatment.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to this article.

Authors' Contribution

Gamal A. Younis designed the experiment and revised the manuscript. Azza Farag shared in the collection of samples and in carrying out the practical part. Rasha M. Elkenany shared in writing the paper and took the responsibility of correspondence to the journal. Rehab El-Shafei collected milk samples and carried out the practical part. All authors approved the final version of the manuscript for publication.

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