Egyptian Journal of Animal Health

P-ISSN: 2735-4938 On Line-ISSN: 2735-4946
Journal homepage: https://ejah.journals.ekb.eg/

Bacteriological and Molecular Studies on *Serratia Marcescens* causing Bovine Mastitis

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Received in 26/7/2023
Received in revised from 23/10/2023
Accepted in 1/11/2023

Keywords:
Serratia marcescens mastitis, antimicrobial resistance, resistance genes

ABSTRACT

Serratia marcescens (*S. marcescens*) is considered an important opportunistic pathogen and has been found to be associated with outbreaks of mastitis among dairy ruminants. Like other various bacterial diseases, multidrug resistance strains pose a serious threat to public health and are considered a great obstacle during treatment of such field problems. The aim of this study was to investigate *Serratia* species, antimicrobial susceptibility, and presence of resistance genes of *S. marcescens* isolates associated with mastitis cases. Eighty mastitic milk samples were collected from different dairy farms located in Menoufia Province. *S. marcescens* was isolated and confirmed in 6 mastitis milk samples out of 80 (7.5%) using culturing and gram staining .VITEK 2 (bioMérieux) System was used to complete the identification of *S. marcescens*. The cultured bacteria were then purified for detection of resistance gene. Further, the resistance of these isolates against antibiotics had been investigated by disk diffusion method and the findings revealed that the isolated strains are sensitive to some antibiotic as cefepime (6\6,100%), ceftazidime (5\6, 83.3%), cefotaxime (4\6, 66.6%), Amoxicillin-Clavulanic acid (6\6,100%), whereas wide differences were observed in the patterns of resistance among the bacterial isolates in particular, Ampicillin (6\6, 100%), chloramphenicol (5\6, % 83.3%), imipenem (4\6, 66.6%). Additionally, The results of resistance genes detection in *S. marcescens* revealed that the most prevalent resistant genes encodings β-Lactams were bla*TEM* (6\6,100%), *bla CTX-M* (6\6, 100%),CYM(CIT) (4 \6,66.6%) , genes encoding chloramphenicol *floR* (5 \6, % 83.3%), and genes encoding carbapenemases *bla IMP* (6\6,100%), *OXA* (2\6, 33.3%), *SME* (2|6, 33.3%).

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DOI: 10.21608/EJAH.2024.325122

INTRODUCTION.

According to **Yang et al. (2018),** one of the most common and expensive diseases affecting the dairy cattle business is bovine mastitis. According to **Zadoks et al. (2001),** the etiopathology of this illness typically involves three variables: exposure to microbes, host defense systems, and environmental factors. *Serratia marcescens*, which is regarded as an important opportunistic pathogen and has been found to be associated with outbreaks of mastitis in dairy cows, is considered an environmental pathogen that has received little attention despite significant progress being made in controlling contagious mastitis pathogens through improved milking **hygiene (Friman et al. 2019)**.

Serratia species are rod-shaped Gram-negative bacteria, which were recently classified under the new family of the order *Enterobacterales: Yersiniaceae* **(Adeolu et al. 2016)**. It is aerobic, motile and produces a red water insoluble pigment besides some heat resistant enzymes **(Bi et al., 2016)**. Approximately 20 different *Serratia* spp. have been described **(Parte AC 2018**). *Serratia* spp., in particular *S. marcescens*, and *Serratia liquefaciens* are ubiquitous environmental bacteria capable of causing opportunistic infections in humans and many animal species **(Mahlen, 2011)** including mastitis in dairy cows (**Schukken et al. 2012**) as well as they are capable of causing spoilage at different points of milk processing **(Desimo et al. 2014).**

Antibiotic therapy is the main method for treating most of the bacterial infections including *S. marcescens* **(Tavares-Carreon et al. 2023).** However, the therapeutic effectiveness has been attenuated by emerging resistant strains **(Iguchi et al. 2014).** As a result, the common multidrug-resistant nature of *S. marcescens* complicates the treatment of its infections **(González-Juarbe et al. 2015).**

Indeed, lines of studies revealed an alarming increase in *S. marcescens* resistance to the commonly used beta-lactams **(Abbas et al. 2020)**. Antimicrobial resistance of *S. marcescens* is mainly attributed to different resistance determinants, such as genes of extended-spectrum beta-lactamase (eg, bla *SHV, blaTEM*, and bla *CTX*) and carbapenemases (eg, bla *OXA-48*, *KPC*, and *NDM*) for beta-lactam resistance and bacterial effector proteins (eg, *sdeB, sdeD*, and *sdeY*) for multidrug resistance. Moreover, the pathogenicity of *S. marcescens* is mediated by an arsenal of virulence factors including hemolysin, lipase, protease, prodigiosin, and motility **(Khayyat et al. 2021).**

Accordingly, the aim of the current study is to detect the *S. marcescens* antibiotic resistance besides identification of resistance genes associated with *S. marcescens* bovine mastitis that might pave the way for controlling such crucial field problem especial in dairy industry.

2. MATERIALS and METHODS

2.1. Ethical approval

All procedures, including the handling and collection of milk samples, were approved by the Benha University ethical committee for animal studies (BUFVTM 05-06-23). The owners of cattle were informed, and permission was taken from them for collection of milk samples.

2.2. Sample Collection

In this study, 80 milk samples of bovine mastitis were collected from different commercial dairy herds in Menoufia Province. Clinical examinations of the udder of lactating cows were conducted according to **Massé et al., 2020**. In short, the symmetry of one quarter of each cow's udder was checked. Then, possible fibrosis, inflammation, swelling, visible damage, tissue atrophy, and swelling of lymph nodes was tested through palpation. The viscosity and appearance of milk secretion from each quarter segment for the presence of clots, thin sections, blood, and water secretions, in order to determine clinical mastitis (**Tezera and Aman 2021**). As well as apparently normal milk was subjected to CMT for detection of subclinical mastitis as described by **Balamurugan and Ranjith (2018)**. Eighty samples (63 and 17 from clinical and subclinical) respectively were collected asepti-

cally and transmitted in cold condition .

Prior to milk samples collection, the udder was washed directly with tap water to remove dirt then dry with clean towel, the teat dip in Iodine solution 1:1000 and leave to dry than the teat was dip in 70% alcohol than dry, before sample taken one or two streams of milk discarded. Milk was collected in sterile vial (test tube 10 ml). These samples were transferred in an ice box directly within an hour to Animal health research institute to be bacteriologically examined with a minimum delay.

2.3. Identification of *Serratia marcescens*

2.3.1 Cultural and microscopical characteristics

The examined milk samples were streaked into nutrient agar then cultured on MacConkey agar, Xylose lysine deoxycholate agar by streaking method.

Further, light microscopy and gram staining were carried out to differentiate whether the bacteria are Gram-negative or Gram-positive. The isolates were subcultured twice and the fresh clinical isolates were subcultured once on MacConkey agar plates for 18 to 24 h at 37°C, before they were tested in the VITEK 2 system. A bacterial suspension was adjusted to a McFarland standard of 0.5 in 2.5 ml of a 0.45% sodium chloride solution by using a Densicheck (bioMérieux). Afterward, the VITEK 2 system ID-GNB card and the bacterial suspension were manually loaded into the VITEK 2 system (bioMérieux, Marcy l'Etoile, France). The VITEK 2 system reported the results automatically with software release 2.01.

2.3.2 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *S. marcescens* to 12 antimicrobial agents was determined via disk diffusion method on Mueller–Hinton agar (MHA; Oxoid, United Kingdom) according to the Clinical and Laboratory Standards Institute **(CLSI, 2018).**

Antimicrobial agents tested in this study include Ampicillin (10 µg), cefepime (30 µg) ceftazidime (30 µg), cefotaxime (30 µg), imipenem (10 µg), gentamicin (10 µg), tetracycline (30 µg), levofloxacin (5 µg), chloramphenicol (30 µg), trimethoprim/ sulfamethoxazole (1.25/23.75 µ ،) Amoxicillin-Clavulanic acid (20/10 µg) and Amikacin (30 µg).

Escherichia coli ATCC 25922 used as quality control strain. The results were logged as susceptible or resistant by the measurement of Diameter of the inhibition zone in millimeter. Multidrug resistance was defined as isolates that were resistant to at least 3 classes of the tested antimicrobial agents. **(Drieux et al. 2008**)

2.3.3 Detection of resistance genes

2.3.3.1 DNA extraction.

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

2.3.3.2 Oligonucleotide Primer.

Primers used were supplied from **Metabion (Germany)** are listed in table (1).

2.3.3.3 PCR amplification.

Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentrations, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied bio system 2720 thermal cycler.

2.3.3.4. Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE

buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A generuler 100 bp ladder (Fermentas, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
bla$_{TEM}$	ATCAGCAATAAAC-CAGC CCCCGAAGAAC-GTTTTC	516	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Colom *et al*., 2003
BlaCTX-M	ATG TGC AGY ACC AGT AAR GTK ATG GC TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Archambault *et al*, 2006
floR	TTTGGWCCGCTMT-CRGAC SGAGAARAAGAC-GAAGAAG	494	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Doublet *et al*., 2003
CIT (CMY2)	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	462	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Pérez-Pérez and Hanson, 2002
blaIMP	CATGGTTT-GGTGGTTCTTGT ATAATTTGGCG-GACTTTGGC	488	94˚C 5 min.	94˚C 30 sec.	53˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Xia et al., 2012 Xia et al., 2012 Xia et al., 2012
OXA	TTTTCTGTTGTTT-GGGTTTT TTTCTT-GGCTTTTATGCTTG	519	94˚C 5 min.	94˚C 30 sec.	48˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
SME	AAC-GGCTTCATTTTTGTT TAG GCTTCCGCAA-TAGTTTTATCA	820	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	

3. RESULTS

3.1. Cultural and microscopical characteristics

The results of bacterial isolation revealed that out of 80 milk samples, six samples belonged to *Serratia marcescens* representing 7.5 %. Further, the culture characters showed different morphological features of bacteria on different media, after incubation at 37 °C for 24 hours, *S. marcescens* is generally easy to characterize and differentiate from other *Enterobacteriaceae* as most strains are red pigmented on nutrient agar as showed in **Figure (1),** On MacConkey agar, colonies were lactose fermenter and appear red due to the ability of *Serratia marcescens* to produce pigment as showed in **Figure (2).** Microscopic examination of the isolated bacteria showed Gram negative rods. A total of 6 bacterial isolates with gram-negative bacillus-like morphology from positive subcultures were investigated by VITEK 2 system which identified all tested isolates as *S. marcescens*, the analysis time was about 6.30 hours with probability rate 99%.

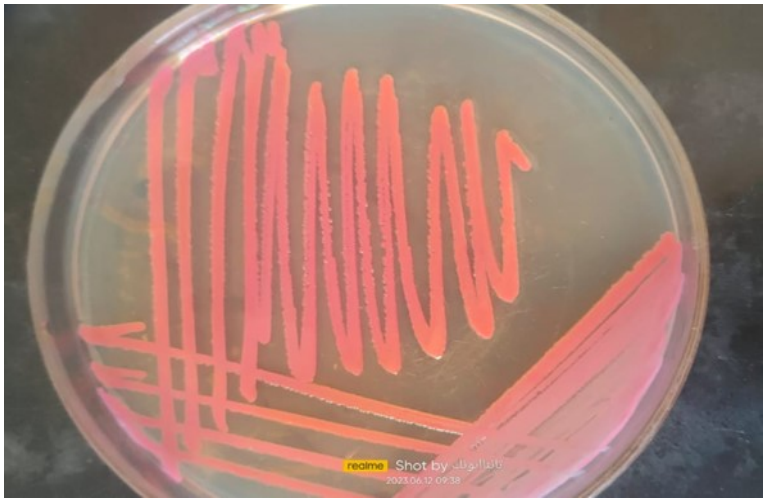

Figure (1): *Serratia marcescens* on nutrient agar showing red pigment due to prodigiosin production.

Figure (2): *Serratia marcescens* on MacConkey agar colonies appeared are lactose fermenter and appear red colonies.

3.2. Antibiotics Susceptibility Testing.

The results of antibiotics susceptibility test showed that *Serratia marcescens* were resistant to Ampicillin (6\6, 100%), chloramphenicol (5 \6, % 83.3%), imipenem (4\6, 66.6%), tetracycline (3\6, 50%) were ineffective against most of the bacterial isolates tested. However, tested isolates were susceptible to cefepime (6 \6,100%), ceftazidime (5\6, 83.3%), cefotaxime (4\6, 66.6%), and Amoxicillin-Clavulanic acid (6\6,100%). While intermediate to gentamicin (5\6, 83.3%), trimethoprim/ sulfamethoxazole (4\6, 66.6%), levofloxacin (6 \6, 100%) and Amikacin (3\6, 50%). Besides, 4 (4/6, 66.6%) of *S. marcescens* isolates were found to be multidrug resistance as shown in **Figure (3) and table (2).**

Table 2. Results of antibiotic resistance expressed as numbers and present of sensitive, Intermediate and resistant antibiotic to *S. marcescens*.

Antibiotic	Sensitive		Intermediate		Resistant	
	Number	%	Number	%	Number	%
Ampicillin (10 µg)	0	0 %	0	0 %	6	10 %
Chloramphenicol (30 µg)	1	16.6%	0	0 %	5	83.%
Imipenem (30 µg)	2	33.3%	0	0 %	4	66.%
Tetracycline(30 µg)	1	16.6 %	2	33.3%	3	50%
Cefepime (30 µg)	6	100%	0	0%	0	0%
Ceftazidime (30 µg)	5	83.3%	1	16.6 %	0	0%
Cefotaxime (30 µg)	4	66.6%	2	33.3%	0	0%
Amoxicillin-Clavulanic acid (30 µg)	6	100%	0	0%	0	0%
Gentamicin (30 µg)	1	16.6 %	5	83.3%	0	0%
Trimethoprim/ sulfamethoxazole (30 µg)	2	33.3%	4	66.6%	0	0%
Levofloxacin(5µg)	0	0%	6	100%	0	0%
Amikacin (30 µg)	3	50%	3	50%	0	0%

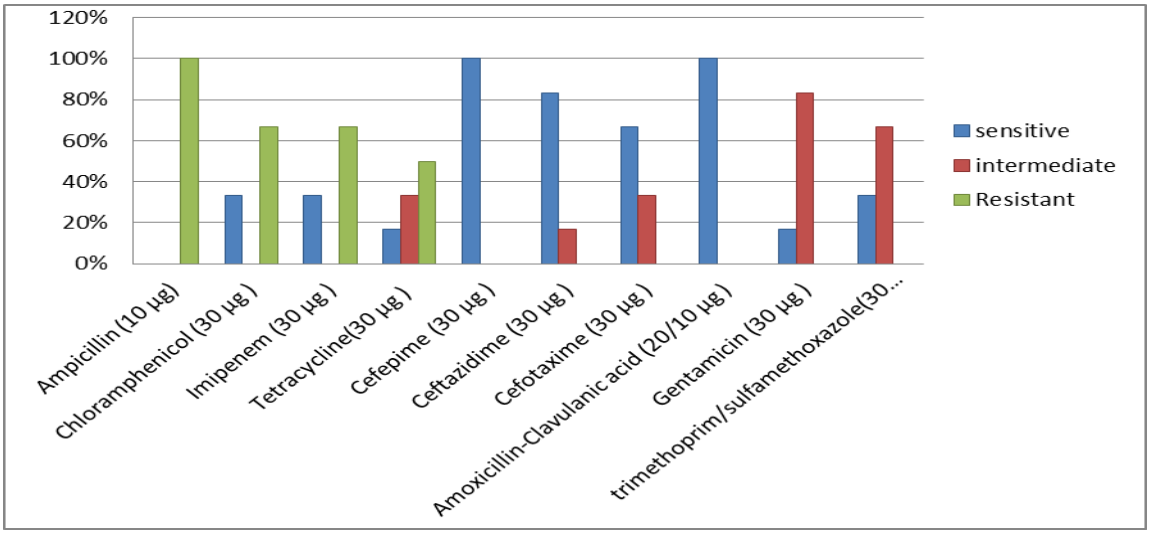

Figure (3) Results of antibiotics Susceptibility test in 6 isolates of *S. marcescens*

3.3. Screening of resistance genes

Resistance encoding genes of the *S. marcescens* isolates had been detected Table(3). The results of resistant genes encoding extended-spectrum beta-lactamase showed that all *S. marcescens* isolates carried *bla TEM* (6 \6,100%), *bla CTX-M* (6\6,100%) and *CIT* (*CMY*)(4\6 ,6.66%). as shown in **figures (4 ,5, 6)**. The results of resistance gene encoding chloramphenicol *floR* (5\6, 83.3%), as shown in **figures (7).** Finally, the results of genes encoding carbapenemases *bla IMP* (6\6,100%), SME (2\6, 33.3%), OXA (5|6, 83.3%) as shown in **figures (8, 9 and 10).**

Table 3. results of resistance genes detection

Sample	*blaTEM*	*BlaCTX-M*	*floR*	*blaIMP*	*OXA*	*CIT (CMY2)*	*SME*
1	+	+	-	+	+	+	-
2	+	+	+	+	-	+	+
3	+	+	+	+	+	-	-
4	+	+	+	+	-	+	+
5	+	+	+	+	-	+	-
6	+	+	+	+	-	-	-

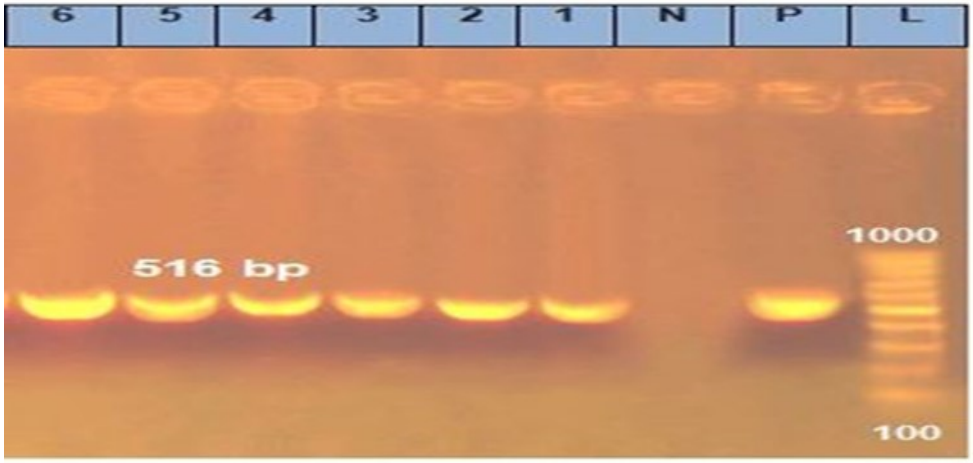

Figure (4): Results of resistant genes encoding extended-spectrum beta-lactamase (*blaTEM*). P: positive control at (516 bp), N: negative control. Lanes (1-6) represent samples and all lanes are considered positive.

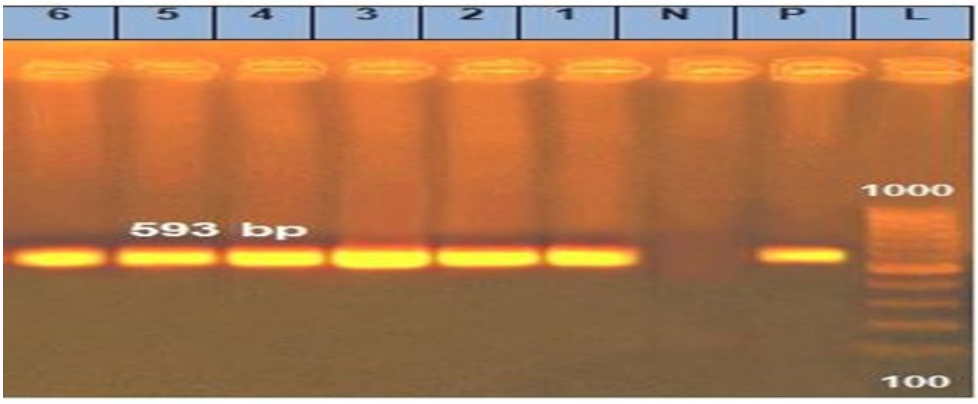

Figure (5): Results of resistant genes encoding extended-spectrum beta-lactamase (*CTX-M*) P: positive control at (593 bp), N: negative control. Lanes (1-6) represent samples and all lanes are considered positive.

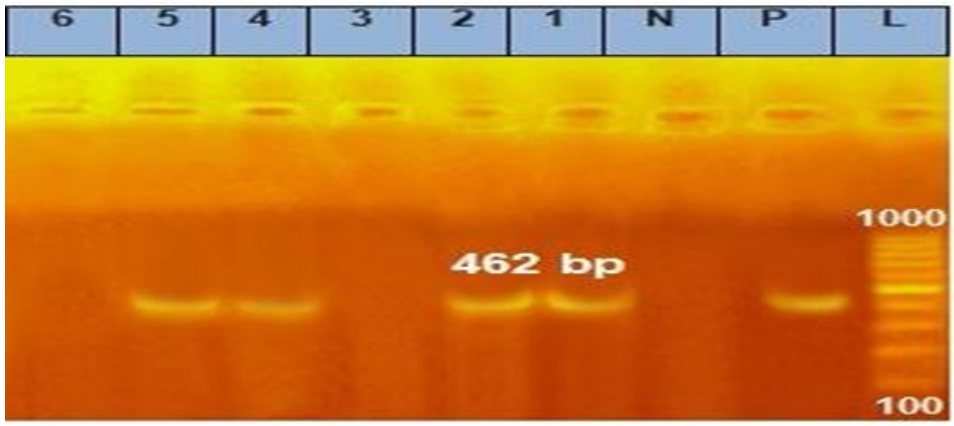

Figure (6): Results of resistant genes encoding extended-spectrum beta-lactamase *CIT*(*CMY*). P: positive control at (462 bp), N: negative control. Lanes (1-6) represent samples and lanes 1,2,4,5 are considered positive.

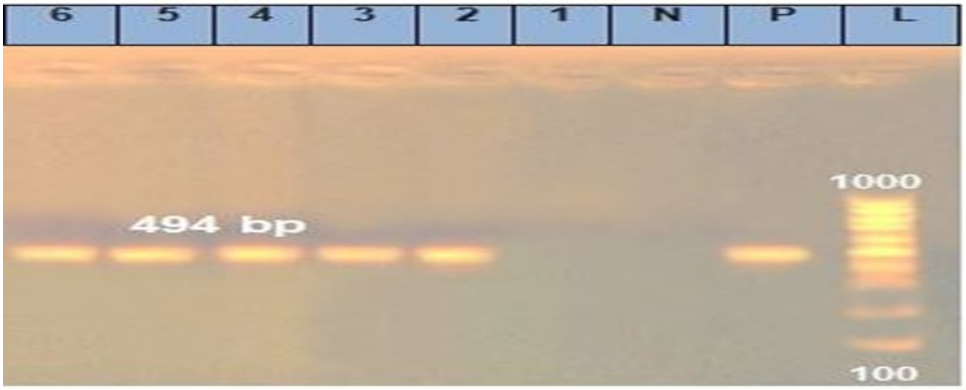

Figure (7): Results of resistance genes encoding chloramphenicol (*floR*). P: positive control at (494 bp), N: Negative control. Lanes (1-6) represent samples and lanes 2, 3, 4, 5, 6 considered positive

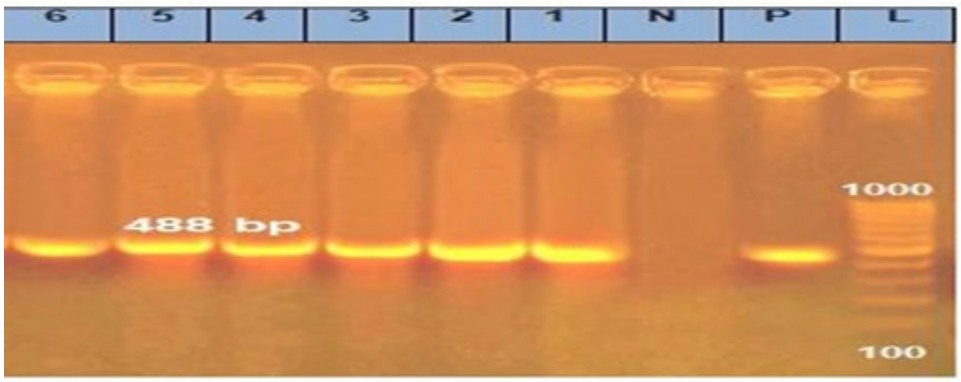

Figure (8): Results of resistance genes encoding carbapenemases (*IMP*). P: positive result at (488 bp), N: negative control. Lanes (1-6) represent samples and all lanes are considered positive.

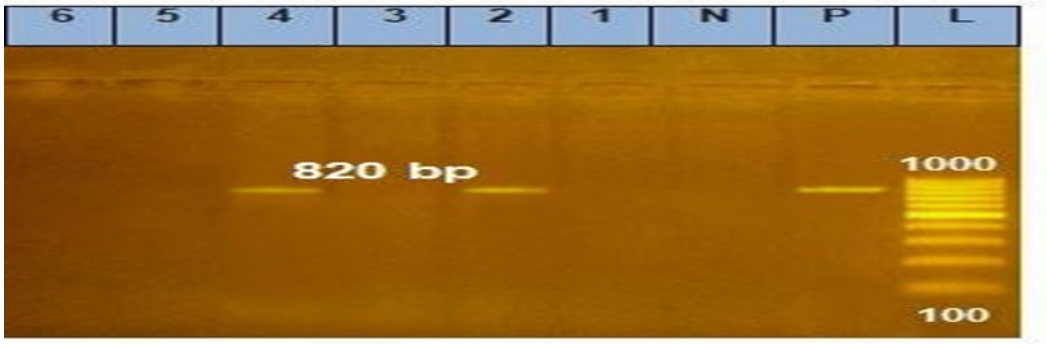

Figure (9): Results of resistance genes encoding carbapenemases (*SME*). P: positive control at (820 bp), N: negative control. Lanes (1-6) represent samples lans 2, 4 are considered positive.

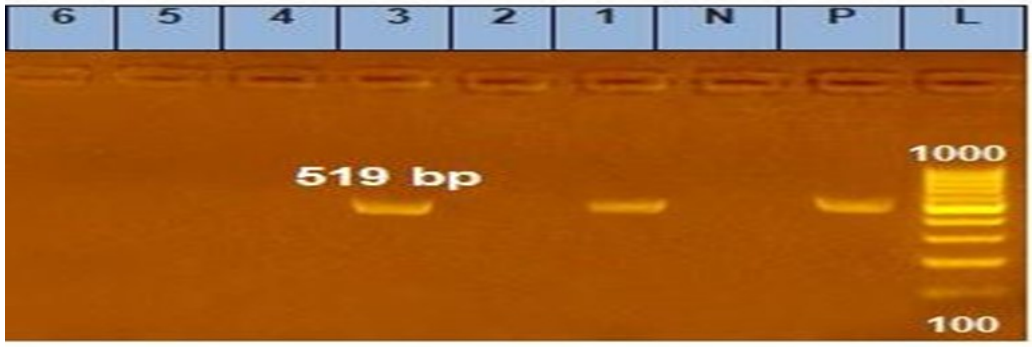

Figure (10): Results of resistance genes encoding carbapenemases (*OXA*). P: positive control at (519 bp), N: Negative control. Lanes (1-6) represent samples and lanes 1, 3 are considered positive

DISCUSSION.

Serratia marcescens is considered an important opportunistic pathogen and has been found to be associated with outbreaks of mastitis in dairy cows. In the current study**,** bacterial isolation of 80 milk samples showed that six samples were belong to *Serratia marcescens* represented 7.5% from milk samples ,this finding higher than **Di Guardo et al. (1997)** who found that 4 out of 120 (3%) cow affected by *Serratia marcescen*s mastitis and a previous study reported in China that 1.5% of the bovine mastitis samples were positive for *S. marcescens* in bovine mastitis **(Bi et al. 2016).** Also higher than **Friman et al. (2019)** who revealed that 45 *S. marcescens* isolate out of 1000 (4.5%) reported in Korea, but lower than 35–39% in outbreaks of mastitis in Finland.

Antibiotic therapy is the chemotherapy of choice for treating infections caused by *S. marcescens.* However, owing to the global problem of antibiotic resistance, the therapeutic effectiveness of antibacterial agents has been diminished. The prolonged and extensive use of these antimicrobials on dairy farms may be the cause of the high resistance **(Swinkels et al. 2015**). Additionally, a rise in drug-resistant strains can result from increased exposure to antimicrobial medications **(Dos Santos et al. 2016)**. The chloramphenicol-resistant bacteria that farm veterinarians carry are subsequently transferred to *Serratia marcescens* by plasmid-mediated transfer. Farm veterinarians may be exposed to chloramphenicol settings The last line of defense among -lactam antibiotics for treating infections brought on by multidrug-resistant Gram-negative bacteria is carbapenems. Unfortunately, with the increased clinical usage of carbapenems, carbapenem-resistant bacteria have arisen and now pose a serious threat to human health. **(Chen et al. 2022)**. The results of antibiotics susceptibility test showed that *Serratia marcescens* were resistant to Ampicillin (6\6, 100%), chloramphenicol (4\6, %

66.6), imipenem (4\6, 66.6%), tetracycline (3 \6, 50%), similar results were reported and revealed that both *S. marcescens* and other Enterobacteriaceae isolates from bovine mastitis frequently exhibited resistance to these antimicrobials (**Ahmed & Shimamoto 2011; Hawkey & Choy 2015 and Yang et al. 2018)**. Moreover**, Wilfert et al. (1970**) showed that *Serratia Spp.* was highly resistant to *Cephalosporin and the Polymyxin B*, but all of the isolates were susceptible to Gentamicin.

Regarding the genotypic resistance profiles of *S. marcescens*, the resistant genes against β-lactams, chloramphenicol and carbapenem were detected. In this study we found that all *S. marcescens* isolates carried *blaTEM* combined with *bla CTX-M* and showed resistance to at least one of the tested β-lactams. Similarly, *floR* gene was found in all of chloramphenicol-resistant isolates. These findings were consistent with other reports that these genes were frequently observed in Enterobacteriaceae isolates from food producing animals in many countries **(Cao et al. 2020),(Bischoff et al. 2005).** On the other hand, Carbapenem resistance, mediated by acquired carbapenemase genes, has been increasingly reported **(Lee et al. 2005),** nearly Similar results were obtained in our study in which *IMP*, *OXA* and *SME* genes represent (100% , 33.3% ,33.3%) respectively. In addition the results of antibiotics susceptibility testing is closely related with resistance genes screening, All isolates were resistant to Ampicillin (100%), and the genes encoding β-lactamase *blaTEM*, *bla CTX-M* and *CIT (CMY2)* were found with percentage (100%, 100%, 66.6%) respectively. Besides that, 5 isolate were resistant to chloramphenicol (83.3%) and the *floR* gene was also detected in (83.3%) of isolates. Carbapenem like imipenem was found resistant with percentage of 66.6%, this is nearly close to detected genes (*blaIMP, OXA,SM,*) (100%, 33.3%,33,3%).

CONCLUSION

In conclusion, the occurrence of *S. marcescens* causing bovine mastitis is considered low in comparison with other Enterobacteriace, but the high frequencies of phenotypic and genotypic resistance to Beta-lactam, carbapenemases, chloramphenicol as well as the multidrug resistance remind the authorities to pay special attention to the antimicrobial agents used in dairy industry. Importantly, the emergence of multi-drug resistant *S. marcescens* poses an alarming threat to public health due to the transmission of resistant determinants through the food chain. Further investigations are to be conducted to under-stand the pathogenicity of the individual virulent factor.

Acknowledgments

The authors would like to thank Prof.Dr\ Nahla Ahmed Shawkey Abo El-Roos, Director of Animal Health Research Institute Shebin El-kom branch for giving the opportunity to do the research and providing invaluable guidance throughout this research.

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