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Genetic Diversity of Multiple Antibiotic Resistance *Streptococcus agalactiae* Isolated from Bovine Mastitis and Retail Markets Milk by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR)

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Abstract

Public health is at risk because *Streptococcus agalactiae* is increasingly linked to incidences of bovine mastitis in Egypt. In this study, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) were used to explore the genotyping of several antibiotic resistant *Streptococcus agalactiae* isolated from bovine mastitis and retail milk. Also, antimicrobial resistance genes were detected. Two hundred and sixty-six (46.7%) strains were isolated from samples of milk obtained from cases of bovine mastitis in dairy farms and retail markets representing 34 (5.96%) *Streptococcus agalactiae* and 232 (40.7%) other *Streptococcus* species strains based bacterial identification. By using disc diffusion assays, it was examined the susceptibility of all *Streptococcus* isolates to twelve antimicrobial agents. The highest prevalence of resistance of *Streptococcus* species was observed against ampicillin (65.5%), amoxicillin (56%), tetracycline (52.5%), ofloxacin (47.8%), and nitrofurantoin (46.9%). High proportion of the *S. agalactiae* isolates were resistance to amoxicillin (83%), followed by tetracycline (82.4%), nitrofurantoin (64.7%), azithromycin (61.8%), and ampicillin (50%). Most (88.2%) of *S. agalactiae* showed multiple antibiotic resistances (MAR) phenotypes with MAR index of 0.1-0.7 and 28 different MAR patterns. The results of genetic antimicrobial resistance of *S. agalactiae* strains revealed amplification of bla_{TEM} (23%) and bla_{CTX} (26%) genes in β-lactam-resistant strains, *erm*(B) gene (20%), *mef*(A) (35%) in macrolides resistant isolates, and *tet*(M) in (44%) tetracycline resistant strains. Using ERIC-PCR, the present study showed the genetic diversity and heterogeneity among *S. agalactiae* strains (n=34) that were classified into 19 distinct ERIC-PCR groups (A–S). Between them, ERIC O (20.6%, 7) was the most widespread group. These results indicated that milk samples served as source of MAR *S. agalactiae*, consequently posing threats to public health, so the improvement of the hygiene regimen on the farms and promotion of the wise use of antimicrobials are necessary. The obtained findings showed that milk samples were a source of MAR *S. agalactiae*, endangering public health. As a result, it is essential to enhance farm hygiene practices and promote the responsible use of antimicrobials. Additional research on the epidemiology of *S. agalactiae* is required to add bacterial genetic information in order to help in rational vaccine strategy in the future.

KEYWORDS
Bovine mastitis, ERIC-PCR, multiple antibiotic resistances, *Streptococcus agalactiae*

INTRODUCTION

Bovine mastitis is a multifactorial, complicated disease which occurs because of animals, the environment, and pathogens. Cow susceptibility to streptococcal mastitis is influenced by several host and environmental factors. Poor hygiene during milking and the absence of infected cows from milking in the last are the primary vectors of the disease. One of the most serious issues that have a significant impact on dairy farm productivity and cow welfare is clinical mastitis. Bovine mastitis can lead to a lot of change in the microbiological, physical, and chemical characteristics of the milk, such as a decrease in milk quantity and quality and increase in somatic cell count which are recorded as most obvious marks of clinical mastitis of cows in dairy farms. Clinical signs on the udder of the animal may be present which help in more identification and early treatment. Additionally, milk is an exceptional medium which helps in the growth of a lot of microbial organism particularly pathogenic bacteria, but it an essential part of the global human diet. However, consumers' health may be at risk due to the presence of pathogens especially zoonotic pathogen and drug residues of antimicrobial drugs in milk (Bradley, 2002). Milk quality can be decreased by adulteration, contamination during and after milking, and udder infections.

Gram-positive facultative anaerobic cocci *Streptococcus* are members of the family *Streptococcaceae*. There are three primary strains of streptococcal mastitis: *Streptococcus agalactiae* (*S. agalactiae*), *Streptococcus dysgalactiae* (*S. dysgalactiae*), and *Streptococcus uberis* (*S. uberis*); that could result in clinical as well as subclinical mastitis. *S. agalactiae* is primarily found in the udder of cows and is responsible for numerous serious human and animal diseases. According to a recent study (Hernandez *et al*., 2021) , the environment and the gastrointestinal tract of cattle are additional sources which carry *S. agalactiae*. Additionally, *S. agalactiae*, which is a member of the B-*Streptococcus* group (GBS), can cause mastitis in cattle, meningitis, septicemia, pneumonia in infants, and puerperal sepsis in pregnant women.

Penicillin (PEN), amoxicillin (AML)/clavulanic acid, ampicillin (AMP), erythromycin (ERY), and clindamycin (CLI) are the antibi-

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otics which are used mainly to treat bovine mastitis. Gram-positive bacteria, such as *Streptococci*, have unfortunately developed an increased capacity for resisting these antibiotics. Typically, the uncontrolled use of antibiotics affects the sensitive bacteria especially microbial system of them, resulting in mutations that enable antibiotic-resistant bacteria to persist and spread further. As a result, antibiotic resistance increases worldwide, posing a typical danger in the infectious diseases treating.

Multiple antibiotic resistances (MAR) have been linked to low rates of bacteriological cure, despite the most effective antimicrobial treatments (Barkema *et al*., 2006). Multidrug-resistant streptococcal mastitis was found to be most common in dairy cows between the ages of 5 and 7, with parity numbers ranging from 4 to 7, consistent with previous findings. The World Health Organization has established that any antimicrobial drugs usage will lead to develop resistance to antimicrobial drugs from bacteria (WHO, 2015) . Good treatment with effective antimicrobials is especially important to reduce the risk of a fatal outcome, especially for invasive bacterial infections.

The 126 bp intergenic inverted repeats that make up the enterobacterial repetitive intergenic consensus (ERIC) sequence primarily found in Gram-negative bacteria of the Enterobacteriaceae family, such as *E. coli* and *Salmonella* Typhimurium (Hulton *et al*., 1991). Many Gram-negative and some Gram-positive bacteria have been molecularly typed using ERIC-PCR (Elsayed *et al*., 2022) . *S. agalactiae* has been shown to have ERIC sequences, and ERIC-PCR was used as a tool for genotyping (Bishi *et al*., 2008) . The ERIC-PCR based genotyping method is a highly discriminatory molecular typing method that is simpler, quicker, and reproducible, regardless of whether the *S. agalactiae* genome contains ERIC elements or not, and even if ERIC primers operate on the principle of RAPD PCR. In addition, it can be used in group B streptococci epidemiological studies and for genotyping of many clinical isolates with a wide geographic distribution. As a result, the goal of this study was to find out multiple antibiotic resistances (MAR) *Streptococcus agalactiae* which causes bovine mastitis on dairy farms and in retail markets. According to the authors' knowledge, *S. agalactiae* is one of the most important bacteria which cause bovine mastitis; this is the first molecular study to use ERIC-PCR to genotype *S. agalactiae* strains from Egypt's retail milk markets and bovine mastitis.

MATERIALS AND METHODS

Study area

The *Streptococcus* species existence in five dairy cattle farms in the northern governorates of Dakahlia and Damietta, Egypt, was the subject of a study. The hot summer weather in the regions from which this study's samples were taken encourages the growth of numerous infectious microorganisms and flies, which are crucial to the appearance of mastitis.

Sampling

From March to December 2021, 570 samples of dairy milk were obtained from cattle with subclinical mastitis (n=270) or clinical mastitis (n=100) in five different dairy farms and 200 different retail markets in the Dakahlia and Damietta Governorates in northern Egypt. Clinical mastitis cases were identified by abnormal milk secretion and/or the affected quarters of udder inflammation, with or without systemic symptoms. A case of subclinical mastitis had a high somatic cell count but no obvious signs of changing in secretion or swelling of the affected areas. In 10 mL sterile vials, each sample was collected in an aseptic manner and immediately transported to the ice box in the laboratory for bacteriological analysis.

Bacteriological analysis

Plates of Edward's media (Oxoid) enriched with 5% sheep blood were streaked by loopful of tested milk sample then aerobically incubated at 37°C in the incubator . After 24 hours, the plates were observed for detecting pigmentation, colony morphology, and hemolytic characteristics (National Mastitis Council Annual and National Mastitis, 2017). Conventional methods like Gram staining, catalase, bile-esculin hydrolysis, hippurate hydrolysis, and CAMP tests were used to identify probable *Streptococcus* species colonies. For further analysis, the identified isolates were stored at -20°C in 20% glycerol.

Molecular identification

By boiling bacterial colonies (obtained from an overnight culture of brain heart infusion broth) (Difco) suspended in sterile water for 20 minutes, the DNA template was (Hernandez *et al*., 2021). By using of the sklA3 gene, which encodes for fibrinogen binding protein, in PCR amplification it could be confirmed the species identification of *S. agalactiae* (Table 1). Amplification was happened by following instruction of the standard PCR assays in Applied Biosystem, 2720 Thermal Cycler (USA) in a total volume of 25 µL consisted of 12.5 µL of 2× PCR master mix (Promega, Madison, USA), 1 µL of individual primer (Metabion, Germany), 4.5 µL PCR-grade water, and 6 µL DNA template. PCR enhancement conditions were an underlying denaturation cycle (2 min at 95°C), trailed by 35 cycles (at 94°C for 45s, at 55°C for 1min, and at 72°C for 2min) and final extension at 72°C for 7 min. Under UV illumination, the products of PCR were photographed and documented in an ethidium bromide stained 1.5% agarose gel.

Antibiotic susceptibility testing

On Mueller-Hinton agar (Difco), antibiotic susceptibility testing was conducted using the disk diffusion method in accordance with (2019) guidelines. *Streptococcus* isolates were examined for susceptibility to twelve antibiotics (Oxoid, Ltd.) including: cefotaxime (30 µg) ceftriaxone (30 µg), cefaclor (30 µg) belonging to cephalosporines, tetracycline (30 µg) belonging to tetracycline, meropenem (10 µg) belonging to carbapenem, , nitrofurantoin (300 µg) belonging to nitrofuran ,ofloxacin (5µg) belonging to quinolones, ampicillin (10 µg), amoxicillin (25 ug) belonging to β-lactams, azithromycin (15 ug), erythromycin (10 µg) belonging to macrolides and vancomycin (30 µg) belonging to vancomycin. The CLSI (2019) was used to evaluate the results. Multiple antibiotic resistance (MAR) strains are isolates that are resistant to three or more classes of antibiotics (Sweeney *et al*., 2018). The formula provided by Singh *et al*. (2010) is used to calculate the multiple antibiotic resistances (MARs) index for each resistant pattern. Multiple antibiotic resistances (MARs) index for each resistant pattern is calculated by the formula given by Singh *et al*. (2010) . MAR index= Number of resistance (isolates classified as intermediate based on inhibition zone were considered as sensitive for MAR index) antibiotics/ Total number of antibiotics tested.

Molecular detection of antimicrobial resistance genes

The genotypic resistance profiles of *S. agalactiae* isolates

were determined by simplex PCR assays the following resistance genes: bla_{CTX}, bla_{TEM} for β-lactamases, *erm* (B), *mef* (A) for macrolides, and *tet* (M) for tetracyclines. The details of oligonucleotide sequences (Metabion, Germany) used in this study are given in Table 1. The PCR and electrophoresis were occurred as described above.

Genetic diversity analysis using ERIC-PCR

Genotyping of *S. agalactiae* strains was investigated using ERIC-PCR as described by Bishi *et al.* (2008) with some modifications using dream taq green pcr master mix 2x cat. Briefly, reactions mixtures (25 µl) consisted of 12.5 µL of 2× PCR master mix (Promega, Madison, USA), 1 µL of individual primer (Metabion, Germany), 4.5 µL PCR-grade water, and 6 µL DNA templates. The PCR amplification was performed as follows: an 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. The products of amplification were visualized in ethidium bromide stained 1.5% agarose and analyzed as described. Depending on whether each band was present or not, the ERIC fingerprinting data were converted into a binary code. Dendrogram was generated by the unweighted pair group method with arithmetic average (UPGMA) and Ward's hierarchical clustering routine. Cluster analysis and dendrogram construction were performed with SPSS, version 22 (IBM 2013) (Hunter, 1990). The online tool was used to calculate the similarity index (Jaccard/Tanimoto Coefficient and number of intersecting elements) between all samples (https://planetcalc.com/1664/).

Statistical analysis

Microsoft Excels Spread sheet (Version 15.0) were used to record data and the investigation was happened by SPSS (Statistical Set for Social Science) software version 22. The antibiotic sensitivity patterns were presented in percentages.

RESULTS

Prevalence of streptococcal species in dairy milk

Out of 570 milk samples received during 9 months from 5 dairy farms and different retail markets located in the Dakahlia and Damietta Governorates, Egypt, 266\570 (46.7%) *Streptococci* composing of 34 (5.96%) *S. agalactiae* and 232 (40.7%) other *Streptococcus* species strains were biochemically identified. *S. agalactiae* was identified in 34 bacterial isolates that were Gram-positive cocci, positive for the CAMP reaction, and negative for catalase and esculin activity. Then, 34 *S. agalactiae* strains from five dairy farms were confirmed by species-specific PCR (sklA3 gene amplification). The PCR products for *S. agalactiae* isolates were at 487 bp.

Antimicrobial resistance patterns in Streptococcus isolates

We used twelve antimicrobial agents to test susceptibility of all *Streptococcus* isolates to antimicrobial agents according to the regulations of the executive standard of antimicrobial susceptibility testing issued by the Clinical and Laboratory Standards Institute and the results are shown in Table 3. Most of *Streptococcus* isolates strains had the highest resistance rate to ampicillin and tetracycline (63.5%, each), followed by amoxicillin (59.4%), nitrofurantoin (48%), and ofloxacin (43%). Most *S. agalactiae* isolates strains showed resistance to amoxicillin (83%), followed by tetracycline (82.4%), nitrofurantoin (64.7%), azithromycin (61.8%), and ampicillin (50%), while other *Streptococcus* species revealed high resistance to ampicillin (65.5%), followed by amoxicillin (56%), tetracycline (52.5%), ofloxacin (47.8%), and nitrofurantoin (46.9%). The most effective antibiotics against *S. agalactiae* were ofloxacin (88.2%) and cephalosporin (cefotaxime 85% and ceftriaxone 82.4%), and vancomycin (82.4%), while other *Streptococcus* species were susceptible to meropenem (85.8%) and erythromy-

Table 1. Oligonucleotide primer sequences used for PCR in this study.

Specific for	Gene	Sequence	References
S. agalactiae (sklA3)	*Gsag-S* *Gsag-AS*	*Gsag-S* ATTGATAACGACGGTGTTACTGTCAT *Gsag-AS* AGTAGCGTTCTGTAATGATGTC	Raemy et al. (2013)
β-lactam	bla_{TEM}	F: ATGAGTATTCAACATTTTCGTG R: TTACCAATGCTTAATCAGTGAG	Sundsfjord et al. (2004)
	bla_{CTX-M}	F: SCSATGTGCAGYACCAGTAA R: ACCAGAAYVAGCGGBGC	Ojdana et al. (2014)
Macrolides	*erm*(B)	F: GAAAAGGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTAC	Sutcliffe et al. (1996)
	mef(A)	F: TGGTTCGGTGCTTACTATTGT R: CCCCTATCAACATTCCAGA	Morvan et al. (2010)
Tetracycline	*tet*(M)	F: GTGGAGTACTACATTTACGAG R: GAAGCGGATCACTATCTGAG	Poyart et al. (2003)
ERIC	ERIC1	F: ATGTAAGCTCCTGGGGATTCAC R: AAGTAAGTGACTGGGGTGAGCG	Bishi et al. (2008)

Table 2. Prevalence of Streptococcus species in dairy milk samples (n=570).

Source of sample	Milk samples (No)	Number of Streptococcus species samples		
		S. agalactiae	Other streptococci	Total (%)
Dairy farms	Clinical mastitis (n=100)	24	43	67 (67%)
	Subclinical mastitis (n=270)	6	179	185 (68%)
Retail market	Raw milk (n=200)	4	10	14(0.1%)
Total	570	34	232	266(46.7%)

cin (81%). From this result the antimicrobial agents which were completely active against *Streptococcus* isolates weren't found. *S. agalactiae* isolates were shown to be resistant to two and up to eight antimicrobial agents (Table 4). In addition, multiple antibiotic resistances to three or more antimicrobial agents were detected in 32 out of 34 (94%) *S. agalactiae* isolates with multiple antibiotic resistance index (MARI) of 0.1-0.7 The *S. agalactiae* in this research demonstrated 28 different MAR patterns, reflecting the high prevalence of MAR among *S. agalactiae* isolates in the surveyed Province (Table 4).

Genetic antimicrobial resistance of Streptococcus agalactiae

The genetic antimicrobial resistance of *S. agalactiae* strains

Table 3. Antibiotic susceptibility test results of Streptococcus species isolated from dairy milk samples (n=266).

Antibiotic class	Antibiotic disc	*S. agalactiae* (n=34)		Other *Streptococcus* species(n=232)		Total (n=266)	
		R	S	R	S	R	S
B-lactam	Ampicillin	17(50%)	17(50%)	152(65.5%)	80 (34.5%)	169(63.5%)	97(36.5%)
	Amoxicillin	28(83%)	6(17%)	130(56%)	102(44%)	158(59.4%)	108(40.6%)
Cephalosporines	Cefotaxime	5(15%)	29(85%)	60(25.9%)	172(74.1%)	65(24.4%)	201(75.6%)
	Ceftriaxone	6(17.6%)	28(82.4%)	70(30.2%)	162(69.8%)	76(28.6%)	190(71.4%)
	Cefaclor	13(38%)	21(62%)	86(37%)	146(63%)	99(38%)	167(62%)
Tetracycline	Tetracycline	28(82.4%)	6(17.6%)	122(52.5%)	110(47.5%)	150(56%)	116(44%)
Nitrofuran	Nitrofurantoin	22(64.7%)	12(35.3%)	109(46.9%)	123(53.1%)	128(48%)	138(52%)
Quinolones	Ofloxacin	4(11.8%)	30(88.2%)	111(47.8%)	121(52.2%)	115(43%)	151(57%)
Macrolides	Azithromycin	21(61.8%)	13(38.2%)	81(34.9%)	151(65.1%)	102(38%)	164(62%)
	Erythromycin	7(20.6%)	27(79.4%)	44(19%)	188(81%)	51(19%)	215(81%)
Meropenem	Meropenem	14(41.2%)	20(58.8%)	33(14.2%)	199(85.8%)	47(17.7%)	219(82.3%)
Vancomycin	Vancomycin	6(17.6%)	28(82.4%)	54(23.3%)	178(76.7%)	60(22.6%)	206 (77.4%)

Table 4. Distribution of antibiotic resistance rates of S. agalactiae isolates (n=34)

Antibiotic pattern profile	Antibiotics	No. of isolates	Percentage of resistant isolates (%)	No. of resistance antibiotics	MARI (%)
1	TE, VA	1	2.8	2	0.1
2	AMP, AMX. CEC, OFX	1	2.8	4	0.3
3	AMX, CEC, CRO, CTX, F, TE	1	2.8	6	0.5
4	OFX, TE	1	2.8	2	0.1
5	AMX, AZ, CEC, E, TE, VA	1	2.8	6	0.5
6	AMP, AMX, F, TE	2	5.8	4	0.3
7	AMX, AZ, E, F, MEM	1	2.8	5	0.4
8	AMX, CEC, CRO, CTX, E, F, TE	1	2.8	7	0.6
9	AMX, AZ, CEC, CRO, CTX, E, F, TE	1	2.8	8	0.7
10	AMX, CRO, E, TE	1	2.8	4	0.3
11	AMX, , AZ, E, F, MEM, TE, VA	1	2.8	7	0.6
12	AMP, AMX, AZ, TE	1	2.8	4	0.3
13	AMX, CEC, F, TE, VA	1	2.8	5	0.4
14	AMX, AZ, CRO, TE	1	2.8	4	0.3
15	AMP, AMX, AZ, F, TE	2	5.8	5	0.4
16	AMP, AMX, AZ, CTX, OFX	1	2.8	5	0.4
17	AMX, CEC, CRO, MEM, TE	1	2.8	5	0.4
18	AMP, AMX, AZ, F	1	2.8	4	0.3
19	AMX, AZ, CEC, MEM, TE	1	2.8	5	0.4
20	AMP, AMX, F MEM, TE	2	5.8	5	0.4
21	AMP, AMX, AZ, F, MEM, TE	3	8.8	6	0.5
22	AMP, AMX, AZ, CEC, F, TE	2	5.8	6	0.5
23	AMP, AMX, AZ, MEM, TE	1	2.8	5	0.4
24	AZ, CEC, CTX, E, OFX	1	2.8	5	0.4
25	AZ, E, F, MEM, TE, VA	1	2.8	5	0.4
26	AZ, CEC, F, MEM, TE, VA	1	2.8	6	0.5
27	AZ, F, MEM	1	2.8	3	0.2
28	AMP, AMX, CEC, F, MEM, TE	1	2.8	6	0.5

AMP: Ampicillin; AMX= Amoxicillin; AZ: Azithromycin; CEC: Cefaclor; CRO: Ceftriaxone; CTX: Cefotaxime; E: Erythromycin; TE: Tetracycline; MEM: Meropenem; F: Nitrofurantoin; OFX: Ofloxacin; VA: Vancomycin; MARI: multidrug antibiotic resistance index.

was investigated using simplex PCR. Interestingly, for minimum level all *S. agalactiae* isolates passed one resistance gene. In relation to β-lactam resistance, the bla_{CTX} and bla_{TEM} genes were amplified. The genes bla_{TEM} and bla_{CTX} were determined in 8(23%) and 9(26%) β-lactam-resistant strains, respectively. Regarding macrolides resistance, the *erm*(B) gene was noticed in 7 (20%) isolates, while *mef*(A) gene was exhibited in 12(35%) isolates. All resistant isolates exhibited resistance associated to *erm*(B) and *mef*(A)genes indicating the presence of a target-site modification by a ribosomal methylase. Also, some susceptible strains carried those genes. The efflux genes *tet*(M) were amplified to record tetracycline resistance. A 15 (44%) tetracycline-resistant strains were discovered carrying the gene *tet*(M).

Genetic diversity of Streptococcus agalactiae strains

A 2–6 bands, whose size ranged from 200 to 800 bp which produced from ERIC-PCR amplification were the DNA fragments electrophoretic profile which obtained from the examined 34 *S. agalactiae* strains. The genetic diversity of the various strains was demonstrated 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 generated two groups, ERIC group I and ERIC group II. ERIC group I contained 97.1% of the isolates (33 strains) that were designated alphabetically (A-R), while only one strain belonged to ERIC group II (ERIC S). The visual comparison of the banding patterns showed 19 distinct ERIC profiles (A–S). ERIC O (20.6%, 7) was the most common ERIC type (Table 5), followed by ERIC A (11.8%, 4), ERIC D and ERIC N (8.8%, 3 each). The ERIC-PCR dendrogram (Fig. 1) presented that the genetic diversity and heterogeneity of the *S. agalactiae* obtained from the five farms and retail markets can be seen in their classification into specific genotypes according to sampling site and sample type.

DISCUSSION

Researchers from all over the world have, without a doubt,

Table 5. ERIC-PCR, and antibiotic resistance of Streptococcus agalactiae isolated from dairy milk (n = 34)

	Type of sample	ID	ERIC-PCR type	Antibiotic resistant pattern	Antibiotic Resistant Genes
FARM I	Clinical mastitis	1	O	TE, VA	*tet(M)*
		2	O	AMP, AMX. CEC, OFX	bla_{CTX-M}
		3	O	AMX, CEC, CRO, CTX, F, TE	*erm(B), tet(M)*
		4	P	OFX, TE	bla_{TEM}
		5	O	AMX, AZ, CEC, E, TE, VA	*tet(M)*
		6	O	AMP, AMX, F, TE	bla_{CTX-M}, *mef (A),*
Retail markets	Raw milk	7	N	AMX, AZ, E, F, MEM	*tet(M)*
		8	O	AMX, CEC, CRO, CTX, E, F, TE	$bla_{CTX-M,}$
		9	O	AMX, AZ, CEC, CRO, CTX, E, F, TE	*mef(A), tet(M)*
		10	R	AMX, CRO, E, TE	*mef(A), tet(M)*
FARM II	Subclinical mastitis	11	N	AMX, AZ, E, F, MEM, TE, VA	$bla_{TEM,}$ *erm(B)*
		12	N	AMP, AMX, AZ, TE	*mef(A)*
		13	L	AMX, CEC, F, TE, VA	*mef(A)*
		14	S	AMX, AZ, CRO, TE	$bla_{TEM,.}$ *erm(B), mef (A),*
		15	L	AMP, AMX, AZ, F, TE	$bla_{TEM,.}$ *erm(B), tet(M)*
		16	E	AMP, AMX, AZ, CTX, OFX	$bla_{TEM,.}$ *erm(B), tet(M)*
	Clinical mastitis	17	H	AMX, CEC, CRO, MEM, TE	*tet(M),* bla_{TEM}
		18	F	AMP, AMX, F, TE	*tet(M)*
		19	G	AMP, AMX, AZ, F	bla_{CTX-M}, *mef(A)*
		20	J	AMX, AZ, CEC, MEM, TE	*tet(M)*
		21	K	AMP, AMX, F MEM, TE	bla_{CTX-M}, $bla_{TEM,..}$
FARM III	Clinical mastitis	22	I	AMP, AMX, AZ, F, MEM, TE	bla_{CTX-M}
		23	Q	AMP, AMX, AZ, CEC, F, TE	*mef(A)*
		24	B	AMP, AMX, AZ, F, MEM, TE	$bla_{TEM,}$, *erm(B)*
		25	M	AMP, AMX, AZ, F, TE	*mef)A), tet(M)*
FARM IV	Clinical mastitis	26	D	AMP, AMX, AZ, MEM, TE	*mef)A),*
		27	C	AMP, AMX, AZ, CEC, F, TE	bla_{CTX-M}, *mef (A),*
		28	D	AZ, CEC, CTX, E, OFX	$bla_{TEM,}$,
		29	A	AZ, E, F, MEM, TE, VA	*tet(M)*
		30	A	AMP, AMX, AZ, C, F, MEM, TE	*mef (A),*
		31	C	AZ, CEC, F, MEM, TE, VA	bla_{CTX-M}
		32	D	AZ, F, MEM	*tet(M)*
FARM V	Clinical mastitis	33	A	AMP, AMX, F, MEM, TE	bla_{CTX-M}, *erm(B)*
		34	A	AMP, AMX, CEC, F, MEM, TE	*tet(M)*

AMP: Ampicillin; AMX= Amoxicillin; AZ: Azithromycin; CEC: Cefaclor; CRO: Ceftriaxone; CTX: Cefotaxime; E: Erythromycin; TE: Tetracycline; MEM: Meropenem; F: Nitrofurantoin; OFX: Ofloxacin; VA: Vancomycin; MARI: multidrug antibiotic resistance index.

become interested in studying streptococcal mastitis in cattle, whether it is subclinical or symptomatic. In addition to the financial losses brought on by decreased milk supply and quality, veterinarian treatment, medication, and increased staff costs, their zoonotic relevance has drawn attention (Keefe, 1997; Hogeveen *et al*., 2011). Milk can be a source of multidrug-resistant streptococci, notably *S. agalactiae*, and acts as a medium for bacterial growth. This investigation showed that milk samples from bovine mastitis and retail markets included 46.7% *Streptococci*, including 5.96% *S. agalactiae* and 40.7% other *Streptococcus* species. *S. uberis*, *S. agalactiae*, *S. dysgalactiae*, and *Enterococcus* spp. are very dangerous mastitis pathogens.

A previous study revealed that the well-known mastitis pathogens *S. uberis*, *S. dysgalactiae*, *S. agalactiae*, and Enterococcus spp were among some Gram-positive, catalase-negative cocci (PNC) strains (36%) (Wyder *et al*., 2011). In contrast, Huma *et al*. (2022) detected that prevalence of *Streptococcus* spp. (10.45%) isolated from milk was low. The obtained microbiological data were consistent with those reported the low isolation rate of *S. agalactiae* (4.3%) in Denmark and (5.9%) in Brazil (Tomazi *et al*., 2018b; Chehabi *et al*., 2019). Also, Emam *et al*. (2021) detected *S. agalactiae* in 4%, 8%, and 10% of examined milk of Bulk tank, individual milk samples, and retail milk samples, respectively. Conversely, our *S. agalactiae* isolation rates were significantly lower than those of Abd El-Razik *et al*. (2021) (12.8%) in Egypt, Zeryehun *et al*. (2013) (21.2%) in Ethiopia and Han *et al*. (2022) (33.6%) in China. (Elmoslemany *et al*., 2009). recorded that this significant disparity in *S. agalactiae* prevalence mainly because of bulk milk samples contaminated by bacteria that came from environment, milking equipment in addition to germs found in the milk of affected quarters. Hence, timely and precise identification of mastitis in cattle can assist prevent it from happening or at the very least lower the costs associated with the disease's effects, which could become worse with any delay.

Antibiogram research for bacteria causing mastitis is crucial because by this exploration we can reduce antimicrobial resistance, know the most suitable antibiotic therapy, and reduce the expected risk of public health. Hence, the isolates were tested against twelve different bacteria in the current investigation as Egyptian dairy farms always did to determine the most suitable antibiotic for treating infectious diseases and bovine mastitis. The present work revealed the high resistance of *Streptococcus* isolates to ampicillin, tetracycline, amoxicillin, nitrofurantoin, and ofloxacin. The *S. agalactiae* isolates showed resistance to amoxicillin, tetracycline, nitrofurantoin, azithromycin, and ampicillin, while other *Streptococcus* species revealed high resistance to ampicillin, amoxicillin, and ofloxacin. These results were expected as these antibiotics are mainly used to treat diseases which caused by Gram-negative bacteria and *S. agalactiae* have its place in the Gram-positive bacteria group. To treat udder infections which caused by *Streptococcus* species, the most used antimicrobial agents are β-Lactams and macrolides (Denamiel *et al*., 2005). An intrinsic antibiotic resistance of *Streptococcus* species was almost similar to the previously recorded results (Porter and Kaplan, 2011; Rüegsegger *et al*., 2014; Saed and Ibrahim, 2020; Hernandez *et al*., 2021; Kabelitz *et al*., 2021). Ceniti *et al*. (2017) detected that antimicrobial resistance against tetracycline and ampicillin in *Streptococcus* samples which collected from cases of bovine mastitis in Canadian dairy farms was at high level. The cephalosporin is commonly used for dry cow therapy and intramammary clinical mastitis treatment. The present study showed an alarm on emergence of streptococcal resistance to cephalosporines (cefaclor 37%, ceftriaxone 28.8%, and cefotaxime 24.4%). This finding is somewhat similar to a previous study that was detected the resistance of some *S. dysgalactiae* isolates from bovine mastitis to cephalosporines (cephalexin 34.1% and ceftriaxone 13.6%) in China (Zhang *et al*., 2018). The observed increasing level of resistance against the β-lactam antimicrobials might at least partly be explained by the increasing usage of cephalosporins for the treatment of infectious diseases on Egyptian dairy farms. The increasing use of β-lactam antimicrobials might have resulted in a selective pressure toward the more resistant isolates. The increasing usage of β-lactam antimicrobials might lead to more selective pressure toward the more resistant isolates. Yet, this study and others throughout the world found minimal levels of erythromycin resistance (Haenni *et al*., 2018).

Furthermore, the present research showed the high prevalence of MAR *S. agalactiae* isolates (88.2%) with MARI of 0.2-0.6 and 29 different MAR patterns. The multidrug-resistant *Streptococcus* species was previously reported in Egypt and other worldwide (Saed and Ibrahim, 2020; Hernandez *et al*., 2021). Unchecked use of antibiotics typically alters the microbial ecology of susceptible bacteria, which results in mutations and enables the survival and spread of antibiotic-resistant bacteria. The uncontrolled use of antibiotics typically affects the microbial system of sensitive bacteria, causing mutations that enable bacteria to survive and further proliferate as antibiotic-resistant bacteria. As a result, antibiotic resistance rises to dangerously high levels worldwide, posing a constant threat to the ability to treat the common infectious diseases (Uh *et al*., 2001) .

Although low resistance to β-lactams was recorded in *Streptococcus* mastitis isolates recorded in *Streptococcus* mastitis isolates (Saini *et al*., 2012) , the current study detected bla_{TEM} and bla_{CTX-M} in 8(23%) and 9(26%) *S. agalactiae* isolates. Such genes might be associated with phenotypic resistance of *S. agalactiae* to β-lactam and cephalosporines. Moreover, 6 isolates were found to be resistant to ceftriaxone phenotypically, while nine isolate was detected to carry bla_{CTX-M}. This exciting finding might be due to the lack of gene expression without induction as has already been described for *Staphylococcus aureus* (De Oliveira *et al*., 2000) or lack of functionality (Rahman *et al*., 2005) . Clearly, it is evident that phenotypic resistance is not always a sign of the presence of resistance genes, and vice versa.

Tetracycline resistance in streptococci is linked, for instance, to the emergence of tetracycline-resistant genes on conventional dairy farms; *tet*(O), *tet*(M), ribosome protection genes, *tet*(L), *tet*(K) and efflux pump genes (Rubio-López *et al*., 2012; Yassin *et al*., 2017; Kabelitz *et al*., 2021). According to several previous studies, the *tet*(M) gene is the most frequently reported gene conferring resistance to tetracycline (Haenni *et al*., 2018; Hernandez *et al*., 2021; Aiewsakun *et al*., 2022).

Treatment failure is caused by bovine strains of streptococci's widespread resistance to macrolides like azithromycin and erythromycin, and there is a possibility of horizontal transfer of resistance genes between nations (Rato *et al*., 2013; Kaczorek *et al*., 2017; Tomazi *et al*., 2018a).

The *erm* gene, which codes for a ribosome methylase, and the *mef* gene, which codes for a membrane-bound protein with an active efflux pump, are the two major mechanisms causing resistance to macrolides. The other studies similarly provide high-level resistance to antibiotics like lincosamide and streptogramin B, as well as macrolides (Ko *et al*., 2004) . According to Emaneini *et al*. (2014) The current study hypothesises that the frequent discovery of the genes *erm*(B) (20%) and *mef*(A) (35%), which were discovered in streptococci isolates, may be related to the extensive and indiscriminate use of such antibiotics globally (Emaneini *et al*., 2014; Hernandez *et al*., 2021). Gram-positive cocci that are resistant to erythromycin shouldn't be treated with 16-limbed macrolides (macrolides having a 16-membered lactone circle), due to the widespread distribution of the *erm* (erythromycin ribosome methylase) gene and the possible development of complete cross-resistance (Werckenthin *et al*., 2005) .

According to Guérin-Faublée *et al*. (2002), mastitis treatment is frequently initiated prior to the pathogen's antimicrobial susceptibility test results. The emergence of pathogens that are resistant, such as the ones found in this study, requires the use of susceptibility tests to select the most affected antimicrobial agents (Minst *et al*., 2012) . Control of farm animal disease frequently shares active substances with human medications. Furthermore, the antibiotics usage in excess is linked to the multidrug-resistant foodborne pathogens development (van Duin and Paterson, 2016). Another important thing is that vaccination is

one of the most likely ways to stop GBS infections.

S. agalactiae from the five farms and retail markets were found to be genetically diverse and heterogeneous by ERIC-PCR analysis. The varied origins of the sampling locations suggested high genetic diversity. By examining the prevalence of genotypes among the sampling locations using ERIC-PCR, the probable *S. agalactiae* contamination routes in the dairy farm and retail markets were found. This research found low similarity in the genotypes of *S. agalactiae* among the five farms, presumably as a result of their geographic locations. Moreover, different strains of the same genotype were susceptible to different antibiotics. For instance, the strain (n=1) with a similar ERIC O type exhibited various antibiotic resistance patterns and different antibiotic resistance genes.

We employed ERIC-PCR to 34 strains for the first time in Egypt because there is little information available on the epidemiology of Egyptian isolates of *S. agalactiae* and because this approach has not been widely used to genotype *S. agalactiae*. In Poland, group B *Streptococcus* genotyping using the ERIC-PCR fingerprinting reaction has been reported. The scientists found 13 genotypes among the 120 strains tested from different clinical samples based exclusively 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 prevalence of *Streptococcus agalactiae* strains in Egyptian retail milk markets and bovine mastitis was the subject of this study. Several antimicrobial resistance profiles were discovered among *Streptococcus agalactiae* strains by antimicrobial analysis The results of this study are the first genotyping data on *S. agalactiae* strains isolated from bovine mastitis cases in dairy farms and retail markets milk in Egypt. Diagnostic, preventative, and therapeutic approaches and the possibility of a vaccine to control bovine mastitis are based on these data. In order to investigate the molecular mechanisms that are responsible for the development of antimicrobial resistance, these data will be helpful. The future challenge of monitoring the spread of MAR strains is also presented by these data.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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