P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2026.011

Molecular Detection of Linezolid Mutations among Methicillin Resistant *Staphylococcus Aureus* Isolated from Bovine Mastitis and Human Sources in Jordan

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Article History: 25-251 Received: 07-Sep-25 Revised: 02-Dec-25 Accepted: 06-Dec-25 Online First: 17-Jan-26

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) is rapidly overcoming the current array of antibiotics. Resistance to linezolid usually develops in gram-positive bacteria because of mutations in domain V of 23S rRNA, 50S ribosomal proteins L3, L4, and L22, and acquisition of the cfr gene. The study aimed to detect the pattern of sensitivity and resistance of MRSA isolates to linezolid, as well as to detect and characterize various mutations associated with linezolid resistance. A total of 113 MRSA isolates (26 from mastitis, 38 from university students, 49 from farm workers) were studied for resistance against 13 antimicrobials by the disc diffusion method, and were investigated for their resistance against linezolid antibiotic using E-test strips. Briefly, to detect the G2576T point mutation, the 23S rRNA gene (domain V region) was amplified by PCR, digested with NheI, and subjected to high-resolution melting (HRM). PCR and sequencing of rplC, rplD, rplV, and cfr genes were carried out to identify the presence of linezolid's various mutations. All isolates showed resistance toward penicillin, oxacillin, and cefoxitin, and a significant difference in resistance percentages between isolates was detected only in erythromycin, gentamycin, and linezolid. Further, (76.9%) of isolates exhibited sensitivity toward linezolid. Among the linezolid-resistant MRSA isolates, only one isolate contained the cfr gene, while other isolates contained various mutations in the 23S rRNA gene and in 50S ribosomal proteins L3, L4, and L22.

Keywords: Methicillin-Resistant *Staphylococcus aureus* (MRSA); Linezolid-resistance; Bovine mastitis; Human samples; High resolution melting; DNA sequencing.

INTRODUCTION

Staphylococcus aureus (*S. aureus*), including (MRSA), is a gram-positive cocci, belongs to the family Staphylococcaceae, order Bacillales, and the *Staphylococcus* genus (Taylor and Unakal 2019; Hameed et al. 2025; Roșu et al. 2025). MRSA was first discovered in 1961 (Jevons et al. 1963). This sort of bacterium has two issues: the first one is the hard-to-treat MRSA infections, and the second issue is its ability to acquire resistance to the newest antibiotics (Aljeldah et al. 2022). Nevertheless, antibiotic resistance in *S. aureus* is an ancient phenomenon, and the emergence of antibiotic-resistant strains of *S. aureus*, especially in MRSA, is a real issue in clinical trials. Like all other antibiotics to date, resistance to the newest antibiotics like daptomycin and linezolid, that used to treat MRSA infections, has just developed (Jones et al. 2008a, b; Ali et al. 2024; Mustafa et al. 2025; Yang et al. 2025).

Linezolid (LZD) (Zyvox – Pfizer) is a bacteriostatic oxazolidinone antibiotic used to treat Gram-positive bacterial infections, such as vancomycin-resistant enterococci and MRSA (Roger et al. 2018; Wu et al. 2019; Turner et al. 2025) and was first approved for commercial use in 2000 by the Food and Drug Administration (Boncu et al. 2020). LZD binds to 23S rRNA in the catalytic site of the bacterial 50S ribosome to inhibit the initiation phase of protein synthesis (Wu et al. 2019; Kramer et al. 2019; Sun et al. 2025), which either stops development or results in bacterial destruction by disrupting the translation of messenger RNA (mRNA) into proteins within the ribosome (Mittal et al. 2019). In 1999, resistance to LZD was first discovered in bacteria, while LZD-resistant *S. aureus* was isolated for the first time in 2001 (Kumari et al. 2019). MRSA's first LZD-resistant strain was identified during an outbreak in a Spanish hospital (Yoo et al. 2020).

Cite This Article as: Iraqi NA, Tarazi Y and Ababneh M, 2026. Molecular Detection of Linezolid Mutations among Methicillin Resistant *Staphylococcus Aureus* Isolated From Bovine Mastitis and Human Sources in Jordan. International Journal of Veterinary Science 15(3): 664-674. https://doi.org/10.47278/journal.ijvs/2026.011

LZD resistance is usually produced by Gram-positive bacteria as a result of mutations in the domain V of the 23S rRNA, 50S ribosomal proteins (L3, L4 and L22), and acquisition of the cfr gene (Yoo et al. 2020; Wali et al. 2022; Nandivarmane et al. 2024; Yang et al. 2025). Detection of LZD resistance in MRSA depended on phenotypic methods (disk diffusion and E-test) (Qi et al. 2006) as well as molecular techniques like PCR (AbdAlhafiz et al. 2023), restriction fragment length polymorphism (RFLP) (Gawryszewska et al. 2017), high-resolution melting (HRM) (Tong and Giffard 2012), and sequencing (Tsiodras et al. 2001; Johnson et al. 2002; AbdAlhafiz et al. 2023). Therefore, our project aims to tackle MRSA isolates from bovine mastitis and human sources in Jordan, with the goal of detecting mechanisms of LZD resistance among these isolates using molecular techniques.

MATERIALS AND METHODS

MRSA isolates

One hundred and thirteen MRSA isolates (mecA gene-positive) were obtained from our Microbiology Research Laboratory, Department of Basic Medical Veterinary Sciences, Faculty of Veterinary Medicine, Jordan University of Science and Technology (JUST), Irbid, Jordan. Eighty-seven isolates were isolated from humans: 38 from JUST university students, 49 from bovine-associated personnel and the remaining 26 were isolated from milk samples that were taken from cows with mastitis.

Antimicrobial susceptibility testing

All isolates were tested according to the agar test diffusion standard Method (CLSI, 2017), using Mueller-Hinton agar (Oxoid). The plates were incubated for 18-24 hours at 37℃ (Winn et al. 2006). MRSA isolates were tested for thirteen antibiotics; oxacillin (OX, 1µg), cefoxitin (FOX, 30µg), penicillin (P, 10 units), tetracycline (TE, 30µg), erythromycin (E, 15µg), linezolid (LZD, 30µg), gentamycin (CN, 10µg), clindamycin (DA, 2µg), doxycycline (DO, 30µg), chloramphenicol (C, 30µg), ciprofloxacin (CIP, 5µg), sulphamethoxazole-trimethoprim (STX, 25µg), and Vancomycin (VA, 30µg) and were determined according to National Committee of Clinical Laboratory Standards (NCCLS) guidelines (CLSI 2017).

E-test method was performed for all sensitive and resistant isolates to disk diffusion test of the above-mentioned antibiotics using the LZD E-test strips from Oxoid, UK, with a concentration gradient corresponding to 256–0.015µg/mL, which was utilized with Mueller-Hinton agar (Oxoid) as described by the manufacturer. After 18-24 hours of incubation at 37°C (MIC), endpoints were read.

DNA extraction

Bacterial DNA was extracted from the tested bacteria using the QIAamp® DNA MiniKit Cat. No. 51304 (Qiagen, Germany) following the manufacturer's instructions.

Plasmid extraction

The plasmid DNA extraction was performed on the LZD-resistant MRSA isolates in order to be sure that the cfr gene is carried on the plasmid by using QIAprep® Spin Miniprep Kit Cat. No. 27104 (Qiagen, Germany) according to the manufacturer's protocol.

PCR amplification of the domain V region of the 23S rRNA and other genes

In this study, specific primers were used to amplify a region of the 23S rRNA gene and to detect cfr, rplC, rplD, and rplV genes as previously reported (Gabriel et al. 2012; Lee et al. 2017; Yoo et al. 2020). The PCR was performed by adding 12.5µL master mix, 1µL of each forward and reverse primers, 2µL template DNA, 8.5µL nuclease free-water to reach a total volume of 25µL. Briefly, the amplification was carried out under specific conditions as mentioned in Table 1. The PCR products were run on a 1.5% agarose gel and were visualized and photographed under UV light with a 100bp molecular ladder.

Restriction fragment length polymorphism (RFLP)

The PCR products were cleaved using the restriction enzyme NheI (TaKaRa Bio, Japan) (Zhang et al. 2015) as indicated by the conditions prescribed by the manufacturer. Post digestion, the Digested DNA was run on a 3% agarose gel and was visualized with ethidium bromide staining.

Real Time – HRM

The purified genomic DNA of MRSA isolates was used in the HRM protocol. A Type-it® HRM™ PCR Kit (Cat. No. 206544) was obtained from Qiagen (Germany). Immediately after DNA extraction, specific primers (5'-TGTCGGCTCATCGCATCCTG-3' and 5'-TCTCAAATTTCCTACGCCCACGAC-3') were used for amplification (Gabriel et al. 2012). The HRM-PCR reaction was prepared in a final volume of 25µL consisting

Table 1: PCR conditions for the tested bacteria genes.

PCR Conditions	23S rRNA	cfr	rplC	rplD	rplV
Initial denaturation	94℃ for 5 min	94℃ for 2 min	94℃ for 10 min	94℃ for 10 min	94℃ for 3 min
Denaturation	94℃ for 30 sec	94℃ for 10 sec	94℃ for 30 sec	94℃ for 30 sec	94℃ for 45 sec
Annealing	55℃ for 30 sec	55℃ for 30 sec	55℃ for 30 sec	55℃ for 30 sec	54℃ for 45 sec
Extension	72℃ for 1 min	72℃ for 30 sec	72℃ for 1 min	72℃ for 1 min	72℃ for 1 min
Final extension	72℃ for 10 min	72℃ for 7 min	72℃ for 10 min	72℃ for 10 min	72℃ for 10 min
*Number of cycle	32 cycles	30 cycles	35 cycles	35 cycles	35 cycles

* Number of cycle for denaturation, annealing, and extension steps.

of 12µL HRM-PCR master mix, 1.75µL of each primer, 2µL DNA, and 7µL Nuclease-free water. The optimized cycling protocol for HRM analysis was carried out under specific conditions: An initial PCR activation step at 95°C for 5 minutes, followed by 40 cycles involving denaturation at 95°C for 10 sec, annealing at 55°C for 30 sec, and extension at 72°C for 10 sec (Gabriel et al. 2012). This was followed by HRM ramping from 65°C− 95°C for 2s with 0.2°C increments. HRM was run on the Qiagen Rotor-Gene Q 5plex HRM machine (Qiagen, Germany).

Real Time – HRM analysis

ScreenClust software was used from Qiagen to analyze the results of HRM-PCR and to differentiate MRSA isolates into clusters according to their HRM results.

Sequencing of genes and their analysis

The PCR products for each gene were sent to Macrogen (South Korea) in order to be sequenced using the Big Dye termination technique. The sequences were edited using the Editseq interface of the DNASTAR software, then aligned using the MegAlign interface of the DNASTAR software.

Statistical analysis

Chi-square test was used to detect the significant difference in the percentages of antibiotic resistance patterns among groups. P-value (P≤0.05) was considered statistically significant.

RESULTS

Disc diffusion assay

In the disc-diffusion approach, the diameters of the inhibition halo were measured after 18-24h of incubation (Fig. 1). Then, these diameters were compared with the CLSI reference. The results are shown in Table 2. Nearly all isolates exhibited resistance to penicillin G (P), oxacillin (OX) and cefoxitin (FOX), while most isolates exhibited resistance toward tetracycline (TE) (68.1%) and erythromycin (E) (53.9%). On the other hand, the moderate rate of resistance was observed for ciprofloxacin (CIP), chloramphenicol (C), clindamycin (DA), linezolid (LZD), gentamycin (CN), doxycycline (DO), 14.2, 17.7, 17.7, 22.1, 25.7, and 30.0%, respectively, followed by a lower rate of resistance for sulfamethoxazole-trimethoprim (STX), and vancomycin (VA), 7.1, and 3.8%, respectively Table 2. A statistically significant difference between the resistance pattern of MRSA isolates was only detected for erythromycin, gentamycin, and linezolid, with P-value (0.007421, 0.0417, and 0.024448, respectively) (P≤0.05) as mentioned in Table 2.

Table 2: Results of antibiotic resistance (% resistant) among 113 MRSA isolates collected from different sources, CLSI (2017)

Antimicrobial Class	Antimicrobial Agent (breakpoint, mm)	Antibiotic resistance (%resistant) and No. isolates				P-value (X^2)*
		Mastitis	Human Sources (N=87)		Total	
		Milk (n=26)%	Farm Workers (n=49)%	University Students (n=38)%	N=(113)%	
β-lactamases	Penicillin (≤28)	(26)100	(49)100	(38)100	(113) 100	–[b]
	Oxacillin (≤12)	(26)100	(49)100	(38)100	(113) 100	–[b]
Cephalosporins	Cefoxitin (≤21)	(26)100	(48)98	(37)97.4	(111) 98.2	–[b]
Fluoroquinolones	Ciprofloxacin (≤ 15)	(5) 19.2	(6)12.2	(5)13.2	(16) 14.2	0.694445
Tetracyclines	Tetracycline (≤ 14)	(17)65.4	(30)61.2	(30)78.9	(77) 68.1	0.200399
	Doxycycline (≤ 12)	(11)38.5	(17)34.7	(6)15.8	(34) 30.0	0.078103
Macrolides	Erythromycin (≤13)	(21)80.8	(22)44.9	(18)47.4	(61) 53.9	0.007421[a]
Lincosamides	Clindamycin (≤ 14)	(6)23.1	(9)18.4	(5)13.2%	(20) 17.7	0.585898
Oxazolidinones	Linezolid (≤ 20)	(9)34.6	(5)10.2	(11)28.9	(25) 22.1	0.024448[a]
Phenicols	Chloramphenicol (≤ 12)	(5)19.2	(9)18.4	(6)15.8	(20) 17.7	0.926804
Glycopeptides	Vancomycin (≤ 21)	(1) 3.8	(0) 0.0	(0) 0.0	(1) 0.88	–[b]
Aminoglycosides	Gentamycin (≤ 12)	(6)23.1	(18)36.7	(5)13.2	(29) 25.7	0.0417[a]
Folate-Pathway Inhibitors	Sulphamethoxazol-trimethoprim (≤ 10)	(2)7.7	(6)12.2	0(0)	(8) 7.1	–[b]

*P-value: Chi-square value of the difference between the prevalence of antibiotics resistant in bovine mastitis and human sources groups. a: statistically significant at P≤0.05; b: Chi-square was not performed when there was zero number of samples; N: total number; n: number of samples; *CLSI: Clinical and Laboratory Standards Institute.

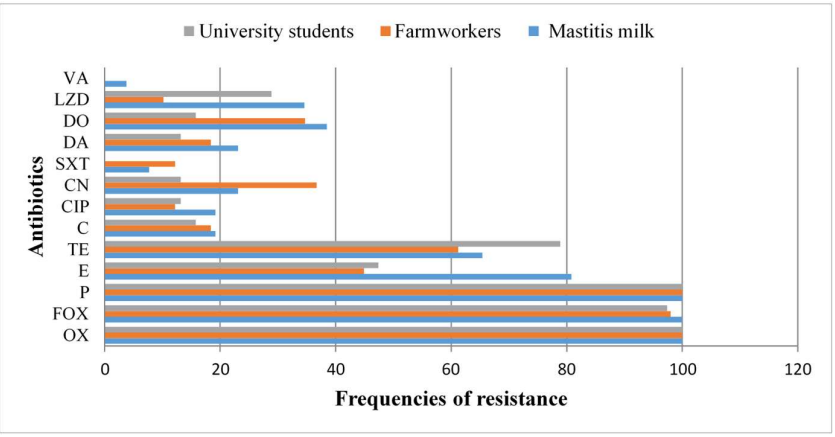

Fig. 1: Antimicrobial resistance pattern of MRSA isolates from bovine mastitis and human sources. Oxacillin (OX); cefoxitin (FOX,); penicillin G (P); erythromycin (E); tetracycline (TE); chloramphenicol (C); ciprofloxacin (CIP); gentamycin (CN); sulfamethoxazole-trimethoprim (STX); clindamycin (DA); doxycycline (DO); linezolid (LZD); and vancomycin (VA).

Minimum inhibitory concentration assay (MIC)

E-test method for linezolid antibiotic was carried out with all isolates of MRSA; the obtained results, as described in Table 3, were compared with the CLSI criteria. According to this test, the MRSA isolates were reported to be 25.7% resistant and 76.9% susceptible.

Detection of the cfr gene using PCR

Among the MRSA-resistant strains, the cfr gene was detected only in one milk mastitis isolate, as shown in Fig. 2.

Detection of linezolid resistance mutations by PCR-RFLP analysis and HRM-PCR

After screening for a G2576T point mutation by PCR-RFLP with NheI restriction enzyme in all tested isolates, no G2576T mutation was detected in any of these isolates. Also, all MRSA isolates were examined using real-time HRM in order to detect the presence of a G2576T mutation and compare it with the sequencing technique. The findings for HRM were analyzed according to their melting pattern using a screen Clust software from Qiagen, where a G2576T mutation was not detected.

Sequencing of the domain V region of the 23S rRNA gene

After sequencing the PCR products on both strands for cfr, 23S rRNA, rplC, rplD, and rplV genes, and comparing the linezolid-resistant MRSA isolates with linezolid-susceptible MRSA isolates. We found that the linezolid-resistant isolates included more than one mutation in 23S rRNA (Fig. 3), rplC (Fig. 4), rplD (Fig. 5), and rplV (Fig. 6) genes are summarized in Table 4 while only one isolate from mastitis milk carried the cfr gene (Fig. 2).

Table 3: Results of E-test method for linezolid antibiotic (% resistant) against 113 MRSA isolates, CLSI (2017)

Mastitis milk (n= 26)	Human Sources N= 87		Total N=113	P-value (X^2)*	MIC interpretive categories (µg/ml) according to CLSI	
	Farm Workers (n= 49)	University Students (n= 38)			S	R
11(42.3%)	5 (10.2%)	10 (26.3%)	26 (25.7%)	0.005987^a	≤ 4	≥ 8

*P-value: Chi-square for the prevalence of LZD-resistance in 3 tested groups; a: Statistically significant at P≤0.05; R: resistant; S: susceptible; CLSI: Clinical and Laboratory Standards Institute; MIC: Minimum inhibitory concentration; N: Total number; n: number of samples.

Table 4: Sequencing results of the 23S rRNA gene mutations, ribosomal protein mutations and the acquisition of cfr gene

23S rRNA gene mutations	Ribosomal Protein Mutations from LZD-resistant MRSA isolates			cfr
	L3	L4	L22	
T42C (3/25)	Asn 93 Lys (3/26)	Met 121 Leu (1/24)	Ala 29 Val (3/24)	
A151T (4/25)	Asn 109 Lys (4/26)			
A169C (7/25)	Met 189 Arg (1/26)			
G190C (3/25)	Lys 221 Asn (1/26)	Arg 123 Cys (1/24)	Ala 30 Gly (1/24)	
T205C (1/25)	Lys 222 Arg (1/26)			+ (1/26) *This isolate was the only one that carried on the plasmid.
G230A (1/25)	Phe 126 Leu (1/26)			
G246C (7/25)	Thr 206 Ala (1/26)	Ala 134 Val (1/24)	Ala 42 Thr (1/24)	
A267C (3/25)	Phe 110 Leu (1/26)			
A371C (1/25)	Lys 232 Asn (1/26)			
G376A (3/25)				

*It should be noted that we did not find any mutation in all resistant isolates; in other words; mutations were only found in one isolate or at most seven isolates of the total (fully resistance to LZD: MIC $> 256\mu g/ml$); *According to 23S rRNA gene sequencing results, only one isolate from mastitis milk source contains one mutation while most of the mutations were detected in university students isolates; *Concerning L3 protein, most of the mutations were detected in mastitis isolates; * Concerning L4 & L22 proteins, all the mutations were detected in university students isolates; * Alanine (Ala); Arginine (Arg); Asparagine (Asn); Cysteine (Cys); Glycine (Gly); Leucine (Leu); Lysine (Lys); Methionine (Met); Phenylalanine (Phe); Threonine (Thr); and Valine (Val).

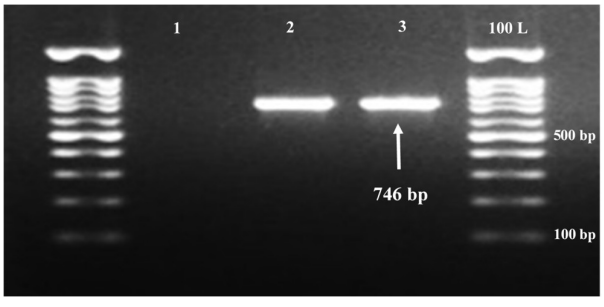

Fig. 2: Detection of cfr gene form linezolid-resistant MRSA isolate using (1.5%) agarose gel electrophoresis. Lane L: DNA ladder (100-1500) bp; Lane 1: negative control; Lane 2: positive control; Lane 3: cfr gene positive from mastitis milk isolate (746) bp.

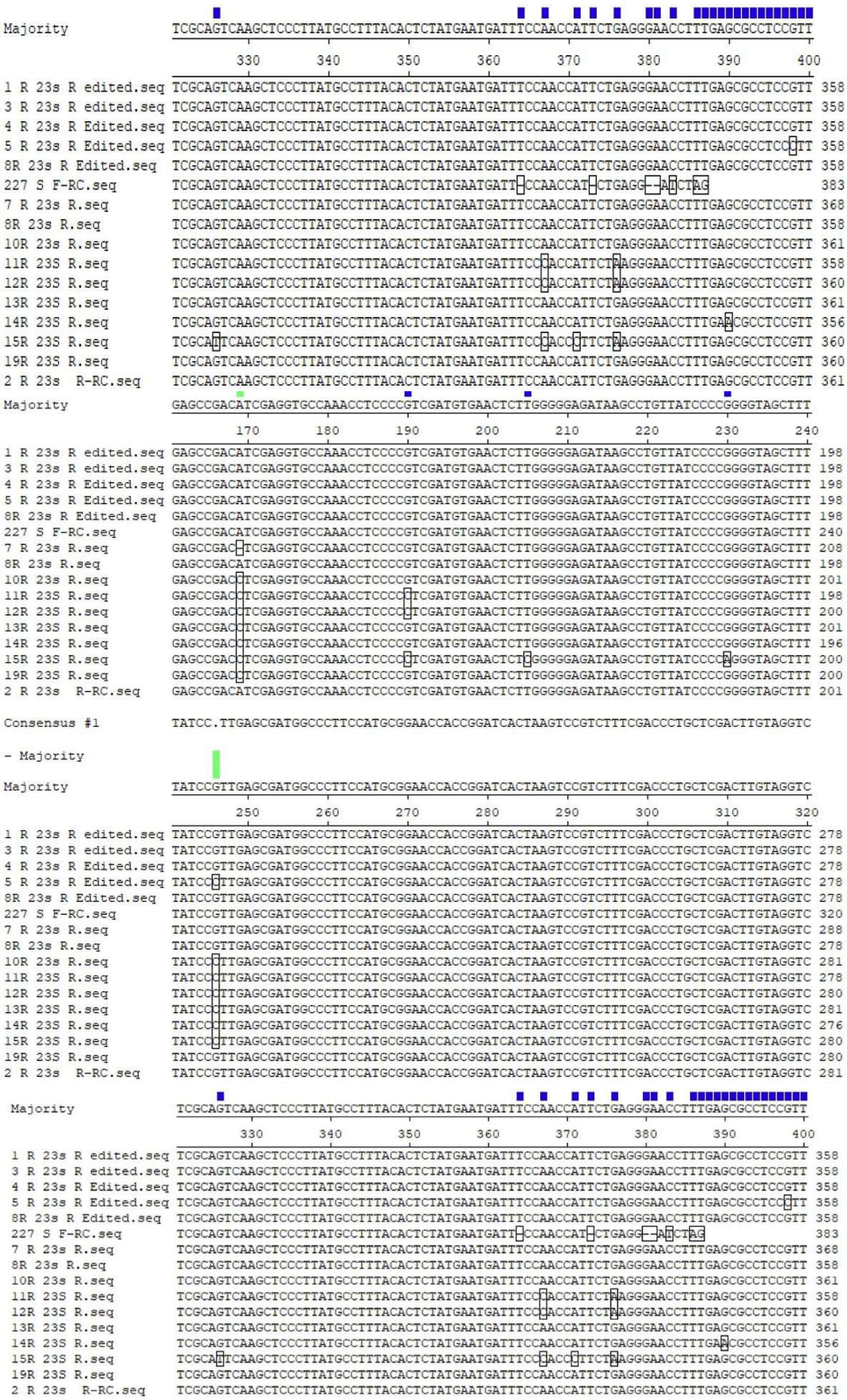

Fig. 3: Alignment of the 23S rRNA nucleotides from resistant and susceptible isolates of MRSA.

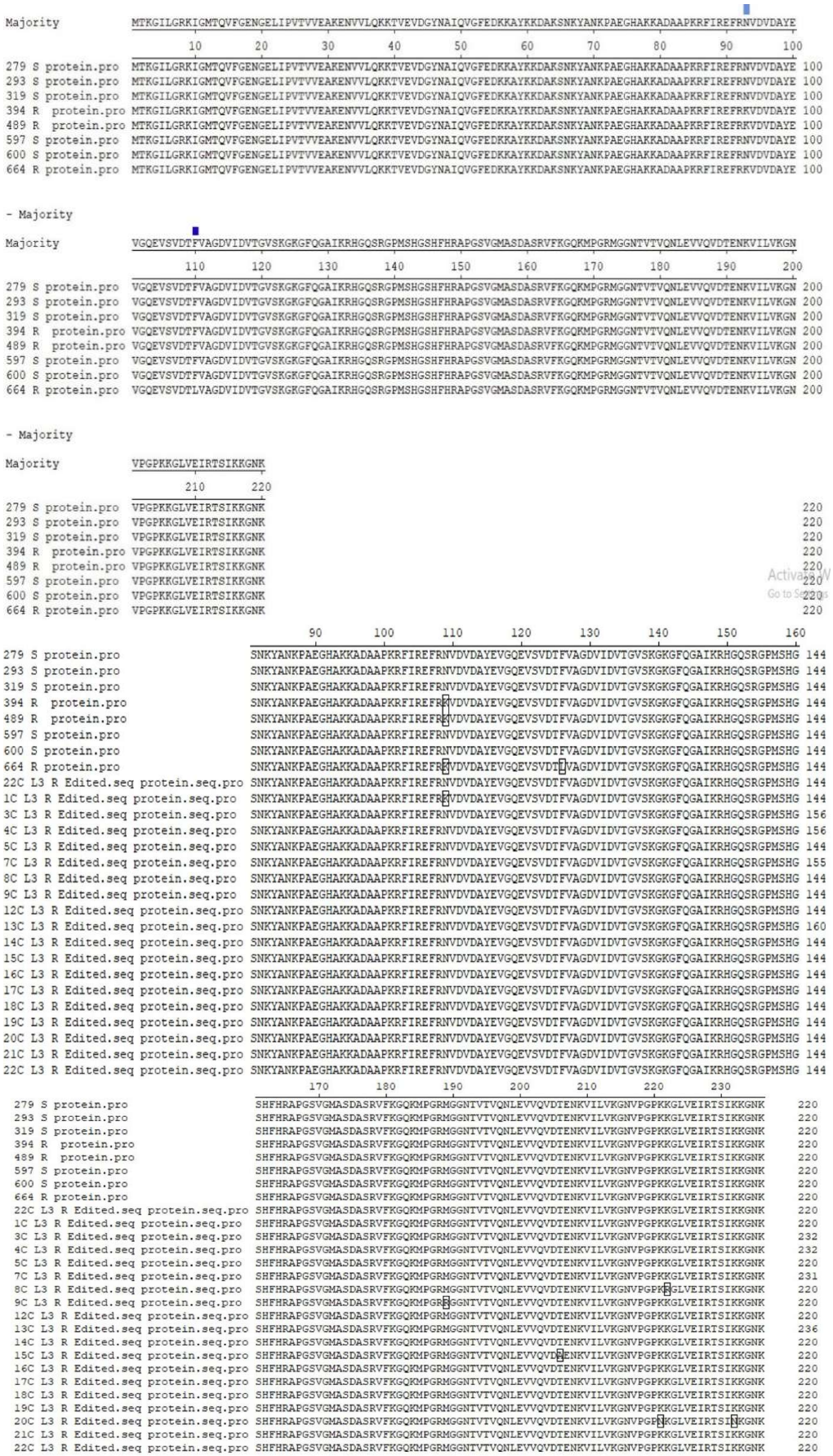

Fig. 4: Alignment of the L3 protein from resistant and susceptible isolates of MRSA.

Fig. 5: Alignment of the L4 protein from resistant and susceptible isolates of MRSA

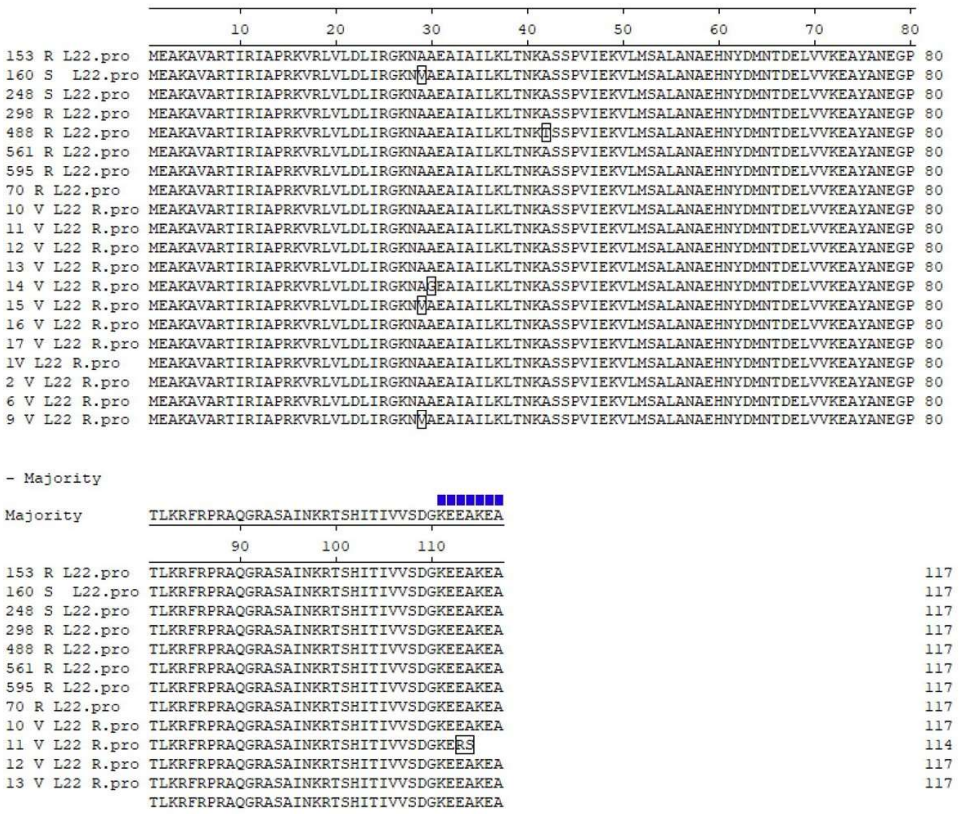

Fig. 6: Alignment of the L22 protein from resistant and susceptible isolates of MRSA.

DISCUSSION

Multidrug-resistant S. *aureus,* including MRSA is one of the major threats worldwide since MRSA shows consistently developing resistance amid recent antibiotics such as LZD which is an important drug used to treat MRSA infections (Abreu et al. 2019). Resistance to LZD is actually very slow due to two reasons: the first reason is *S. aureus* strains have multiple copies (5-6 copies) of 23S rRNA gene (Yoo et al. 2020). Thus, the number of mutated copies of rRNA gene is based on the dosage of LZD and also the prolonged exposure to this antibiotic (Yoo et al. 2020). The second reason is the catalytic site of the 50S ribosome that known as peptidyl transferase center (PTC) which is very conserved region because the occurrence of the mutations depends on altering the far nucleotides (Prosdocimi et al. 2020). In general, the rRNA mutations depending on the type of mutation and the number of mutated alleles. As we mentioned before, the development of resistance to LZD attributed to three mechanisms: mutations in the domain V region of the 23S rRNA gene, acquisition of the cfr gene (Wali et al. 2022) and mutations in 50S ribosomal proteins L3, L4, and L22 encoded by rplC, rplD, rplV genes respectively (Wu et al. 2019; Yoo et al. 2020). Multiple studies have indicated the prevalence of LZD-resistant MRSA around the world. In India, the reported LZD resistance level in MRSA was about 5.7% in 2001 (Tsiodras et al. 2001). In 2012, a study from India reported that resistance to LZD in MRSA was 58.33% (Thool et al. 2012). Meanwhile, previous studies in Jordan revealed that there were no MRSA isolates that are resistant to LZD, and these studies are similar to many studies in other countries (Al-Tamimi et al. 2018; Obaidat et al. 2018). In our research, we examined the prevalence of LZD resistance in 113 MRSA strains isolated from humans and bovine mastitis in Jordan and that is by detecting mutations using phenotypic and genotypic techniques. In this research, the antibiotic susceptibility test results showed widespread resistance among all isolates from three different sources against at least two of the tested antibiotics (Table 2). These findings are in congruence with previously published results (Alekish et al. 2020; Al-Salihi et al. 2023). Remarkably, all MRSA isolates from three different sources showed an almost complete rate of resistance to penicillin (P), oxacillin (OX) and cefoxitin (FOX), and this is in line with other studies in Jordan (Al-Tamimi et al. 2018; Obaidat et al. 2018). In our study, the results of antibiotic resistance percentages of MRSA isolates in bovine mastitis, farmworkers, and university students were compared. Phenotypically, MRSA isolates in 3 groups exhibited resistance to many non-β-lactam antibiotics, including ciprofloxacin, tetracycline, clindamycin, erythromycin and sulphamethoxazole-trimethoprim. This is described by several authors (Abreu et al. 2019; Iramiot et al. 2020). To be noted, penicillin, oxacillin, and cefoxitin were used in order to confirm that our isolates are MRSA (phenotypically resistant). As we mentioned before, all MRSA isolates in 3 groups were almost completely resistant to these 3 antibiotics (not statistically significant). In addition, clear differences in the antibiotics resistance percentages between MRSA isolates in bovine mastitis, farmworkers, and university students was observed for ciprofloxacin (19.2%, 12.2%, and 13.2%, respectively), doxycycline (38.5%, 34.7%, and 15.8%, respectively), clindamycin (23.1%, 18.4%, and 13.2%, respectively), chloramphenicol (19.2%, 18.4%, and 15.8%, respectively), sulfamethoxazole-trimethoprim (7.7%, 12.2%, and 0%, respectively), and tetracycline (65.4, 61.2, and 78.9%, respectively) (although not significantly). Otherwise, our results showed significant differences in antibiotic resistance among isolates from bovine mastitis, farmworkers, and university students. The resistance rates to erythromycin were 80.8%, 44.9%, and 47.4%, respectively (P=0.007421). For gentamycin, the resistance rates were 23.1%, 36.7%, and 13.2% (P=0.0417), while for linezolid they were 34.6%, 10.2%, and 28.9% (P=0.024448). As all P-values were ≤ 0.05, these findings indicate statistically significant variations in resistance percentages among the three groups. The observations indicated that the erythromycin resistance rate was significantly higher in mastitis milk isolates than in human isolates, while the gentamicin resistance rate was significantly higher in farmworkers isolates than in isolates from mastitis milk and students. Surprisingly, the LZD resistance percentage in farmworkers was significantly lower than isolates from both mastitis milk and students. As previously discussed, in some cases, the use of antibiotics such as gentamicin, erythromycin, and others either in the treatment of humans or animals may result in the development of resistance to them (Iramiot et al. 2020). According to our results, as is evident, the rates of resistance towards antibiotics in bovine isolates are the highest (10 antibiotics out of 13) comparing with human sources isolates, as expected and this is due to the excessive and uncontrolled use of antibiotics in bovine (Alekish et al. 2013; Eidaroos et al. 2025) while farmworkers isolates showed higher resistance rates towards 6 antibiotics than students' isolates and this is logical due to the close contact with infected cows that may result in the transmission of multi-drug resistance MRSA from cows to humans (Alekish et al. 2020; Titouche et al. 2024). Concerning E-test results, 26 MRSA isolates (25.7%) showed a relatively low percentage of resistance towards LZD, which was identified from 113 MRSA isolates, including 11 from milk mastitis isolates, 10 from healthy volunteer students, and the remaining 5 from farmworkers (Table 3). This result is comparable to the previous study in Jordan (20.9%) (Obaidat et al. 2018), but lower than other previous studies: 85.7% (Sarma and Ahmed 2010) from India. In general, elevated rates of linezolid resistance (Suzuki et al. 2023) and other antibiotics in MRSA isolates are either due to antimicrobial misuse and overuse or as a result of the transmission of multidrug-resistant MRSA strains from animal-to-human (zoonotic) or from person-to-person, which is an alarming situation for both human and animal health. Due to the significance of the antibiotic susceptibility test results, it has been decided to obtain genotypic support for our findings. The present research describes the appearance of mutations in the 23S rRNA, rplC, rplD, and rplV genes and also the acquisition of cfr gene in all MRSA isolates, using genotypic techniques. Unfortunately, very few studies have been performed worldwide to examine the presence of LZD mutations among cow mastitis, and CA-MRSA. Also, there are still no studies in Jordan, which make the comparison of the various studies findings very difficult. As previously

described, the presence of the cfr, rplC, rplD, and rplV genes in the MRSA isolates was determined using PCR. Based on molecular techniques results, none of the bovine and human MRSA isolates contains the most common point mutation (G2576T) in the domain V of the 23S rRNA gene which means there is a 100% correlation between molecular techniques (RFLP- PCR, HRM, and sequencing). Our resistance profile findings showed that cfr-positive MRSA isolate was resistant to all tested antibiotics (Table 2) except erythromycin because macrolides (erythromycin) bind near the PTC region in the peptide exit tunnel, and this means they do not bind directly to A2503 of 23S rRNA, so erythromycin is not affected by the cfr-mediated methylation of A2503 nucleotide (Long et al. 2006). Our results also showed a high incidence of multidrug resistance in cfr-positive MRSA isolates compared with other cfr-negative MRSA isolates, which is in agreement with the resistance profile of these isolates and similar to other observations in China (Li and Webster 2018). In addition, we found that the MIC of LZD (MIC> 256mcg/mL) in cfr-positive MRSA isolates was markedly higher than in cfr-negative MRSA isolates. This result is in alignment with other recent studies done by Ruiz-Ripa et al. (2020). Besides, previous studies reported that there may be a correlation between the cfr gene and other LZD mutations (Baos et al. 2013; Ruiz-Ripa et al. 2020), but our data show no detection of any of the LZD mutations in the cfr-positive MRSA isolate. In this study, sequencing analysis revealed the amino acid changes in the ribosomal proteins L3, L4, and L22 in addition to the appearance of various mutations in the 23S rRNA gene in our tested isolates (Table 4). To the best of our knowledge, no prior research has been conducted regarding these mutations in the ribosomal proteins and in the 23S rRNA gene among MRSA isolates. Thus, our current study was innovative in being the first study that revealed novel mutations in ribosomal proteins and the 23S rRNA gene in MRSA isolates from bovine mastitis and human sources. It is worth highlighting the results obtained in this study for bovine mastitis and CA-MRSA because most of the previous studies on linezolid resistance are focused on swine and HA-MRSA.

Conclusion

This study demonstrated the presence of MRSA, an important livestock, community, and hospital-associated pathogen. Our tested MRSA isolates were recovered from human sources and bovine mastitis cases, suggesting that it has the ability to spread from animals to humans.

The MRSA isolates in our research were phenotypically multi-resistant. Our findings also reflect very low resistance toward LZD (10.2%), which was detected by the phenotypic and genotypic techniques. Thus, it can be concluded that the presence of the cfr gene and novel mutations in L3, L4, and L22 ribosomal proteins and the 23S rRNA gene played an important role in LZD resistance. Consequently, canny use of LZD should be undertaken to reduce the development and spread of resistance among MRSA.

DECLARATIONS

Funding: The study was funded by the Deanship of Research at Jordan University of Science and Technology under project #20190500.

Acknowledgement: The authors express a deep thanks to the Deanship of Research at Jordan University of Science and Technology for its financial support of this work.

Conflict of Interest: The authors declare that they have no conflict of interest.

Data Availability: All the data generated during study are presented in the article.

Ethics Statement: The research did not directly include any human participants or animals and involved only in vitro experiments. Ethical approval was not required for this study.

Author's Contribution: NAI and YT did the planning and design of the study. They also oversaw sample collection, managed laboratory operations, and conducted formal data analysis. The manuscript was written by NAI, and it was revised with assistance from YT and MA. The final version of the manuscript has been reviewed and approved by all authors.

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