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Linezolid resistant *Staphylococcus hominis* isolated in a paediatric Romanian hospital

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Abstract

In February 2013, one Staphylococcus hominis strain found linezolid-resistant by routine investigations was isolated from bloodstream of a patient hospitalized in the Paediatric/ Neonatal Intensive Care Unit (NICU) of the Clinical Emergency Children Hospital, Bucharest, Romania. The isolate was received at the Reference Laboratory for Nosocomial Infections and Antimicrobial Resistance within the "Cantacuzino" National Institute of Research, Bucharest, Romania, for linezolid-resistance confirmation and further investigation. The isolate was confirmed multidrug-resistant, being susceptible to trimethoprim-sulfamethoxazole, quinupristin-dalfopristin, vancomycin. Minimum inhibitory concentration (MIC) of linezolid by E-test was 192 mg/L. Molecular biology techniques revealed that this isolate harboured a cfr gene and, also, had a G2603T mutation (S. aureus numbering, GenBank accession no. X68425.1) in domain V region of 23S rRNA gene.As far as we know, this is the first report of a linezolid-resistant strain of staphylococci in Romania.

Keywords: *Staphylococcus hominis*,linezolid resistance, *cfr* gene, domain V region of 23S rRNA gene, G2603T mutation

1. Introduction

Gram-positive microorganisms are a predominant cause of serious infections across the world (MENICHETTI [1]). Currently, serious infections caused by both methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-resistant coagulase negative staphylococci (MR-CoNS), as well as vancomycin-resistant enterococci (VRE) and multidrug-resistant (MDR) Streptococcus pneumoniae are often treated with vancomycin or linezolid (SHINABARGER [2]). CoNS, which can be part of normal skin microbiota are commonly implicated in catheter infections, infectious endocarditis, prosthesis infections and osteoarticular infections, among others. Their treatment with linezolid, especially in Intensive Care Units (ICUs), has considerably increased in recent years. As with other families of antibiotics, it has been reported that the increase in their use is related to an increase in the detection of resistant isolates (KELLY & al. [3]).

Linezolid is a synthetic antibiotic [(S)-N-({3-[3-fluoro-4-(morpholin-4-yl) phenyl]-2-oxo-1,3-oxazolidin-5-yl}methyl)acetamide], assigned as the first member of oxazolidinone class of

antibiotics, discovered in the 1990s and approved for clinical use in 2000 by the U.S. Food and Drug Administration (FDA).

Linezolid can inhibit bacterial protein synthesis by binding to the 50S subunit of the bacterial ribosome via interaction with the domain V region of 23S rRNA of Gram-positive bacteria, thereby preventing formation of the N-formyl methionyl-tRNA-mRNA-70S initiation ribosomal tertiary complex. It stops the growth of bacteria by disrupting their production of proteins. The exerted effect is bacteriostatic, not bactericidal and linezolid differs from other protein synthesis inhibitors like chloramphenicol, macrolides, lincosamides and tetracyclines, which inhibit peptide elongation. Thus, linezolid activity benefit of the advantage that it can prevent the synthesis of staphylococcal and streptococcal proteins as concomitant effect with bacterial cell growth inhibition.

Its pharmacokinetic and pharmacodynamic properties have led to its increasing use for the main indications, i.e., nosocomial and community-acquired pneumonia, complicated skin and soft tissue infections, osteomyelitis (ANEZIOKORO & al. [4]), sepsis (PISTELLA & al. [5]) etc.

At the introduction of linezolid, it was claimed that the resistance would be rare and that it would be not prone to cause cross-resistance (ZHOU & al. [6]).

Initialy, it was believed that linezolid is not affected, as other protein synthesis inhibitors, by rRNAmethylases that modify 23S rRNA (LIVERMORE [7]).

However, the first alert with linezolid-resistant methicillin-resistant S. aureus (MRSA) was reported in 2001, in North America (TSIODRAS & al. [8]). After this year, linezolid-resistant staphylococci and enterococci have been increasingly reported (CHEN & al. [9]; GU & al. [10]; POTOSKI & al. [11]).

In the United States of America (U.S.A.), linezolid susceptibility of Gram-positive clinical isolates has been monitored and tracked since 2004 through a program named the Linezolid Experience and Accurate Determination of Resistance (LEADER). The results showed that the resistance has remained stable and extremely low (JONES & al. [12]). A similar surveillance network called the "Zyvox Annual Appraisal of Potency and Spectrum Study" or ZAAPS has been conducted in European medical centers (FLAMM & al. [13]).

Three resistance mechanisms to linezolid have been described until now:

a. Various mutations at the central loop of one or more alleles of the domain V region of 23S rRNA gene (DE ALMEIDA & al. [14]; LINCOPAN & al. [15]; SORLOZANO & al. [16]).

b. Methylation of RNA by two different enzymes - RlnM, with enhanced methyltransferase activity following a codon insertion in the methyltransferase gene rlmN in S. aureus (GAO & al. [17]) and 23S rRNAmethyltransferase produced as a result of chloramphenicol-florfenicol resistance (cfr) gene acquisition (CAMPANILE & al. [18]); 23S rRNAmethyltransferasemethylates the adenosine at position 2503 in 23S rRNA (E. coli 23S rRNA gene numbering).

The cfr gene confers resistance to five classes of antimicrobial agents, i.e., phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, a phenotype that has been termed PhLOPSA (LONG & al. [19]), it is plasmid borne or chromosomally located (KEHRENBERG & al. [20]). It can be horizontally transmitted between species and this mode of transmission is difficult to prevent and stop (KEHRENBERG & al. [20]). The cfr gene was originally identified in CoNS from animals (KEHRENBERG & SCHWARZ [21]; SCHWARZ & al. [22]). It has also been found in a very limited number of S. aureus and CoNS strains from humans (CAI & al. [23]; SHEN & al. [24]; RAJAN & al. [25]; CUI & al. [26]).

c. Mutations or deletions in genes encoding the 50S ribosomal subunit proteins L3 (DE ALMEIDA & al. [14]), L4 and L22 (encoded by the rplC, rplD and rplV genes) (LONG and VESTER [27]).

Little attention has been given to CoNS in research carried out in Romanian laboratories so far, though other authors reported the ability of these organisms to be involved in severe infections and to acquire linezolid resistance (MENDES & al. [28]). They represent a neglected reservoir of cfr-mediated resistance, frequently reported among patients in many regions, including North America (USA, Mexico), South America (Brazil), Europe (Greece, Spain, Italy, France, Ireland), and Asia (India) (GU & al. [10], POTOSKI & al. [11]). Such strains have rarely been reported in China (ZHOU & al. [6]; CHEN & al. [9]; YANG & al. [29]).

In several hospital wards, especially ICUs, the antibiotic pressure, due to both adequate and inadequate use of antibiotics and the difficulty in detecting certain resistance phenotypes led to a major increase in bacterial resistance rates worldwide and favored the development of multiple resistances in microorganisms such as Staphylococcus spp.

The first autochthonous strain of CoNS suspected to be linezolid resistant received at the Reference Laboratory for Nosocomial Infections and Antimicrobial Resistance for confirmation was the target of this study which aimed to elucidate the molecular basis of this resistance phenotype.

2. Materials and Methods

2.1. Clinical data

In January 2013 a male patient, 4 month of age was admitted in the Paediatric ward and then in the NICU of the Clinical Emergency Children Hospital, Bucharest, a 426-bed emergency paediatric hospital in Southeast Romania.

The patient suffered from bronchopneumonia, acute bronchiolitis, generalized tonic-clonic seizures and laryngeal stridor.

Complete Blood Count (CBC) showed that the patient has leukocytosis (22.60/ 10^3/ ul)andneutrophilia (82%).

Two types of samples were collected at admission for bacteriological investigation: tracheobronchial aspirate and stool. Sputum was found positive for *Stenotrophomonasmaltophilia* and stool for *Pseudomonas aeruginosa*.

Empirical therapy was instituted with a combination of antibiotics targeting both Gram-negative and positive bacteria. The patient received several succesive regimens of antibiotics, all including linezolid.

In February 2013, a Gram-positive coccus was isolated from a blood sample processed using the BacT/ ALERT 3D automated system (bioMérieux, Marcy-l'Etoile, France), subcultured on Columbia plate containing 7% sheep blood and identified as CoNS by colony morphology, Gram staining, catalase testing and coagulase assays. Antimicrobial susceptibility testing was performed in order to establish the optimum treatment. The results indicated that the isolated strain was multidrug-resistant, including linezolid. In March 2013, the patient developed a cardiac arrest and died.

2.2. CoNS linezolid resistance confirmation

Strain identification

In April 2013 the clinical CoNS isolate was sent to the Reference Laboratory for Nosocomial Infections and Antimicrobial Resistance within the "Cantacuzino" Institute, Bucharest, Romania, for linezolid resistance confirmation and further investigation. It was

cultivated on 7% sheep blood agar Columbia (Oxoid) and Mannitol Salt agar (Oxoid). Characteristic colonies were identified using an automated method, Vitek2 system (bioMérieux, France).

Phenotypic antimicrobial susceptibility testing

Kirby-Bauer disk diffusion method was performed following the EUCAST 2013 guidelines [30], using the following antibiotics: benzylpenicillin (P, 1 U), cefoxitin (FOX, 30 µg), erythromycin (E, 15 µg), clindamycin (DA, 2 µg), kanamycin (K, 30 µg), gentamicin (CN, 10 µg), tobramycin (TOB, 10 µg), ciprofloxacin (CIP, 5 µg), tetracycline (TE, 30 µg), rifampicin (RD, 5 µg), chloramphenicol (C, 30 µg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), quinupristin-dalfopristin (QD, 15 µg), linezolid (LZD, 10 µg). The MICs of linezolid and vancomycin were determined by E-test (AB Biodisk, Solna, Sweden). Resistance to linezolid was defined as an MIC > 4 mg/L. Sensitivity to vancomycin was defined as an MIC <= 4 mg/L.

Molecular detection of linezolid resistance

DNA extraction

Total genomic DNA was obtained by using NucleoSpin Tissue Macherey-Nagelkitaccording to manufacturer's instructions.

Polymerase chain reaction and sequencing of cfr and domain V region of 23S rRNAgenes

For *cfr* gene (746 bp) detection we used the primers described by Kehrenberg and Schwarz, 2006 ([21]). The specificity of amplification was verified by amplicon sequencing using the same primers.

To amplify the domain V region of 23S rRNA gene we used the primers described by Pillai et al., 2002 ([31]). Amplicon sequencing was used to identify potential mutations, that could explain the linezolid resistance. DNA sequence obtained for domain V of 23S rRNA gene was aligned with the corresponding nucleotide sequence from a linezolid susceptible *S. aureus* reference strain (GenBank accession number X68425.1) (BENSON & al. [32]) using BioEdit Sequence Alignment Editor.

Sequence of domain V region of 23S rRNA was checked by Sanger method using an Applied Biosystems DNA sequencing ABI PRISM 3130 Genetic Analyzer.

3. Results and Conclusions

The strain was identified as *Staphylococcus hominis* by Vitek2 system.

The isolate was susceptible to trimethoprim-sulfamethoxazole, quinupristin-dalfopristin, vancomycin (MIC value = 3 mg/L) (Figure 1) and resistant to benzylpenicillin, cefoxitin, erythromycin, clindamycin, kanamycin, gentamicin, tobramycin, ciprofloxacin, tetracycline, rifampicin, chloramphenicol, linezolid.

According to the MIC value the isolate displayed high-level resistance to linezolid (192 mg/L) (Figure 2). By sequencing and comparing the domain V region of the 23S rRNA gene sequence with the sequence of *S. aureus* 23S rRNA gene (GenBank accession no. X68425.1), a single nucleotide point mutation was detected in the 2603 position, substituting nucleotide G with T (Figure 4).

This mutation was found previously as being involved in linezolid resistance (ZHOU [6]; CHAMON & al. [33]).

Figure 1.Susceptibility test by E-test forvancomycin.

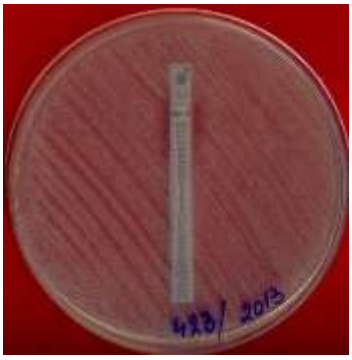

Figure 2. Susceptibility test by E-test for linezolid.

An amplicon of the expected size was obtained by PCR for *cfr* gene (Figure 3). The sequence obtained corresponded to *cfr* gene sequence deposited at the National Center for Biotechnology Information (NCBI) database (BENSON & al. [32]).

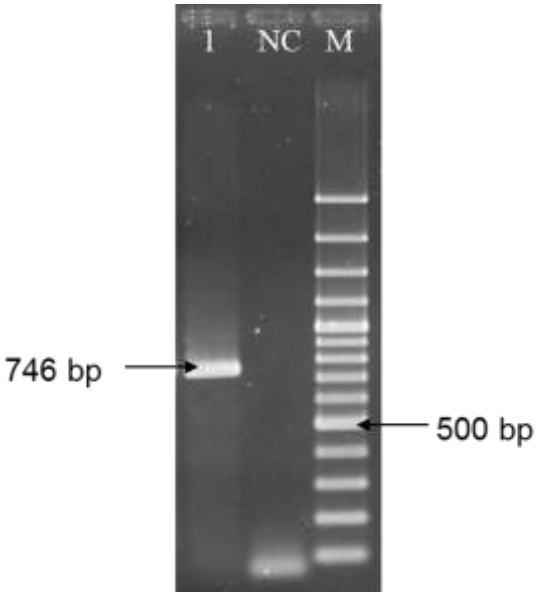

Figure 3.Agarose gel electrophoresis for *cfr* gene (original photo)
1 – *S. hominis* 423/ 22633 strain; NC – negative control (with H_2O); M – 100 bp DNA Ladder Promega.

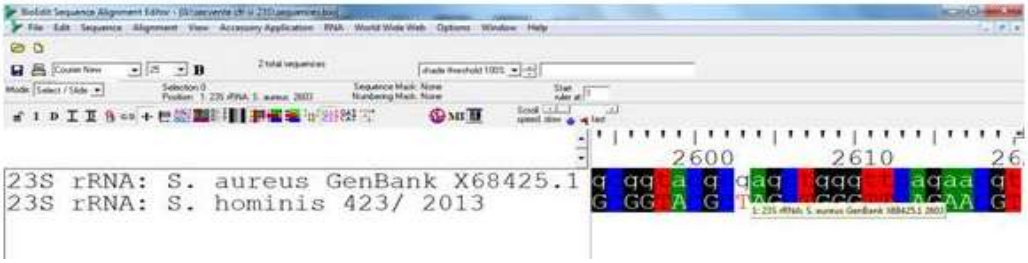

Figure 4.The single nucleotide point mutation in the 2603 position in the domain V region of 23S rRNA gene (BioEdit Sequence Alignment Editor).

The previous administration of linezolid is known to be associated with the development of resistance in CoNS. There are also reports of colonization or infection by linezolid-resistant CoNS in patients with no previous exposure to the antibiotic (POTOSKI & al. [11]) and of environmental contamination with methicillin-resistant staphylococci with reduced susceptibility to glycopeptides. Many studies show that tigecycline, as compared to vancomycin and linezolid, is safer and more effective in hospitalized patients with serious infections caused by MRSA (FLORESCU & al. [34]). Moreover, tigecycline resistance was rare in isolates causing clinically significant infections.

In Romania, reports on antimicrobials consumption indicated that some of antimicrobials reserved for hospital use, linezolid included can be purchased from community pharmacies [35]. This could represent a premise of eroding linezolid efficiency on Gram-positive cocci (enterococci, staphylococci), which is even more unwanted as linezolid is considered the ultimate solution for vancomycin resistant enterococci. In 2013, the incidence of *Enterococcus faecalis* strains with reduced susceptibility to linezolid was as high as 8.2%, which is the highest in Europe [POPESCU – unpublished data].

Additionally, linezolid was recently officialy included in the WHO 5th category of drugs recommended for the therapy of XDR *Mycobacterium tuberculosis*, which could have a significant impact in a country with high prevalence of tuberculosis (TB) like Romania (LANGE & al. [36]).

On the other hand, transmission of resistance genes and/or resistant strains have to be taken into consideration. Sorlozano et al., ([16]) firstly reported G2603T mutation in the domain V region of 23S rRNA gene of *S. hominis* strains isolated in 2010 from infections evolving in an ICU in Granada, Spain, but on our knowledge, no other reporting in Europe of this mutation in *S. hominis* was registered until now. However, many groups of researchers, from Brasil (LINCOPAN & al. [15]; CHAMON & al. [33]; CIDRAL & al. [37]) and the other from China (ZHOU & al. [6]) reported this mutation in *Staphylococcus epidermidis, Staphylococcus haemolyticus, S. hominis*and *Staphylococcus capitis*, respectively. One study reported an outbreak of linezolid-resistant *S. haemolyticus* strains in an Italian intensive care unit (MAZZARIOL & al. [38]). Yalcin& al. reported the first investigation of linezolid resistance in *S. epidermidis* ([39]) and a recent study from Mexico showed *S. epidermidis* and *S. haemolyticus* linezolid-resistant strains (MARTINEZ-MELENDEZ & al. [40]). Another study showed a nosocomial spread of linezolid-resistant *S. hominis* with G2576T mutation in domain V of 23S rRNA gene, in two hospitals from Majorca (RUIZ DE GOPEQUI & al. [41]).

In our case, the source remained unknown, despite of the isolation of this linezolid-resistant CoNS, because the hospital did not implement screening but for *Staphylococcus aureus*. Moreover, irrespective of the fact that we have no data to support the *S. hominis* strain involvement in sepsis, as it was isolated from a single bloodculture, previously mentioned propensity towards genetic transfer of resistance makes public health impact significant.

Nowadays, linezolid-resistant *Staphylococcus* is still sporadic. The prolonged hospital stay, especially in ICUs, frequent interventions, the adequate or inadequate use of antibioticsand the difficulty in detecting certain resistance phenotypes/ genotypes may accelerate the dissemination of linezolid resistant *Staphylococcus*.To preserve the therapeutic efficacy of this reserve antimicrobial for a longer period of time, rational use of linezolid and surveillance of resistance in staphylococci using the most rapid and sensitive methods are recommended.

This is the first case of linezolid-resistant bacteria received and confirmed by the Reference Laboratory for Nosocomial Infections and Antimicrobial Resistance in Romania. The rapid identification, the prevention of dissemination along with rational use of antibiotics are important bundles contributing together in preventing the selection of drug-resistant pathogens. As far as we know, this is the first report of a linezolid-resistant strain of staphylococci in Romania and the second report of G2603T mutation in the domain V region of 23S rRNA gene of *S. hominis* in Europe.

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Conflict of interest

None declared.

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