TRIPLEX REAL-TIME PCR FOR DETECTION OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS AND PANTON-VALENTINE LEUKOCIDIN IN NORTH-EAST ROMANIA

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TRIPLEX REAL-TIME PCR FOR DETECTION OF METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS AND PANTON-VALENTINE LEUKOCIDIN IN NORTH-EAST ROMANIA (Abstract): The aim of the present study was to investigate S. aureus isolates for the presence of methicillin-resistance and Panton-Valentine leukocidin (PVL) genes and to further characterize positive strains by means of antibiotic resistance patterns. Material and Methods: We used a triplex Real-Time PCR method for simultaneous detection of nuc, mecA and pvl genes in clinical isolates from 188 patients admitted to “Sf. Parascheva” Infectious Diseases Hospital Iaşi, during a 3 year period (2008-2010). Results The study revealed a relatively high rate of PVL-producing strains (23.93%), mainly community-associated (CA-MRSA) (51.11%). Most pvl-positive CA-MRSA isolates were resistant to erythromycin (91.3%), but none was resistant to clindamycin, fluoroquinolones, rifampicin, chloramphenicol or fusidic acid. Conclusions: Antibiotic susceptibility testing showed a high rate of multidrug-resistance among strains classified as CA-MRSA (54.83%), but not among PVL-producers (4.44%). Although resistance to fusidic acid was previously proposed as a marker for PVL-producing CA-MRSA, our data suggest that we cannot rely on resistance to fusidic acid to screen for PVL-producing CA-MRSA in our setting. Keywords: MRSA, MECA, PVL.

Staphylococcus aureus is an important hospital and community-acquired pathogen that causes a broad spectrum of diseases, for which the currently available treatments are at risk of becoming less effective, as this bacterium is able to rapidly acquire resistance to antibiotics. The mobile element SCCmec carries mecA gene encoding a low-affinity PBP2a that confers resistance to beta-lactam antibiotics (1). Methicillin-resistant S. aureus (MRSA) is a common agent of nosocomial infections (HA-MRSA) and is now increasingly implicated in infections among persons in the community without established risk factors for MRSA acquisition (CA-MRSA) (2). S. aureus pathogenicity is related to a wide variety of virulence factors that allow it to colonize, invade and evade the immune system. These factors include Panton-Valentine leukocidin (PVL), a pore-forming cytotoxin, encoded by lukF-PV and lukS-PV genes. The toxin targets polymorphonuclears and contributes to tissue necrosis. Infec-
tions caused by PVL-producing strains are characterized by an increased systemic inflammatory response, decreased survival in patients with community pneumonia and increased severity of localized lesions (3). PVL has been linked to skin and soft tissue infections (SSTIs), necrotizing pneumonia, bone and joint infections and was associated with CA-MRSA infections that affect young healthy individuals with no exposure to healthcare centers (4).

Our aim was to evaluate the prevalence of PVL-producing MRSA strains in “Sf. Parascheva” Infectious Diseases Hospital Iaşi, using a triplex RT-PCR technique that allows rapid and simultaneous detection of nuc, pvl and mecA genes. This study constitutes a preamble to the use of multiplex RT-PCR directly on clinical specimens. We also analyzed the phenotypic antimicrobial resistance profiles among CA-MRSA, HA-MRSA and methicillin-susceptible S. aureus (MSSA). Multi-drug resistance (MDR) among MRSA was defined as concurrent resistance to three or more non-beta-lactam antimicrobial agents.

MATERIAL AND METHODS
Clinical isolates. In total, 188 non-duplicate S. aureus isolates from “Sf. Parascheva” Infectious Diseases Hospital Iaşi were evaluated in the present study. The strains were isolated from pus (102 strains), blood-cultures (BC) (46 strains), cerebrospinal fluid (CSF) (6 strains), sputum (18 strains) or from multiple specimens (16 strains), from 2008 to 2010.

Triplex Real Time PCR. The isolates were analyzed for the presence of mecA, pvl and nuc genes using a triplex RT-PCR method previously described (5). Nucleic acid was extracted using GenElute Bacterial Genomic DNA kit (Sigma Aldrich, Germany). Primers and probes were synthesized based on oligonucleotide sequences previously described by McDonald et al. (6), and were used in concentrations of: 0.30 µM mecA primers, 0.1 µM mecA probes, 0.40 µM pvl primers, 0.1 µM pvl probes, 0.05 µM nuc primers, 0.05 µM nuc probes. Reactions were performed with Brilliant Multiplex QPCR Master Mix (Agilent Technologies, USA) under the following thermal cycling conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. Simultaneous amplification of target genes was detected with Stratagene MX3005P system (Agilent Technologies, USA). S. aureus ATCC 25923, 33592 and 49775 were used as controls of amplification.

Antibiotic susceptibility testing. Antimicrobial susceptibility patterns were evaluated using ATB STAPH 5 strips (bioMérieux, France) and disk diffusion method, as recommended by CLSI guidelines 2008-2010. We tested the sensitivity to the following antibiotics: penicillin (P), oxacillin (OX), cephoxitin (FOX), clindamycin (CM), erythromycin (E), tetracycline (TE), gentamicin (G), rifampicin (RIF), trimethoprim/sulfamethoxazole (SXT), fluoroquinolones (FQ), chloramphenicol (C), linezolid (LZD), teicoplanin (TEC) and fusidic acid (FUS). Inducible resistance to CM was assessed by double-disk (E and CM) diffusion method (D-zone test). Minimum inhibitory concentrations (MICs) for vancomycin (VA) and OX were determined using E-test method (bioMérieux, France).

RESULTS
We detected mecA gene in 86 isolates (45.74%), while phenotypic testing failed to detect methicillin-resistance in 5 cases.
Thus, the sensitivity of 1 µg oxacillin disk testing was 95.34% and the sensitivities of 30 µg cephoxitin disk testing and ATB STAPH 5 strips were 97.67%, compared to meca detection as gold standard. MICs for OX varied between 0.094-256 µg/ml, with a heteroresistance rate of 21.27% of the total number of strains. Of the total number of tested strains, 45 were pvl-positive (23.93%), representing 33.72% of MRSA isolates. Most pvl-positive strains were isolated from pus (82.22%), the rest being isolated from BC (6), CSF + BC (1) and CSF + BC + sputum (1). Clinical evolution for those patients was as follows: death (2 cases), aggravated (1), unchanged (6), improved (26) and healed (10). Out of all pvl-positive strains, 16 were MSSA, with 6 different resistance patterns, the most common being resistance to P (8 strains); one strain was susceptible to all antibiotics (tab. I).

### TABLE I
**Antibiotic resistance patterns for MSSA pvl-positive strains**

<table>
<thead>
<tr>
<th>Resistant strains</th>
<th>P</th>
<th>G</th>
<th>CM</th>
<th>E</th>
<th>TE</th>
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<tr>
<td>8</td>
<td>+</td>
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<tr>
<td>3</td>
<td>+</td>
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<tr>
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</table>

Among PVL-producing MRSA (29 strains), 7 resistance profiles were described, the most common being single-drug resistance to E (22 strains); of those strains, two were MDR (tab. II). None of the pvl-positive strains showed inducible CM resistance and all of them were susceptible to C, LZD, TEC, VA and FUS. Among PVL-non producing strains, 44 different resistance profiles were detected, most of them being MSSA resistant to P (27.97%) and MRSA multidrug-resistant to E, CM and TE (11.18%).

### TABLE II
**Antibiotic resistance patterns for MRSA pvl-positive strains**

<table>
<thead>
<tr>
<th>Resistant strains</th>
<th>G</th>
<th>CM</th>
<th>E</th>
<th>TE</th>
<th>FQ</th>
<th>SXT</th>
<th>RIF</th>
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Community-onset infections were defined as those episodes with a positive culture within 48h of admission, without health care-associated risk factors (history of hospitalization in the past year, surgery, renal dialysis, residence in a long-term care facility, presence of percutaneous medical devices or positive MRSA culture within the preceding 1 year). Based on this definition, 32.97% of the total number of isolates and 72.09% of MRSA strains were considered CA-MRSA; out of those, 37.09% were PVL-producing strains. A comparison of antibiotic resistance between CA-MRSA and HA-MRSA strains reveals a higher resistance rate for TE, G, RIF and FQ in the case of HA-MRSA isolates, while resistance rates to SXT, C and FUS were similar (fig. 1).

Out of 86 MRSA strains, 54 were MDR, the majority being classified as CA-MRSA (34/54), with the most frequent resistance profile: E, CM and TE (10 strains). Most
MDR strains classified as HA-MRSA were additionally resistant to G, RIF and FQ (9 strains). Only 2 pvl-positive strains were MDR, representing 3.7% of MDR strains.

**DISCUSSION**

MRSA rate determined by this study (45.74%) is concordant with previously published data by Dorneanu et al (47%) (7) and with reports from EARS-Net, placing Romania on one of the first places on the incidence of invasive MRSA infections (39.1%) (8). Correct identification of methicillin-resistance is needed for effective monitoring of MRSA spread. Since conventional methods of antibiotic susceptibility testing sometimes fail to detect hetero-resistant strains, molecular techniques were developed as standard tests for MRSA confirmation. These techniques are not accessible for routine testing in clinical laboratories, so evaluation of sensitivities for most frequently used phenotypic methods is imposed. Our data regarding MRSA detection were similar to those reported by Anand et al. (9) and Pottumarthya et al. (10), showing a sensitivity of 30 µg cephoxitin disk testing of 100% and 99.0% respectively, while a weaker concordance was observed when 1 µg oxacillin disk was used (sensitivity 87.5% and 94.7% respectively).

The emergence and community dissemination of new and virulent MRSA strains harboring PVL constitutes a public health concern (11, 12). Some authors anticipate the replacement of HA-MRSA strains by community strains infiltrating the hospital environment due to their colonization properties (13). The majority of CA-MRSA causes SSTIs, but certain cases can progress to invasive tissue infections and bacteremia (14). Usually, these strains are sensitive to most non-beta-lactam antibiotics and have a low level of methicillin-resistance, often expressed heterogeneously. Our study found an oxacillin heteroresistance rate in CA-MRSA of 45.16% and a prevalence of MDR among these strains of 54.83%, with resistance to: TE (94.11% of MDR strains), E (91.17%), CM (79.41%), G (44.11%), FQ (41.17%), RIF (38.23%), SXT (5.88%), C (5.88%), FUS (5.88%). These data are concordant with observations that indicate a blurring of the boundaries between community and hospital, with CA-MRSA becoming more resistant to antibiotics (15).

The role of PVL toxin to the virulence of *S. aureus* has been disputed due to inconclusive results of *in vivo* studies using mice. But epidemiological data and studies on rabbit model, whose neutrophils are as
sensitive to PVL action as human cells, are linking necrotizing skin infections with PVL-production (16). More than 250 articles published during 2002-2007 identified an association between PVL and CA-MRSA disease outbreaks (17). Hedin et al. (18) showed that PVL is a stable genetic marker for CA-MRSA infections, 96% of pvl-positive strains being CA-MRSA, while 81% of pvl-negative strains were HA-MRSA. Fang et al.(19) reported that 56% of 104 CA-MRSA strains carried pvl genes and a similar rate (66%) was detected by Berglund et al.(20). Our study showed a relative high rate of pvl-positive isolates (23.93%), when compared to the results of Holms et al. (21) that noted a low rate of PVL-producers among S. aureus isolates (1.6%).

Most of pvl-positive strains analyzed were CA-MRSA (23 / 45, 51.11% respectively). Only two PVL-producing CA-MRSA strains were MDR, showing resistance to E, G, TE, one strain being also resistant to SXT. Most pvl-positive CA-MRSA isolates (21/23) were resistant to E, but none was resistant to CM, FQ, RIF, C or FUS. The 100% sensitivity to FUS among those strains is discordant with reports suggesting that fusidic acid resistance can be used for identification of PVL-producing CA-MRSA (22). In our study none of the PVL-producing strains showed resistance to FUS.

CONCLUSIONS

Multiplex RT-PCR is a useful tool for rapid detection of MRSA and identification of genes encoding the synthesis of virulence factors. The introduction of this technique in our laboratory allowed us to verify the results of phenotypic testing, which showed good sensitivity in the detection of MRSA (95.34 – 97.67%). Detection of pvl genes revealed a relatively high rate of PVL-producing strains (23.93%) that were associated mostly with CA-MRSA strains and a particular antibiotic resistance profile with resistance to E and sensitivity to CM, FQ, RIF, C and FUS. According to our data, resistance to fusidic acid, although previously proposed as a marker for PVL-producing CA-MRSA, cannot be used to screen for those strains in our setting. Antimicrobial susceptibility testing showed a high rate of MDR among CA-MRSA (54.83%), but not among PVL-producers. This raises the problem concerning the tendency of community strains to spread into hospitals and gain resistance.

ACKNOWLEDGMENTS

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REFERENCES


