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Brief Report

Ribosomal Proteins Mutations of *Staphylococcus haemolyticus* Causing Linezolid Resistance in Egyptian ICU Patients

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ABSTRACT

Linezolid resistance has since emerged gained a lot of attention worldwide as linezolid is considered one of the last resort antibiotics against multiple-drug resistant bacterial strains, yet in Egypt, little is known about the situation. As various linezolid resistance mechanisms have been identified in staphylococci, we investigated the molecular characteristics of staphylococci showing reduced susceptibility to Linezolid at Kasr-El-Eini Teaching Hospital. Thirty isolates were examined by disc diffusion method. Of the 30 isolates, two were confirmed as linezolid resistant and screened for the presence of mutations by sequencing the ribosomal proteins (L3, L4, and L22). Strain SZ-2 harbored a L3 M156T. H146P and V154L amino acids alternations, while in strain SZ-7, a L3 R138V, R144V, G152D and V154L mutations were detected. Novel mutations M105L and E191R in L4 protein were detected in strain SZ-2 and strain SZ-2, respectively. No isolates contained L22 protein alterations.

Keywords: Linezolid, Resistance, Ribosomal proteins, Mutations, *Staphylococcus haemolyticus*.

1. Introduction

Linezolid-resistant bacterial isolates have become a worrisome clinical problem as they pose themselves as a problematic pathogen in healthcare settings worldwide, especially in intensive care units (ICU). As their pathogenic significance increases, it becomes important to learn about the epidemiology and pathogenic potential of individual species (Long and Vester, 2012; Usha *et al.,* 2013). Management of such bacterial infections is thus challenging because of the associated risk factors and the multiple drug resistance, which narrows therapeutic options (Surekha *et al.,* 2011).

Linezolid is an oxazolidinone antibiotic potent against multidrug-resistant Gram-positive bacteria and is considered as one of the last resorts for humans against resistant bacteria (Yoo *et al.,* 2020; Nguyen *et al.,* 2020; Gostev *et al.,* 2021). Activity of linezolid results from binding to the 23S rRNA in the 50S ribosomal subunit. Thus, it acts as an inhibitor of protein synthesis by binding to the ribosomal peptidyl transferase center (PTC) affecting tRNA positioning and eventually stopping the growth of bacteria (Long and Vester, 2012; Sadowy, 2018).

S. haemolyticus, an emerging pathogen causing nosocomial infections, has a remarkable flexible genome characterized by the abundance of insertion sequences, and resistance to several antibiotics (Ahmed *et al.,* 2015; Hosseinkhani *et al.,* 2016). In the absence of appropriate diagnosis and management of infections, resistant strains of this pathogen can spread to other hospitals, and probably to the community (Hosseinkhani *et al.,* 2016).

Among the most frequently reported mutations associated with resistance to linezolid are the mutation in genes encoding the 50S ribosomal proteins L3, L4 and L22 of PTC of the ribosome (Kehrenberg *et al.,* 2005; Pfaller *et al.,* 2017). The molecular rationale for how alteration of such proteins cause resistance to linezolid has not been fully understood. Mutations of amino acid residues

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of these ribosomal proteins impact the PTC conformation and stability as they interact directly with the nascent peptide constriction site. Since the mechanism of linezolid inhibition is dependent on components of the nascent chain, it is crucial to understand the role of amino acid alterations in L3, L4 and L22 proteins causing resistance in bacterial isolates. Notably, mutations within L3, L4 and L22 have also been implicated in disrupting macrolide-dependent ribosome stalling (Halfon, 2019).

At Kasr-El-Eini Teaching Hospital, the escalating concern lies in staphylococcal isolates, which exhibit an extraordinary predisposition to develop resistance to all available antibiotics including linezolid. In pursuit of viable treatment alternatives, investigating the amino acid alterations in L3, L4 and L22 proteins as a possible mechanism associated with resistance to linezolid was undertaken.

2. Materials and Methods

2.1. Sample collection, identification and antimicrobial susceptibility testing of bacterial isolates

Over two years; from March 2020 to March 2022, a total of 30 clinical isolates of staphylococci showing reduced susceptibility to Linezolid were collected from Kasr El-Eini Teaching Hospital. They were isolated from different specimens, including pus and blood. Screening of isolates for linezolid resistance was done using the disc diffusion method. Linezolid concentration used was 30 µg. The antimicrobial discs were obtained from Oxoid Ltd (Basingstoke, Hampshire, England, UK) and processed according to the manufacturer's instructions.

The identification of isolates was confirmed using conventional techniques such as cultivation on mannitol salt agar (Kateete *et al.,* 2010) and deoxyribonuclease (DNase) agar (Boerlin *et al.,* 2003). The antibiotic susceptibility test of isolates was performed on Mueller Hinton agar using the Kirby Bauer disc diffusion method (Hudzicki., 2009) as per Clinical and Laboratory Standard Institute (CLSI, 2023) guidelines using antibiotic Linezolid 30 µg. Subsequently, identification of the two most resistant bacterial isolates was confirmed by 16S rRNA gene sequencing (White *et al.,* 1990).

2.2. Mechanisms of linezolid resistance among the isolates

The two most resistant *Staphylococcus haemolyticus* strains (namely SZ-2 and SZ-7) were selected and screened for the presence of mutations in rplC, rplD and rplV genes encoding for the ribosomal proteins L3, L4 and L22 , respectively, by PCR and DNA sequencing as described previously (Yoo *et al.,* 2020; Maarouf *et al.,* 2021; Han *et al.,* 2022; Zhou *et al.,* 2023). Amplification by PCR was done was done using primers listed in Table 1. PCR mixtures and PCR conditions were set as described previously (Yoo *et al.,* 2020). Amplicons were sequenced on both strands and aligned with the corresponding sequences from linezolid-susceptible *S. haemolyticus* JCSC 1435.

3. Results and Discussion

Twenty-four of the total 30 isolates were found to be totally susceptible to linezolid. The two most resistant isolates to linezolid from the collection were selected and identified to be S. haemolyticus isolates (named SZ-2 and SZ-7). Targeted genes were amplified using the primers and PCR conditions described in Table 1. All the amplicons were sequenced on both stands, and the sequences were BLASTx against the amino acid sequences of the corresponding genes from S. haemolyticus JCSC1435.

Table 1: Primers are used for PCR amplification of targeted genes.

Target genes	Primer name	Sequence (5′–3′)	Product size (bp)	PCR conditions	Reference
rplC	L3_rplC_F	AACCTGATTTAGTTCCGTCTA	822	94°C for 10 min 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for1 min	Lee *et al.* (2017)
	L3_rplC_R	GTTGACGCTTTAATGGGCTTA			
rplD	L4_rplD_F	TCGCTTACCTCCTTAATG	1200		
	L4_rplD_R	GGTGGAAACACTGTAACTG			
rplV	L22_rplV_F	CAACACGAAGTCCGATTGGA	350		
	L22_rplV_R	GCAGACGACAAGAAAACAAG			

Strain SZ-2, with a linezolid inhibition zone diameter (IZD) less than 1 mm (zone diameter breakpoint according to CLSI 2023 ≤ 20 mm), was found to have three (M156T, H146P and V154L)

mutations in L3 protein (Table 2). In L4 protein, one mutation (M105L) was detected. The isolate did not possess any mutation in L22 ribosomal protein. Similarly, strain SZ-7 with a linezolid IZD less than 1 mm exhibited some mutations in the ribosomal protein-encoding genes. In L3 protein, four (R138V, R144V, G152D and V154L) amino acid changes were detected, and only one mutation (E191R) in L4 protein while in L22 protein, no mutations were detected for this isolate.

Four of the L3 amino acid alterations found in this study (M156T, V154L, R138V and G152D) were reported previously as a main source of resistance to linezolid (Tewhey *et al.,* 2014; Yoo *et al.,* 2020; Nguyen *et al.,* 2020; Gostev *et al.,* 2021). L3 protein interacts closely with the PTC in the 50S subunit (Billal *et al.,* 2011) and has been identified as a key contributor to linezolid resistance. Our findings also align with (Tewhey *et al.,* 2014), who identified H146Q as a contributor to resistance in linezolid. Intriguingly, our study reveals a substitution of Histidine (H) with Proline (P) at the same position in *S. haemolyticus*, suggesting the potential resistance capacity of the strain. Furthermore, our study reveals a novel R144V mutation in L3 of strain SZ-2 that was not reported before. Although the impact on linezolid resistance is unclear and to conclusively establish the connection between this new amino acid alteration and heightened linezolid resistance, additional research is essential.

Table 2: Mutations detected in the ribosomal proteins of the resistant isolates *(S. haemolyticus strain SZ-2 and S. haemolyticus strain SZ-7)*.

Strain	ICU	Clinical sample	Mutations in L3 protein	Mutations in L4 protein	Mutations in L22 protein	MIC (µg/mm) Linezolid	Linezolid IZD (CLSI 020)
SZ-2	Yes	Blood	M156T, H146P, V154L	M105L	-	30 µg	< 1 mm
SZ-7	Yes	Blood	R138V, R144V, G152D, V154L	E191R	-	30 µg	< 1 mm

Among the main L3 mutations causing linezolid resistance in bacteria, G152D was extensively studied. The mutation G152D was previously reported to be involved in resistance to pleuromutilins (Gentry *et al.,* 2007) and more recently in clinical LRSA isolates (Endimiani *et al.,* 2011). Although the role of the L3 G152D mutation in linezolid resistance needs to be further studied, previous studies demonstrated that this mutation could be acquired without linezolid exposure (Yoo *et al.,* 2020). M156T, V154L, R138V and G152D mutations and other reported mutations in adjacent amino acids have been implicated in linezolid resistance owing to their proximity to the binding cleft of linezolid in L3 protein (Long and Vester, 2012).

Part of the ribosomal protein L4 is positioned in close proximity to the PTC within the tunnel through which nascent peptides exit the ribosome (Long and Vester, 2012). Since linezolid inhibits bacterial protein synthesis by binding to the ribosome's PTC, mutations in L4 can affect the drug's binding site or overall ribosomal function. This alterations This alterations could confer resistance to linezolid by reducing the drug's efficacy, allowing bacteria to evade its inhibitory effects and survive treatment (Ban *et al.,* 2000, Harms *et al.,* 2001; Gostev *et al.,* 2021). In L4 protein of both studied strains, no mutations detected in the highly conserved region (63KPWK/RQKGTGRAR74), which is usually involved in oxazolidinone resistance. However, our investigation uncovers the presence of M105L and E191R in L4 of SZ-2 and SZ-7, respectively, novel amino acid alterations not previously reported. While their impact on linezolid resistance remains uncertain, previous documented mutations in L4, involving alterations in nearby amino acids (Farrell *et al.,* 2004, Prunier *et al.,* 2005, Wolter *et al.,* 2005), warrant further exploration. To definitively establish the link between these novel amino acid alterations and increased linezolid resistance, additional studies are imperative.

Although mutations in the ribosomal proteins were reported in several staphylococcal species form many countries around the world such as Spain (Morales *et al.,* 2010), the USA (Bonilla *et al.,* 2010), Brazil (Gales *et al.,* 2006), Mexico (Mendes *et al.,* 2010), Japan (Ikeda-Dantsuji *et al.,* 2011) and Korea (An *et al.,* 2011), this is the first report of these mutations in *S. haemolyticus* isolated in Egypt. This first emergence of LRSH in intensive care units in our hospital during a short period is very worrying, requiring serious measures and proper surveillance in the healthcare settings to prevent the intrahospital dissemination of resistant strains. The novel H146P and R144V mutations in L3 protein and M105L and E191R in L4, whether they play a role in linezolid resistance is uncertain and needs

further confirmation. Nevertheless, this revelation may have the potential to contribute valuable insights into investigating the mechanism of linezolid resistance caused by mutations in the ribosomal proteins. IZD = Inhibation zone diameter; Mutations according to *Staphylococcus* numbering (BAE04111, BAE04112, BAE04116).

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The authors declare that there are no conflicts of interest.

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