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ORIGINAL ARTICLE

The Multi-drug Resistance *cfr* Gene: an Evolving Mechanism for Linezolid Resistance in *Staphylococcus aureus*

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ABSTRACT

Background: *The emergence of linezolid resistance conveyed by the cfr (chloramphenicol-florfenicol resistance) gene in Staphylococcus aureus possess an utmost importance attributable to its ease of spread.* ***Objectives:*** *This study was undertaken for (1) evaluation of the susceptibility of S. aureus clinical isolates from Mansoura University Hospital (MUH), Mansoura, Egypt to linezolid, (2) detection of the risk factors associated with the emergence of linezolid-resistant S. aureus (LRSA) isolates, and (3) detection of the cfr gene encoding for linezolid resistance.* ***Methodology:*** *During the study period, clinical samples were collected from patients admitted to MUH. Antimicrobial susceptibility was assessed by the Kirby-Bauer's disc diffusion method. The minimum inhibitory concentrations (MICs) of linezolid were determined by agar dilution (AD) method. Polymerase chain reaction (PCR) was used for detection of the cfr gene encoding for linezolid resistance.* ***Results:*** *Out of 197 S. aureus isolates, an overall linezolid resistance of 8.1% was perceived by AD method. Among the risk factors associated with acquisition of LRSA, only hospital stay > 2 weeks retained significance in the logistic regression model (P = 0.002). The cfr gene was detected in 37.5% of LRSA isolates by PCR.* ***Conclusion:*** *The existent study disclosed that the presence of cfr gene is a leading mechanism conferring linezolid resistance in S. aureus. Thereby, prudent consumption of linezolid and ongoing surveillance of cfr-positive strains are crucial to prevent the dissemination of cfr-harboring strains in healthcare-settings.*

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a Gram-positive coccus that frequently occur in grape-like clusters.[1] Although 30% of healthy adults are colonized with this bacterium,[2] it is associated with many infections including skin and soft-tissue infections, infective endocarditis, pneumonia, meningitis and bloodstream infections.[3] Furthermore, toxic shock syndrome and food poisoning are examples of toxin-mediated diseases by *S. aureus*.[4]

Linezolid, the main representative of the oxazolidinone antibiotics, was approved in 2000.[5] It has a broad spectrum of activity against a variety of Gram-positive bacteria including methicillin-resistant *S. aureus* (MRSA).[6] This antibiotic inhibits protein synthesis by binding to the 23S ribosomal RNA (rRNA) of the 50S subunit of bacterial ribosomes.[7]

Even though this antibiotic is one of limited surrogate options for management of vancomycin-resistant MRSA, linezolid resistance developed a few years after its introduction owing to point mutation in the 23S rRNA gene or mutations in the L3 or L4 ribosomal proteins.[8] In addition, a non-mutational mechanism of resistance has been reported in bovine *Staphylococcus sciuri* isolate.[9] It involves acquisition of the chloramphenicol-florfenicol resistance (*cfr*) gene which also confers resistance to phenicols, lincosamides, oxazolidinones, pleromutilines as well as streptogramin A (PhLOPSA phenotype).[10] The *cfr* gene mediates linezolid resistance by coding for Cfr methyltransferase that catalyzes enzymatic methylation of adenosine in position 2503 of the 23S rRNA.[11]

Up till now, there is a shortage of literature from Egypt regarding the prevalence of LRSA isolates and their underlying genetic backgrounds. Therefore, this study was organized for (1) evaluation of the susceptibility of *S. aureus* clinical isolates from Mansoura University Hospital (MUH), Mansoura, Egypt to linezolid, (2) detection of the risk factors associated with the emergence of linezolid-resistant *S. aureus* (LRSA) isolates, and (3) detection of the *cfr* gene encoding for linezolid resistance.

METHODOLOGY

Study Design

This prospective cohort study was designed at MUH, Mansoura, Egypt in collaboration with the Department of Medical Microbiology and Immunology,

Faculty of Medicine, Mansoura University, Egypt. The study was conducted over a period of 12 months (January to December 2016).

Sample collection and processing

Clinical samples were collected from patients admitted to MUH. The samples were transported immediately to the Microbiology Diagnostics and Infection Control Unit (MDICU) at the Department of Medical Microbiology and Immunology for processing. All media used in the study were purchased from Oxoid (Oxoid Ltd., Basingstoke, UK). Identification of *S. aureus* isolates was done based on colony morphology, Gram staining characters, catalase, coagulase and DNAse tests.[12]

Antibiotic susceptibility testing

Antibiotic susceptibility was determined by the Kirby-Bauer's disc diffusion method and results were interpreted according to the published criteria of the Clinical and Laboratory Standards Institute (CLSI).[13] The antibiotic discs tested (Oxoid Ltd., Basingstoke, UK) included penicillin G (P; 10 Units), amoxicillin/clavulanic acid (AMC; 20/10 µg), cefadroxil (CFR; 30 µg), cefuroxime (CXM; 30 µg), gentamicin (CN; 10 µg), chloramphenicol (C; 30 µg), erythromycin (E; 15 µg), trimethoprim/ sulfamethoxazole (SXT; 1.25/23.75 µg), fusidic acid (FD; 10 µg), clindamycin (DA; 10 µg), ciprofloxacin (CIP; 10 µg), imipenem (IPM; 10 µg) and linezolid (LZD; 30 µg). The American Type Culture Collection *S. aureus* ATCC 25923 was included for quality control purposes.

Screening of *S. aureus* isolates for methicillin resistance

Methicillin resistance was detected by disc diffusion method using cefoxitin (FOX; 30 µg, Oxoid Ltd., Basingstoke, UK).[14] Briefly, Muller-Hinton agar (MHA) plates were inoculated with a suspension of the test *S. aureus* strains. A cefoxitin disc (30 µg) was placed and plates were incubated at 35ºC for 24 hours. Isolates with zone diameters of ≤ 21 mm were considered as MRSA, while those with zone diameters of ≥ 22 mm were considered to be sensitive.[13] *S. aureus* ATCC 25923 which is methicillin-susceptible was used for quality control.

Determination of the minimum inhibitory concentrations (MICs) of vancomycin and linezolid

The MICs of vancomycin and linezolid (Sigma-Aldrich, Italy) were determined by agar dilution (AD) method. As per the guidelines of the CLSI,[13] MHA plates which had concentrations of 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µg/ml of the aforementioned drugs were prepared. Few colonies of *S. aureus* isolates were picked with the help of a wire loop and emulsified in 0.9% normal saline in test tubes. The tubes were then incubated for 2 hours at 37°C.The turbidity of the suspensions were matched against the turbidity of 0.5 McFarland turbidity standard. These suspensions were spot inoculated on MHA plates which had different vancomycin and linezolid concentrations, in addition to a control plate without antimicrobial agent using a micropipette. The plates were incubated at 37°C for 24 hours. The MICs were interpreted as the lowest concentration of the agents that completely inhibited visible growth as judged by the naked eye. MIC values that inhibited 50% and 90% of the isolates were accepted as MIC_{50} and MIC_{90}, respectively. *S. aureus* ATCC 29213 was tested concurrently as a quality control strain in each run of MIC measurements.

Genetic detection of the *cfr* gene by polymerase chain reaction (PCR)

Genomic DNA was extracted from an overnight broth saturated cultures of *S. aureus* isolates using QIAGEN DNeasy Bacterial Genomic DNA Kit (Qiagen GmbH, Hilden, Germany). PCR amplifications of the *cfr* gene were performed using Taq PCR Master Mix (AM, Egypt) and the *cfr* oligonucleotide primers (AM, Egypt) described previously.[15] PCR conditions were as follows: denaturation for 2 minutes at 94°C, 30 cycles of denaturation for 10 seconds at 94°C, annealing for 30 seconds at 55°C, extension for 30 seconds at 72°C and a final extension for 7 minutes at 72°C. The amplified products were checked on agarose gel electrophoresis. Visualization of the gel after electrophoresis was done under the ultraviolet trans-illuminator (Fisher Scientific, Pittsburg, CA, USA). Gels were photographed (photo-documentation) using digital camera.

Ethical considerations

The protocol of this study was approved by our institutional review board (R/17.04.101). Written informed consents were obtained from all participants included in this study.

Statistical analyses

All statistical analyses were executed by IBM-SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were described in the form of numbers and percentages. The Chi-square (χ^2) test was used to define the statistical significance of the data. Univariate analyses were used to determine possible associations between various risk factors and LRSA infection. Multivariate logistic regression analysis was used to assess any association between dependent and independent variables. Adjusted odds ratios (OR) with 95% confidence intervals (CI) were determined. *P*-values < 0.05 were considered statistically-significant.

For linezolid susceptibility results, categorical agreement between disc diffusion and AD method was evaluated with the later considered to be the reference method.[13] Categorical agreement between both methods

was achieved when an isolate was classified within the same category (i.e., susceptible or resistant) by both testing methods based on the CLSI breakpoints.[13] Errors were ranked as major errors; disc diffusion are resistant and AD is susceptible, or very major errors; disc diffusion are susceptible and AD is resistant.[16]

RESULTS

Bacterial isolates

A total of 197 consecutive, non-duplicate (single isolate/patient) *S. aureus* isolates were identified. The maximum recovery of these isolates was achieved from wound swabs (41.6%). Sample-wise distribution of the isolates is illustrated in Table 1.

Table 1: Sample-wise distribution of the recovered *Staphylococcus aureus* isolates

Sample	Number	%
Wound swabs	82	41.6
Blood	54	27.4
Sputum	20	10.2
Throat swabs	17	8.6
Urine	12	6.1
ETA	9	4.6
Eye swabs	2	1
CSF	1	0.5
Total	**197**	**100**

Abbreviations: ETA; Endotracheal aspirate, and CSF; cerebrospinal fluid.

Antibiotic susceptibility patterns of the test isolates

Out of 197 *S. aureus* isolates, 179 (90.9%) were found to be linezolid-susceptible by disc diffusion method. Most of the investigated *S. aureus* isolates (83.8%) were also sensitive to imipenem. On the other hand, 48.2% and 47.2% of the test isolates were susceptible to chloramphenicol and clindamycin, respectively. Besides, 52.8% of the isolates were found to be MRSA. Most of these isolates (43.3%) were recovered from blood samples and wound swabs (35.6%). The antibiotic susceptibility profiles of the isolates to other antibiotics are depicted in Table 2.

Table 2: Antibiotic susceptibility profiles of the test *Staphylococcus aureus* isolates by disc diffusion method

Antibiotic	Susceptible isolates N	%
Linezolid (LZD; 30 µg)	179	90.9%
Imipenem (IPM; 10 µg)	165	83.8%
Fusidic acid (FD; 10 µg)	132	67%
Trimethoprim/sulfamethoxazole (SXT; 1.25/23.75 µg)	116	58.9%
Gentamicin (CN; 10 µg)	109	55.3%
Ciprofloxacin (CIP; 10 µg)	98	49.7%
Chloramphenicol (C; 30 µg)	95	48.2%
Cefoxitin (FOX; 30 µg)	93	47.2%
Clindamycin (DA; 10 µg)	93	47.2%
Cefuroxime (CXM; 30 µg)	90	45.7%
Erythromycin (E; 15 µg)	73	37.1%
Amoxicillin/clavulanic AMC (20/10 µg)	48	24.4%
Cefadroxil (CFR; 30 µg)	27	13.7%
Penicillin G (P; 10 Units)	4	2%

The MICs of vancomycin and linezolid by AD method

An overall vancomycin sensitivity of 85.3% (MIC_{50} and MIC_{90}; 1 and 8 µg/mL, respectively) was recognized among *S. aureus* isolates, with MICs ranged from 0.25 to 8 µg/mL. A total of 29 isolates (14.7%) expressed a resistant phenotype with MICs ranged between 16 to 32 µg/ml. The MICs of vancomycin for the test isolates are shown in Fig. 1. Most of VRSA isolates (51.7%) were retrieved from wound swabs. In addition, 31.1%, 13.8% and 3.4% were obtained from blood, sputum and urine samples, respectively.

By AD method, 91.9% of the isolates displayed susceptibility to linezolid with MIC_{50} and MIC_{90}; 1 and 4 µg/mL, respectively. A total of 16 isolates were found to be linezolid-resistant, with MICs ranged between 8 to 32 µg/ml. The MICs of linezolid for the test isolates are presented in Fig. 1. Categorical agreement between disc diffusion test and AD method was 99% as 2 *S. aureus* isolates showed a false resistant phenotype by disc diffusion (1% major error), but they were susceptible by AD. Notably, 50% of LRSA isolates were obtained from blood samples, whereas 31.2% and 18.8% were recovered from wound swabs and sputum samples, respectively. In addition, all of LRSA isolates were also resistant to methicillin, meanwhile 62.5% of them were vancomycin-resistant.

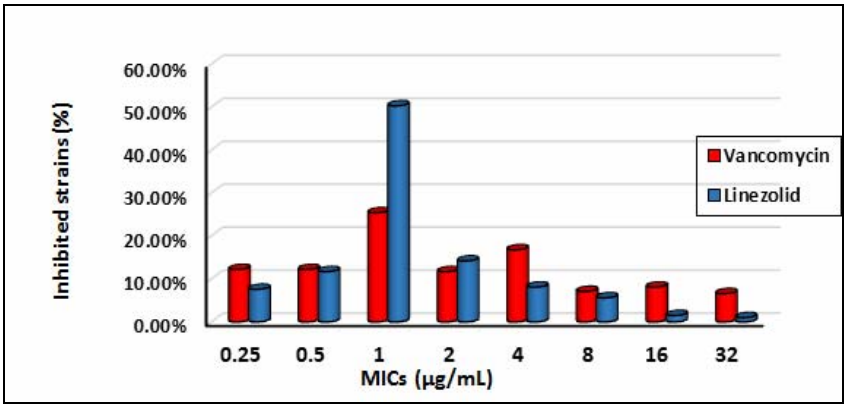

Fig. 1: Linezolid and vancomycin minimum inhibitory concentrations (MICs) for the test *Staphylococcus aureus* isolates by agar dilution method. Data are presented as the percentage of strains inhibited at each MIC (µg/mL).

Risk factors associated with acquisition of LRSA infection

Potential risk factors for acquisition of LRSA infection are enlisted in Table 3. Significant factors included hospitalization > 2 weeks (OR; 30.41, 95% CI; 4.61–37.32, *P* = 0.001), surgical intervention (OR; 4.65, 95% CI; 1.44–14.98, *P* = 0.005) and presence of wound drains (OR; 2.73, 95% CI; 0.97–7.68, *P* = 0.04). In the multivariate logistic regression analysis, only hospital stay > 2 weeks remained as an independent risk factor for LRSA infection (adjusted OR; 27.84, 95% CI; 4.11–33.41, *P* = 0.002).

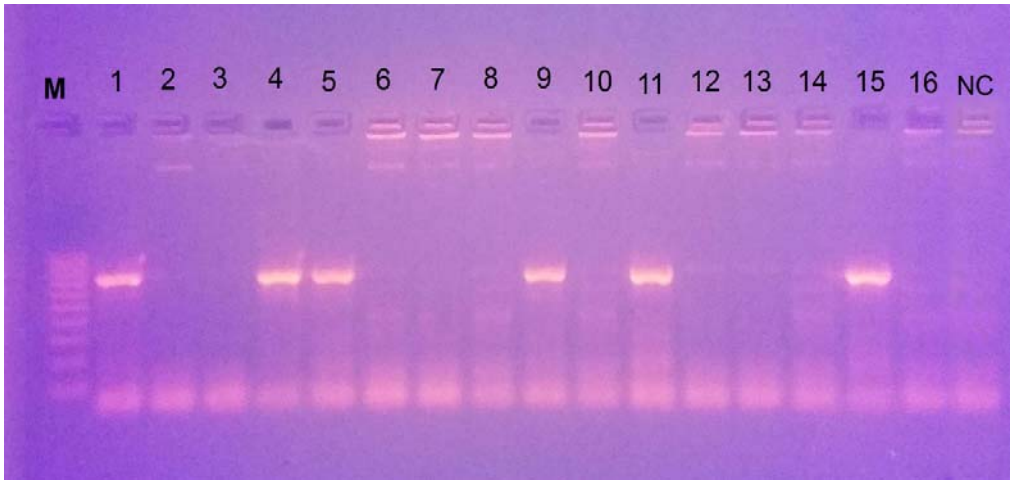

Fig. 2: PCR analysis of the *cfr* gene from linezolid-resistant *S. aureus* (LRSA) isolates. The amplicons (~746 bp) were separated by agarose gel electrophoresis.
M: DNA standard marker: Φ X 174-HaeIII digest marker with fragments ranging in size from 1353 bp to 72 bp.
Lanes 2, 3, 6, 7, 8, 10, 12, 13, 14 and 16: negative result for *cfr* gene.
Lanes 1, 4, 5, 9, 11 and 15: showing amplification of the *cfr* gene.
NC: negative control.

DISCUSSION

The advent of antibiotic-resistant *S. aureus*, particularly MRSA and VRSA isolates, has led to an increased clinical use of the most recently approved antimicrobials, such as linezolid. However, resistance to linezolid was first reported in the United States in an MRSA isolate that was retrieved from a patient treated with this agent, in 2001.[17] Since then, LRSA isolates have been progressively described in Europe as well as in the United States.[18]

In the current work, 9.1% of the test *S. aureus* isolates were resistant to linezolid according to disc diffusion test. Subsequently, MIC determination by AD method yielded that 8.1% of the isolates were linezolid-resistant, with MICs ranged between 8 to 32 µg/ml. In this context, Tian and co-workers from a study done in China quoted that their LRSA isolates had linezolid

MICs from 8 to 32 µg/ml, which is in a range similar to the finding of this work.[19] Nonetheless, lower values were extrapolated from the United States.[20] It seems that different techniques used for evaluation of the MIC might contribute to such discrepancy in results.

In support of our conclusion, Onelum and associates, from a study performed in Nigeria, confirmed that 8.8% of their *S. aureus* isolates were linezolid-resistant.[21] However, Ashour and El-Sharif, from a previous study conducted in Egypt, announced that 15.4% of *S. aureus* isolates recovered from cancer patients were linezolid-resistant which is considerably higher than ours.[22] This significant divergence in results could be traced to the study cohort. Cancer patients are immune-compromised, exposing them to many opportunistic infections. Therefore, prophylactic antibiotics are used routinely, including linezolid, hence promoting the development of resistant strains.

Outstandingly, a lower percentage of LRSA isolates was reported in Iran by Naghavi-Behzad and his group, amounting to 2.9%.[23] Even though, Khalid et al. from a study done in Pakistan, declared that all of their isolates were highly sensitive to linezolid with MICs ranged between 0.023 to 0.75 µg/ml as determined by Etest.[24] Such a substantial incongruity in results could be attributed to the regional differences in antibiotic use policies and infection control strategies.

Likely risk factors associated with acquisition of LRSA infection are poorly defined. In the present study, only prolonged hospital stay > 2 weeks was an independent predictor for infection with LRSA strains (adjusted OR; 27.84, 95% CI; 4.11–33.41, $P = 0.002$), proposing that the hospital setting has a fundamental role in spread of such superbugs. In accord with this finding, Russo and his colleagues observed that hospitalization in the previous 90 days was independent risk factor associated with isolation of LRSA strains.[25] Besides, they perceived that antibiotic therapy in the previous 30 days and antibiotic therapy > 14 days were associated with LRSA infection which contrasts our results (Table 3).

In the contemporary study, existence of the *cfr* gene was demonstrated in 6 out of the 197 *S. aureus* isolates (3%). Consistent with this finding, Kehrenberg and Schwarz identified the *cfr* gene in 3% of their Staphylococcal isolates.[15] Nevertheless, Zeng et al. reported up to 18.6% prevalence of the *cfr* gene in Staphylococcal isolates of animal origin.[26] This high rate could be ascribed to the extensive use of chloramphenicol in animal farms in China.

Unlike chromosomal mutations that infer slow spread of linezolid resistance, the discovery of the plasmid-borne *cfr* gene in staphylococci conveys an enormous peril for physicians. This gene is a part of an integral plasmid that is able of excision and mobilization, thereby, permitting rapid dissemination of linezolid resistance among different bacterial strains.[27]

Strikingly, none of the enrolled patients in the current study had prior linezolid exposure (which could select for the acquisition of the *cfr* gene). It is possible that the emergence of such gene might have been induced by the usage of florfenicol and chloramphenicol in veterinary industry in Egypt. Consequently, this gene may be transmitted from strains of animal origin to human-derived strains and horizontally transferred among dissimilar bacterial species. In favor of this assumption, a recent report described a clinical case caused by a *cfr*-positive livestock-associated (LA-) MRSA CC398 in Belgium.[28]

CONCLUSION

Existence of the *cfr* gene is an emerging mechanism for linezolid resistance in *S. aureus*. Thereby, stringent usage of antibiotics in humans and animals, in addition to timely detection of *cfr* gene are mandatory to prevent further spread of this gene owing to its plasmid location. The major limitation of this work is unexploring of the other genetic mechanisms conferring resistance to linezolid. So, future studies are recommended to decipher this issue.

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