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Molecular characterization of *Streptococcus agalactiae* isolated form dairy cows with mastitis

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Abstract

Mastitis is a significant disease affecting dairy cattle in Egypt, leading to huge economic losses for dairy industries. A highly infectious bacterium that causes bovine mastitis in dairy cows is the *Streptococcus agalactiae*. The current study aim to examine the genotypic traits of *S. agalactiae* recovered from mastitic cows and evaluating any possible relations between these traits and the behavioral properties. Milk and bulk tank milk samples were collected from dairy cows in the El-Sharkia governorate in Egypt that suffering from mastitis in addition to environmental samples. Following bacteriological isolation and molecular confirmation of *S. agalactiae* isolates, PCR was used to identify virulence and antibiotic resistance genes. Additionally, ERIC-PCR was employed to investigate the genotyping of numerous antibiotic-resistant *S. agalactiae* isolates. *S. agalactiae* was recovered in an incidence of 28%, 7.8% and 21.4% from mastitic cow's milk samples, environmental samples and bulk tank milk samples respectively. Cefotaxime, Ceftiofur, Erythromycin, Enrofloxacin, and Vancomycin were all effective against the majority of strains tested. High Tetracycline resistance is caused by overuse of antimicrobials. In addition, we found 17 MDR *S. agalactiae* isolates. The predominant virulence profile of isolated *S. agalactiae* isolates was characterized by the existence of ***fbsA***, ***fbsB***, ***bibA***, ***cfb***, and ***cyl*** genes. Only 37 (95%) isolated strains exhibited the resistance genes. ERIC-PCR results revealed genomic diversity and heterogeneity among *S. agalactiae* isolates. Our work presents virulence factor and antimicrobial susceptibility profiles that are useful for clinical management, prevention, and control of bovine mastitis in dairy cows. Furthermore, we underline the importance of raising knowledge about the accurate use of antimicrobials in dairy farms.

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Introduction

Bovine mastitis is a major hazard to the dairy production worldwide, affecting milk quality and quantity and resulting in huge financial losses (**Abebe *et al.,* 2016**).
Streptococcus agalactiae is an infectious bacterium associated with bovine mastitis. This is a leading cause of udder infection causing clinical and subclinical mastitis that decreases milk yield and lowers milk quality (**Reyes *et al.,* 2017**).

To approach appropriate treatment and control, it is vital to perform antibiotic sensitivity test of related antimicrobials because the consistent utilization of frequently used antibiotics for the treatment of animals or the overuse and misuse of antimicrobial agents have led to the development of resistant forms of previously harmless bacteria (**Seyoum *et al.,* 2018**).

It is crucial to comprehend how virulence factors contribute to bacterial pathogenicity in order to create focused therapies for bovine mastitis. Virulence factors associated with adhesion of *S. agalactiae* include the immunogenic bacterial adhesion (***bibA***) and ***protein C***, covering surface proteins ***Cα (bca)*** and ***Cβ (bac***), while fibrinogen-binding protein A (***fbsA***), fibrinogen-binding protein B (***fbsB***), pilus island 1 (***PI-1***), and pilus island 2 (***PI-2a, PI-2b***) contributing to invasion and colonization. The CAMP factor is the virulence factor linked to the infection stage. The two main virulence factors influencing *S. agalactiae* pathogenicity are β-hemolysin/cytolysin (***cyl***) and CAMP factor (***cfb***). These virulence factors are often used as markers for genotyping of *S. agalactiae* isolates and in its epidemiological studies (**Zastempowska *et al.,* 2022**).

ERIC-PCR has been used to molecularly type a large number of Gram-negative and some Gram-positive bacteria (**Elsayed *et al.,* 2022**). It has been demonstrated that *S. agalactiae* possesses ERIC sequences, and ERIC-PCR was employed as a genotyping technique. (**Bishi *et al.,* 2008**). The ERIC-PCR-based genotyping technique is a highly discriminatory molecular typing method that is highly reproducible, simpler, and quicker, regardless of whether the *S. agalactiae* genome contains ERIC elements or not. This method is also applicable when ERIC primers operate on the principle of RAPD PCR. In addition, it is applicable for the genotyping of numerous clinical isolates with a broad geographic distribution and for epidemiological studies of group B streptococci. As a result, the goal of this research was to study prevalence, multiple antibiotic resistances (MAR), virulence factors of *S. agalactiae* bacteria causes bovine mastitis in dairy farms and its genetic interaction with surrounding environment , as well as to evaluate the genetic diversity among the recovered isolates by ERIC-PCR.

Materials and Methods

Samples Collection and Bacteriological Examination

Milk and Bulk Tank Milk (BTM) samples were collected from El-Sharkia governorate, Egypt, transported and bacteriologically examined according to National Mastitis Council guidelines **(National Mastitis Council Annual and National Mastitis, 2017)**. Also, Different environmental samples were collected and subjected to bacteriological analysis **(ISO, 2012).**

The swabs of workers hands were collected, transported and handled according to **ISO, 2004.** While**,** Fifty grams of bedding and silage samples were collected and examined **(Clegg *et al.,* 1983)** and sixty milliliters of bulk tank milk samples were collected and examined according to **APHA, 2004** and water sample of 100 mL was collected in sterile screw capped bottles of 150 mL capacity and examined **(ISO, 2012).**

After streaking a loopful of the sample on MacConkey agar plates (Oxoid Deutschland GmbH, Germany) and blood agar plates (Oxoid Deutschland GmbH, Germany) with 5% sheep red blood cells, the sample was subcultured on Edwards Medium plates (Oxoid Deutschland GmbH).

Table (1). Source, Type and Number of Collected Samples (N= 192)

Type of Samples		Number of Samples		Total Number of Collected Samples
Animal Samples	Clinical Mastitic Cow's Milk	50	100	192
	Subclinical Mastitic Cow's Milk	50		
Environmental Samples	Bedding	15	64	
	Silage	7		
	Water	16		
	Milking Equipment	11		
	Worker Hands	15		
Bulk Tank Milk	Bulk Tank Milk	28	28	

Identification of Streptococcus Species

All recovered streptococcus isolates were confirmed using three techniques:

(1) Traditional microbiological methods following the NMC Laboratory Handbook on Bovine Mastitis (**Laevens *et al.*, 2017**). Briefly, all isolates were identified based on their colony morphology, hemolysis type, esculin hydrolysis, hippurate hydrolysis, catalase production, and CAMP reaction using *S. aureus* (ATCC 25923).

(2) Automated bacterial identification using the VITEK® 2 compact system (BioMérieux, France) following the manufacturer's instructions.

(3) Molecular PCR using of the sklA3 gene according to **(Hernandez *et al.*, 2021)**. Primer sequence is displayed in **Table 3.** Amplification was carried out through following the protocol of the standard PCR assays in Applied Biosystem, 2720 Thermal Cycler (USA) in a total size of 25 µL consisted of 12.5 µL of 2× PCR master mix (Promega, Madison, USA), 1 µL of single primer (Metabion, Germany), 4.5 µL PCR-grade water, and 6 µL DNA template.

Antimicrobial Sensitivity Test

The antimicrobial sensitivity test was conducted on Mueller-Hinton agar (Difco) using the agar Kirby–Bauer disk diffusion susceptibility method following the procedures of the VET 01 supplement (**CLSI, 2026**). The antimicrobial discs were obtained as commercial Oxoid™ disks with *Streptococcus pneumoniae* ATCC 49619 strains is used as a reference control strain, **Table 2**.

The **CLSI (2026)** was used to assess the results. Multidrug-resistance (MDR) strains are isolates that are resistant to three or more groups of antibiotics (**Sweeney *et al.*, 2018**). Multiple antibiotic resistances (MAR) index for every resistant pattern is calculated by the formula given by **Singh *et al.* (2010)**.

$$\textbf{MAR Index} = \frac{\text{Number of Resistance Antibiotics}}{\text{Total Number of Antibiotics Tested}}$$

N.B.: Isolates categorized as intermediate based on inhibition zone were considered as sensitive for MAR index.

Table (2). Antibiotics Concentration used in Antimicrobial Sensitivity Test on ***S. agalactiae*** Isolates (N=39).

Antibiotic Class	Antibiotic	Disc Name	Disc Concentration
Beta-lactams Penicillin	Penicillin	PEN	10 IU
	Ampicillin	AMP	10 µg
	Amoxicillin + Clavulanic acid	AMC	30 µg
Beta-lactams Cephalosporin	Cefotaxime	CTX	30 µg
	Ceftiofur	EFT	30 µg
Macrolides	Erythromycin	E	15 µg
Quinolones	Enrofloxacin	ENR	5 µg
Glycopeptides	Vancomycin	VA	30 µg
Tetracyclines	Tetracycline	TE	30 µg
Sulfa Drugs	Sulfamethoxazole + trimethoprim	STX	25 µg

Characterization of Virulence Factors

Seven virulence genes were detected via Uniplex conventional PCR. The virulence genes studied were ***bibA, bca, bac, fbsA, fbsB, cfb, and cyl***.

All primer sequences from this study are displayed in **Table 3** (**Boonyayatra *et al.*, 2020**).

The PCR mix (25µL), consisted of 12.5µL of 10x Green Go Taq® Flexi Buffer sourced from PROMEGA in Madison, WI, USA. Moreover, the mixture includes 1µL (10.0µM) of the forward primer, 1µL (10.0µM) of the reverse primer, 2µL of the DNA template, and 8.5µL of Nuclease Free Water. PCR mixtures were amplified in a T100 Thermal Cycler (Biorad®, California, U.S.).

Detection of Antibiotic Resistance Genes

All ***S. agalactiae*** strains were examined for the existence of the following resistance genes: bla_{CTX}, bla_{TEM} for β-lactamases, ***erm (B), mef (A)*** for macrolides, ***tet (M)*** for tetracyclines. PCR was done using specific primers shown in **Table 3**.

Reaction mixture (20 µL) was prepared with 10 µL of 2×Taq Master Mix (Beijing Solarbio Science & Technology Co., Ltd), 1 µL of template DNA, 0.5 µL of each primer (10 µM), and 8 µL of distilled water.

Genetic diversity by ERIC-PCR

Genotyping of ***S. agalactiae*** strains was studied using ERIC-PCR as explained by **Bishi *et al.*, (2008).** Some modifications using dream Taq green PCR master mix 2x cat (**Abd-Elfatah *et al.*, 2023**).

Briefly, reactions mixtures (25 µl) composed of 12.5 µL of 2× PCR master mix (Promega, Madison, USA), 1 µL of single primer (Metabion, Germany), 4.5 µL PCR-grade water, and 6 µL DNA templates.

Table (3). List of Primer Sequences Utilized in This Study:

Gene	Sequence	Specificity	Amplicon Size	Reference
Identification Gene				
Gsag-S ***Gsag-AS***	Gsag-S ATTGATAACGAC-GGTGTTACTGTCAT Gsag-AS AGTAGCGTTCTG-TAATGATGTC	*S. agalactiae* (sklA3)	487 bp	**Raemy *et al.* (2013)**
Initial denaturation (95◦C/ 2 min), followed by 35 cycles (45 seconds at 94◦C), (1 min at 55◦C), and (2 min at 72◦C), concluding with a final extension cycle (7 min at 72◦C).				
Virulence Genes				
bibA	F: AACCAGAAGCCAAGCCAGCAACC	*S. agalactiae* Adhesion	127 bp	**Kayansamruaj *et al.* (2014)**
	R: AGTGGACTTGCGGCTTCACCC			
bca	F: TAACAGTTATGA-TACTTCACAGAC		535 bp	**Emaneini *et al.* (2016)**
	R: ACGACTTTCTTCCGTCCACTTAGG			
bac	F: CTATTTTTGATATTGACAATGCAA		592 bp	**Emaneini *et al.* (2016)**
	R: GTCGTTACTTCCTTGAGATGTAAC			
fbsA	F: GTCACCTTGACTAGAGTGATTATT	*S. agalactiae* Invasion	85 bp	**Kayansamruaj *et al.* (2014)**
	R: CCAAGTAGGTCAACTTATAGGGA			
fbsB	F: TCTGTCCAACAGCCGGCTCC		144 bp	**Kayansamruaj *et al.* (2014)**
	R: TTCCGCAGTTGTTACACCGGC			
cfb	F: GCTGTTTGAAGTGCTGCTTG	*S. agalactiae* Infection and Tissue Damage	288 bp	**Shome *et al.* (2012)**
	R: GACTTCATTGCGTGCCAAC			
cyl	F: TTCTCCTCCTGGCAAAGCCAGC		124 bp	**Kayansamruaj *et al.* (2014)**
	R: CGCCTCCTCCGATGATGCTTG			
Initial denaturation (95◦C/ 5min), followed by 35 cycles (1 min at 95◦C), (1 min at 55◦C), and (2 min at 72◦C), concluding with a final extension cycle (5 min at 72◦C).				
Antibiotic Resistance Genes				
bla$_{TEM}$	F: ATGAGTATTCAACATTTTCGTG	*β-lactam* Resistance	686 bp	**Sundsfjord *et al.* (2004)**
	R: TTACCAATGCTTAATCAGTGAG			
bla$_{CTX-M}$	F: SCSATGTGCAGYACCAGTAA		585 bp	**Ojdana *et al.* (2014)**
	R: ACCAGAAYVAGCGGBGC			
erm (B)	F: GAAAAGGTACTCAACCAAATA	Macrolides Resistance	639 bp	**Sutcliffe *et al.* (1996)**
	R: AGTAACGGTACTTAAATT-GTTTAC			
mef (A)	F: TGGTTCGGTGCTTACTATTGT		500 bp	**Morvan *et al.* (2010)**
	R: CCCCTATCAACATTCCAGA			
tet (M)	F: GTGGAGTACTACATTTACGAG	Tetracycline Resistance	406 bp	**Poyart *et al.* (2003)**
	R: GAAGCGGATCACTATCTGAG			
Initial denaturation (94˚C for 3 min), followed by 34 cycles of amplification (94˚C for 20 s), annealing at specific temperatures for 20 s, extension (72˚C for 45 s), and a final extension step (72˚C for 5 min).				
ERIC-PCR				
ERIC	F: ATGTAAGCTCCTGGGGATTCAC	ERIC	200 – 800 bp	**Bishi *et al.* (2008)**
	R: AAGTAAGTGACTGGGGTGAGCG			
Initial denaturation cycle (40 seconds at 94°C), followed by 4 cycles of low stringency (1 min at 94°C, 1 min at 26°C, and 4 min at 72°C), 35 cycles of high stringency (1min at 94°C, 1.5 min at 52°C, and 8 min at 72°C) and final extension for 10 min at 72°C.				

Results

Results of Bacteriological Isolation

In this study, 28 *S. agalactiae* isolates were recovered from examined milk samples, 5 from different environmental samples and 6 from bulk tank milk samples, **Table 4**.

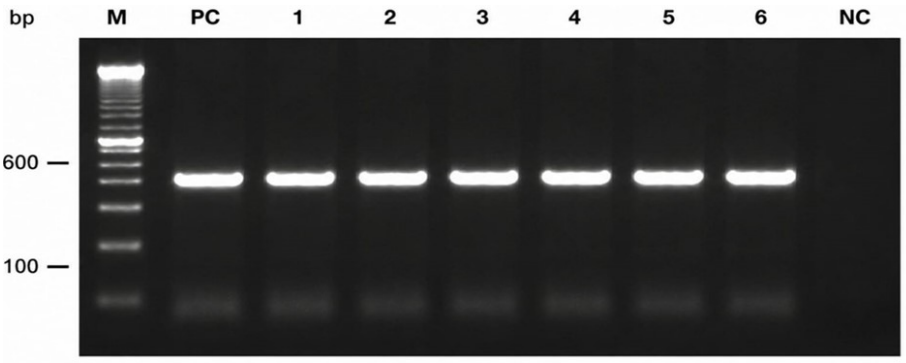
Table (4). Prevalence of Bacteria Isolated from Different Samples (N=192)

Isolated Microorganism	Milk Samples (N=100)		Environmental Samples (N=64)		Bulk Tank Milk (N=28)		Total (N=192)	
	Positive	%	Positive	%	Positive	%	Positive	%
S. agalactiae	28	28	5	7.8	6	21.4	39	20.3
S. uberis	22	22	3	4.6	6	21.4	31	16.1
S. dysgalactiae	23	23	1	1.5	6	21.4	30	15.6

Identification of Isolates

All 39 *S. agalactiae* isolates were screened for their phenotypic features. Additionally, all isolates were positive in the CAMP test but negative in the esculin hydrolysis and catalase tests. The VITEK® 2 identification system successfully confirmed all 39 isolates as *S. agalactiae*. The probability of identification classification, based on the percentage of probability, indicated an excellent probability of identification at 100% (39 out of 39).

All 39 *S. agalactiae* strains were identified by species-specific PCR (sklA3 gene amplification), **Figure 1**.



M= Marker, PC=Positive Control, NC=Negative Control, 1-6= Tested Samples

Figure (1)/ Gel electrophoresis of sklA3 gene amplification products of *Streptococcus agalactiae* (487 bp).

Results of Antimicrobial sensitivity Profiles

Antimicrobial sensitivity test was judged according to the CLSI VET 01 breakpoint zone diameter standard, **Table 5**.

Table (5). Antibiotic sensitivity test results of ***S. agalactiae*** isolates (N=39).

Antibiotic Class	Antibiotic	Sensitive		Resistant	
		Number	%	Number	%
Beta-lactams Penicillin	Penicillin	26	67	13	33
	Ampicillin	21	54	18	46
	Amoxicillin + Clavulanic acid	26	67	13	33
Beta-lactams Cephalosporin	Cefotaxime	34	87	5	13
	Ceftiofur	32	82	7	18
Macrolides	Erythromycin	30	77	9	23
Quinolones	Enrofloxacin	30	77	9	23
Glycopeptides	Vancomycin	35	90	4	10
Tetracyclines	Tetracycline	9	23	30	77
Sulfa Drugs	Sulfamethoxazole + trimethoprim	32	82	7	18

N.B.: Isolates categorized as intermediate based on inhibition zone were considered as sensitive.

S. agalactiae isolates were revealed to be resistant to one and up to six antimicrobial agents. We identified 17 (44 %) strains exhibiting multidrug resistance (MDR) profiles. Many MDR profiles have been categorized, **Table 6**. *S. agalactiae* isolates showed many antibiotic resistance index (MARI) of 0.1-0.6, **Table 6**.

Table (6). Antibiotic resistance rates of *S. agalactiae* isolates (N=39)

Antibiotic Pattern Profile	Antibiotic Resistant Pattern	Number of Resistant Isolates	Percentage of Resistant Isolates (%)	No. of Resistance Antibiotics	MARI (%)
1	PEN, AMP, AMC, EFT, ENR, TE	1	2.6	6	0.6
2	AMP, EFT, ENR	1	2.6	3	0.3
3	AMC, TE, STX	3	7.7	3	0.3
4	PEN, AMP, TE	1	2.6	3	0.3
5	AMP, ENR	1	2.6	2	0.2
6	PEN, AMP, EFT, TE	2	5.1	4	0.4
7	AMC, TE	2	5.1	2	0.2
8	PEN, CTX, STX	1	2.6	3	0.3
9	AMC, ENR, TE	3	7.7	3	0.3
10	PEN, AMP, AMC, CTX	1	2.6	4	0.4
11	AMC, E, ENR	1	2.6	3	0.3
12	PEN, AMP	1	2.6	2	0.2
13	EFT, VA, TE	1	2.6	3	0.3
14	AMP, AMC, E	1	2.6	3	0.3
15	PEN, TE, STX	1	2.6	3	0.3
16	AMP, TE	5	12.8	2	0.2
17	E, VA, TE	2	5.1	3	0.3
18	PEN, TE	1	2.6	2	0.2
19	PEN, ENR	1	2.6	2	0.2
20	CTX, EFT, E, TE	1	2.6	4	0.4
21	AMP, TE, STX	1	2.6	3	0.3
22	PEN, AMP, CTX, ENR, TE	1	2.6	5	0.5
23	PEN, EFT, VA, TE	1	2.6	4	0.4
24	E, STX	1	2.6	2	0.2
25	PEN	1	2.6	1	0.1
26	AMP, E, STX	1	2.6	3	0.3
27	PEN, AMP, CTX, E, TE	1	2.6	5	0.5
28	AMP, E, TE	1	2.6	3	0.3

Molecular Characterization of Virulence Factors

The ***fbsB, bibA, and cfb*** genes were found in all the isolates. The incidence rates of ***fbsA, and cyl*** were 95%, and 87%, respectively. However, the ***bca,*** and ***bac*** genes were not identified. The major virulence profile includes ***fbsA, fbsB, bibA, cfb, and cyl*** which were recognized in 33 isolates. However, the presence of virulence genes did not show a statistically important link with the form of mastitis (clinical and subclinical).

Prevalence of Antimicrobial Resistance Genes

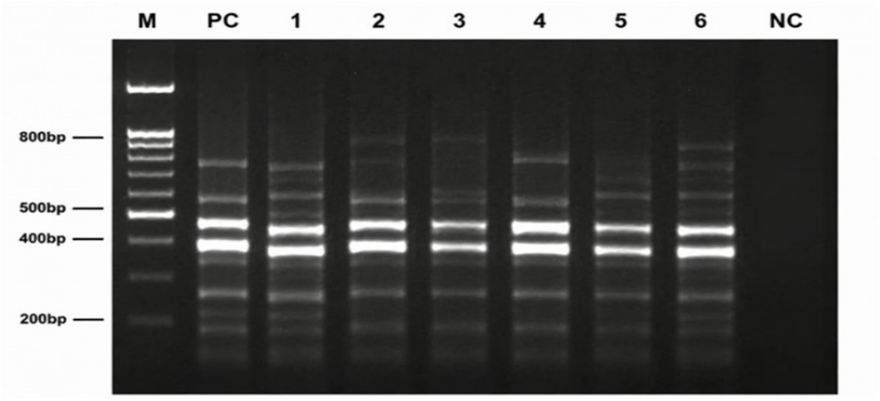
Five resistance genes for β-lactams (***bla_{TEM} and bla_{CTX-M}***), macrolides (***erm (B) and (mef (A)*** and (***tet (M))*** for tetracycline were examined in 39 *S. agalactiae* isolates. Only 37 isolated strains exhibited the resistance genes, **Table 7**.

Genetic diversity of *Streptococcus agalactiae* isolates

A 2–6 band, whose size ranged from 200 to 800 bp which resulted by ERIC-PCR amplification, were the DNA fragments electrophoretic profile which obtained from the screened *S. agalactiae* isolates, **Figure 2**. The genomic diversity of the different isolates was validated by the varying positions and intensities of the amplified PCR products. Nearly all strains shared the amplified bands found at 400 and 500 bp. ERIC PCR pattern produced two groups, ERIC group I and ERIC group II. ERIC group I contained 77 % of the isolates (30 strains) that were designated alphabetically (A-R), while 9 strains belonged to ERIC group II (ERIC S-W). The visual comparison of the banding patterns showed 23 different ERIC profiles (A–W). ERIC W (15%) was the most common ERIC type, as shown in **Table 7**. The ERIC-PCR dendrogram showed that the genetic diversity and heterogeneity of the *S. agalactiae* obtained from the farms and environment can be seen in their classification into specific genotypes according to sampling site and type.

Table (7). ERIC-PCR, and antibiotic resistance of *S. agalactiae* isolates (N=39).

Type of Sample	Sample ID	Antibiotic resistant pattern	Antibiotic Resistant Genes	ERIC-PCR type
Milk Sample	1	PEN, AMP, AMC, EFT, ENR, TE	***bla_{TEM}, bla_{CTX-M}, tet(M)***	V
	2	AMP, EFT, ENR		F
	3	AMC, TE, STX	***tet(M)***	W
	4	PEN, AMP, TE	***bla_{CTX-M}***	L
	5	AMC, TE, STX	***tet(M)***	W
	6	AMP, ENR	***bla_{CTX-M}***	G
	7	PEN, AMP, EFT, TE	***bla_{TEM}, tet(M)***	R
	8	AMC, TE	***tet(M)***	W
	9	PEN, CTX, STX	***bla_{CTX-M}***	J
	10	AMC, ENR, TE	***tet(M)***	B
	11	PEN, AMP, AMC, CTX	***bla_{TEM}***	M
	12	AMC, E, ENR	***mef(A)***	A
	13	PEN, AMP	***bla_{TEM}***	U
	14	EFT, VA, TE	***tet(M)***	P
	15	AMP, AMC, E	***bla_{TEM}***	E
	16	AMC, TE	***bla_{CTX-M}***	C
	17	PEN, TE, STX	***bla_{CTX-M}***	H
	18	AMP, TE	***tet(M)***	N
	19	AMC, ENR, TE	***tet(M)***	A
	20	PEN, AMP, EFT, TE	***bla_{TEM}***	S
	21	E, VA, TE	***erm(B)***	Q
	22	PEN, TE	***tet(M)***	I
	23	AMC, TE, STX	***bla_{TEM}***	D
	24	PEN, ENR		T
	25	CTX, EFT, E, TE	***mef(A), tet(M)***	W
	26	AMP, TE	***tet(M)***	O
	27	AMP, TE, STX	***bla_{CTX-M}, tet(M)***	N
	28	AMP, TE	***bla_{CTX-M}, tet(M)***	P
Environmental Sample	29	PEN, AMP, CTX, ENR, TE	***bla_{TEM}, bla_{CTX-M}, tet(M)***	K
	30	AMC, ENR, TE	***tet(M)***	B
	31	PEN, EFT, VA, TE	***bla_{TEM}***	T
	32	E, STX	***erm(B)***	N
	33	AMP, TE	***bla_{CTX-M}, tet(M)***	N
Milk Tank Samples	34	PEN	***tet(M)***	Q
	35	AMP, TE	***bla_{CTX-M}***	O
	36	AMP, E, STX	***bla_{CTX-M}***	W
	37	E, VA, TE	***erm(B), tet(M)***	Q
	38	PEN, AMP, CTX, E, TE	***bla_{TEM}, tet(M)***	S
	39	AMP, E, TE	***bla_{CTX-M}, erm(B), tet(M)***	W



M= Marker, PC=Positive Control, NC=Negative Control, 1-6= Tested Samples
Figure (2). Agarose gel electrophoresis of characteristic ERIC-PCR patterns; Lane M – 100bp DNA ladder

Discussion

In Egypt, *S. agalactiae* has a great significance as a major causative bacterium of infectious mastitis and its severity arises from the development of multidrug resistant strains (**El-Jakee *et al.*, 2013**).

The bacteriological method using CAMP herein confirmed the identification of 19 (38%) isolates of *S. agalactiae* from cattle diagnosed with subclinical mastitis, which confirms the poor hygienic measures within the examined farms. This finding was comparable with the results obtained by others (**Momtaz *et al.*, 2012 and Elsayed *et al.*, 2016**) that isolated *S. agalactiae* with an prevalence of 12.7%, and 14.7% respectively.

In this study, 28 (28%) *S. agalactiae* isolates were isolated from 100 milk samples. Whereas 5 (7.8%) isolates came from environmental samples and 6 (21 %) isolated from bulk tank milk samples based on biochemical examination, confirmation by VITEK-2 compact system and confirmation by species-specific PCR (sklA3 gene amplification), **Table 4**. Lower incidence of *S. agalactiae* isolated from mastitic cows than our results were reported by **El-Jakee *et al.* (2013) and Elhaig and Selim, (2015)** with incidence rates of 21.7%, 20% respectively. While same incidences of *S. agalactiae* isolation rate from mastitic cows were reported by **Khan and Mohammad, 2005** with incidence rates of 30%. Egyptian investigations also emphasized the importance of environmental streptococci in mastitis epidemiology, particularly under poor hygienic and biosecurity conditions in dairy farms. In the present study, *S. agalactiae* was recovered from environmental samples in an incidence of 7.8%. Lower incidence of *S. agalactiae* isolated from environmental samples was reported by **Youssif *et al.* (2019)**. While higher incidence rate of *S. agalactiae* isolated from environmental samples was reported by **Haggag *et al.* (2018)** that isolated *S. agalactiae* from milking machines swabs, hand swabs and bedding samples with isolation rate 30.23%, 21.47% and 7.02 respectively. The unsanitary measures and various milking tools such as cups, towels, milkers' hands, cross suckling calves; milking machines are all considered potential sources of infection in cows (**Merz *et al.*, 2016**).

The incidence rate *streptococcus agalactiae* in bulk tank samples was 21.4%, higher incidence rate of *streptococcus agalactiae* was reported by **Zadoks *et al.*, (2004)** 31% of 48 examined bulk tank samples and **Cheng *et al.*, (2010)** 27% of examined 100 raw milk samples and lower incidence rate (1.4%) of examined raw milk samples was reported by **Karima *et al.*, (2007).**

It is known that the beta-lactams and tetracyclines continue to be the antibiotics of choice in the treatment of streptococcal infection for several years; the macrolide erythromycin is considered the most significant used alternative for treatment of streptococcal infection, so a significant increase in the rate of resistance to these antibiotics was observed as demonstrated

in **Table 5**. While **Youssif *et al.,* (2021)** demonstrated that all of the examined 30 isolates of *S. agalactiae* were resistant to penicillin, with a lower resistance of isolates was found against ampicillin, amikacin, oxacillin, amoxicillin + clavulanic acid, erythromycin and tetracycline.

The results of screened virulence genes agree with **Wataradee *et al.,* (2023)** who found the virulence factors ***bibA, fbsB,*** and ***cfb*** in all *S. agalactiae* strains. The study of virulence factors revealed that most of the strains (96%) amplified the following virulence genes: ***fbsA, fbsB, bibA, cfb,*** and ***cyl***, which play a role in the phases of intramammary infection caused by *S. agalactiae*, such as adhesion (***bibA***), invasion and colonization (***fbsA and fb***sB), and infection (***cfb and cyl***). Also, our findings agrees with a previous study indicating that all bovine mastitis strains caused by *S. agalactiae* were positive for ***bibA*** (**Heath, 2016**) and ***fbsB*** **and** ***fbsA*** (**Zastempowska *et al.,* 2022**).

Although low resistance to β-lactams was recorded in *S. agalactiae* isolates, the current study detected $\boldsymbol{bla_{TEM}}$ ***and*** $\boldsymbol{bla_{CTX\text{-}M}}$ in 10(26%) and 13(33%) of *S. agalactiae* isolates. Such genes may be associated with phenotypic resistance of *S. agalactiae* to β-lactam and cephalosporines. Moreover, 5 isolates were detected to be resistant to cefotaxime phenotypically and 7 to ceftiofur, while 13 isolate was detected to carry $\boldsymbol{bla_{CTX\text{-}M}}$. This exciting result might be due to the absence of gene expression without induction.

According to many previous studies, the ***tet (M)*** gene is the most frequently reported gene conferring resistance to tetracycline (**Hernandez *et al.,* 2021 and Aiewsakun *et al.,* 2022**). The current study detected ***tet (M)*** in 21(54%) of ***S. agalactiae*** isolates which responsible for tetracycline resistance in 30 (77%) strains of ***S. agalactiae.*** According to **Emaneini *et al.* (2014)** and **Hernandez *et al.,* (2021)** our study hypothesizes that the frequent detection of the genes ***erm (B)*** (10%) and ***mef (A)*** (5%), which were detected in streptococci isolates, may be related to the widespread and unselective use of such antibiotics worldwide.

This study found low similarity in the genotypes of *S. agalactiae* among animals, presumably as a consequence of their geographic localities. Moreover, different strains of the same genotype were susceptible to different antibiotics. For example, the strain with a similar ERIC W type exhibited various antibiotic resistance patterns and different antibiotic resistance genes. We used ERIC-PCR because there is little data available on the epidemiology of Egyptian isolates of *S. agalactiae* and because this method has not been widely used to study *S. agalactiae* which agrees with **Abd-Elfatah *et al.,* (2023)**. In Poland, group B Streptococcus genotyping by the ERIC-PCR fingerprinting reaction has been described. The scientists found 13 genotypes among the 120 strains examined from different collected samples based completely on the visualization of banding patterns (**Dabrowska-Szponar and Galiński, 2003**). Moreover, a previous work in India genotyped 86 strains of *S. agalactiae* into 62 unique ERIC types using clinical samples such as vaginal swabs, urine, and pus from various individuals (**Bishi *et al.,* 2008**).

Conclusion

The genetic diversity of *S. agalactiae* strains, which were isolated from mastitis cases in Egyptian cattle, was illustrated with the pattern of virulence genes. Antimicrobial analysis revealed various resistance profiles among these *S. agalactiae* strains. This study offers genotyping information on *S. agalactiae* strains isolated from bovine mastitic cows in farms and the environment in Egypt. These findings form the basis for diagnostic, preventive, and therapeutic strategies, as well as the potential development of a vaccine to manage bovine mastitis. Additionally, this data will aid in exploring the molecular mechanisms behind the emergence of antimicrobial resistance.

Conflict of Interests

The authors declare that they have no conflict of interest.

Ethical Approval:

No ethical approval was required for this study since all procedures were performed in vitro

under laboratory conditions without the use of live animals or human subjects.

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