Diversity of staphylococcal cassette chromosome mec elements in nosocomial multiresistant Staphylococcus haemolyticus isolates

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Abstract Staphylococcus haemolyticus is the second, most frequently isolated coagulase-negative staphylococcus (CoNS) from patients with hospital-acquired infections, and it is usually resistant to methicillin and other semisynthetic penicillins. The purpose of this study was to characterize staphylococcal cassette chromosome mec (SCCmec) elements and assess the in-vitro activity of antibiotics against 60 S. haemolyticus strains recovered from hospitalized patients. All these strains expressed methicillin resistance and carried a mecA gene. Moreover, all strains possessed a multiresistant phenotype, i.e., exhibited resistance to more than three classes of antibiotics. Eleven strains (18 %) harbored the SCCmec type V, containing ccrC and mec complex C. Three isolates harboring the ccrC gene did not contain a known mec complex. One strain positive for mec complex C was not typeable for ccr. This suggests that ccrC and mec complex C may exist autonomously. Only four strains carried mec complex B, whereas none of the S. haemolyticus harboured mec complex A. A new combination, which is mec complex B-ccrABship, was found in S. haemolyticus. The ccrABship was also identified in two strains of S. haemolyticus in which the mec gene complex was not identified. The results of the present study indicate that in S. haemolyticus the mec gene complex and the ccr genes are highly divergent. However, ccr sequence analysis does not allow the identification of a new allotype, based on a cut-off value of 85 % identity. The ccr genes in the S. haemolyticus strain showed ≥96 % sequence identity to the ccrAB2 genes.

Keywords Staphylococcus haemolyticus • SCCmec • Antibiotic resistance

Introduction

Staphylococcus haemolyticus belongs to the group of coagulase-negative staphylococci (CoNS) and is a part of the normal skin flora and mucous membranes. It is an opportunistic pathogen capable of causing various types of infections, including bacteremia, meningitis, skin infection, prosthetic joint infections, and endocarditis, associated with adherence of staphylococci to medical devices and ability of these bacteria to biofilm formation (Götz et al. 2006; Kristof et al. 2011; Nunes et al. 2005; Rodhe et al. 2006; Szczuka et al. 2015). Other virulence factors, such as exotoxins and enzymes, including nucleases, proteases, lipases, and hemolysins as well as cytotoxic activity through induction of apoptosis, also play a role in the pathogenesis of this species (Kloos and Bannerman 1999; Krzymińska et al. 2012, 2015). Among CoNS, S. haemolyticus has the highest tendency for developing resistance to multiple antibiotics, and it is usually resistant to methicillin and other semisynthetic penicillins (Barros et al. 2012; Krediet et al. 2001, 2004). The methicillin resistance of staphylococci is based on the expression of a modified penicillin-binding protein transpeptidase, with a low affinity for β-lactams, known as PBP2a or PBP2′, which
is encoded by the mecA gene. Notably, a novel PBP2a homolog was described as being encoded by mecC, which shares 70% identity with mecA at the DNA level. The mecC conferred cefoxitin and oxacillin resistance in S. aureus strains (Ballhausen et al. 2014). The mecA gene is carried on a mobile genetic element, called the staphylococcal chromosome mec (SCCmec) integrated into the chromosome at a specific site, located near the origin of replication. To date, eleven SCCmec types have been assigned for Staphylococcus aureus based on the composition of the ccr gene complex and the class of the mec gene complex (Ito et al. 2001, 2004; IWG-SCC 2009). The mec gene complex (A–E) is composed of a mecA gene, regulatory genes (mecRI and mecI), a hypervariable region (HVR), and an associated insertion sequence. The ccr gene complex (type ccrA, ccrB and ccrC) contains recombinase genes encoding recombines responsible for the precise excision and integration of SCCmec within the bacterial chromosome. The ccrA and ccrB genes have been classified into different allotypes, whereas there is only one ccrC allotype (IWG-SCC 2009). Extra ccr allotypes have been also identified, i.e., ccrA5 in S. pseudintermedius, ccrB6 in S. saprophyticus, ccrB7 in S. saprophyticus, ccrA5 in S. cohnii, and ccrABship in S. haemolyticus (IWG 2009; Pi et al. 2009; Zong and Li 2010). The ccrABship products catalyse the mobility of SCCmec and might be responsible for movement of the arginine catabolic mobile element (ACME). This element carries genes encoding a arginine deiminase pathway that converts L-arginine to carbon dioxide, ATP, and ammonia. This metabolic pathway is important for bacteria survival at low pH and for inhibition of the immune response against bacterial infections (Pi et al. 2009). In addition to the ccr and mec gene complex, the SCCmec element contains various other mobile genetic elements (MGE), e.g., plasmid, insertion sequence, and transposon mediating resistance to non-β-lactam antibiotics or heavy metals (Shore and Coleman 2013). It is believed that the increase in resistance of staphylococci to antibiotics is in part due to the presence of the SCCmec, which could be easily transferred between staphylococcal species (Hanssen and Ericson Sollid 2006). The horizontal transfer of a SCCmec type V from MRSH (methicillin-resistant S. haemolyticus) to methicillin-susceptible S. aureus strains resulted in creating MRSA clone (Berglund and Söderquist 2008).

Currently, there is great concern given to the increasing number of methicillin-resistant S. haemolyticus (MRSH) strains that are able to cause severe infections in hospitalized people. These bacteria show a remarkable tendency of developing resistance to multiple antibiotics, as well as the potential to transfer SCCmec elements. This study was undertaken to characterize the staphylococcal cassette chromosome in clinical MRSH isolates, and determine the susceptibility profiles of these isolates.

Material and methods

Bacterial strains Sixty isolates of S. haemolyticus were collected from clinical specimens of patients treated at the Regional Hospital in Poznań. Only isolates considered clinically relevant were included in this study. Isolates were identified by Gram staining, colony morphology, catalase reaction and by biochemical tests using the a Vitek 2 system (bioMérieux, France). The isolates were stored at in medium with 25% glycerol at −70°C.

Susceptibility testing Resistance to β-lactams was determined by the cefoxitin (30 μg) screen test as well as by amplification of mecA gene by PCR technique. Analysis of susceptibility to the following antibiotic agents was also performed using a Vitek 2 system (bioMérieux, France) according to EUCAST recommendations (http://www.eucast.org/clinical_breakpoints): fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin), amino- glycosides (gentamicin and tobramycin), glycopeptides (teicoplanin and vancomycin), macrolides and lincosamides (clindamycin and erythromycin), tetracyclines (tetracycline and tigecycline), and others (linezolid, rifampin, trimethoprim-sulfamethoxazole, fusidic acid and fosfomycin).

Preparation of total DNA for PCR The total DNA was isolated and purified using the Genomic Mini DNA kit (A&A Biotechnology, Gdynia, Poland).

SCCmec analysis The detection of mecA gene was carried out using primers and method as described previously (Zhang et al. 2005). The SCCmec analysis was carried out by identification of the mec complex and crr genes by PCR method according to the criteria set out for S. aureus (Zhang et al. 2005). In addition, the detection of gene lineages of mecA-mecI (class A mec), mecA-IS1272 (class B mec), and mecA- IS431 (class C mec) was performed by PCR technique using four primers assigned by Kondo et al. (2007). The presence of the novel ccr allotype (ccrABship) in S. haemolyticus isolates was checked with primers assigned by Pi et al. (2009). The amplification products were electrophoresed in 1.5% agarose gel. The gels were stained with ethidium bromide, visualized on a UV light transilluminator, and documented with a V.99 Bio-Print system (Vilber Lourmat, Torcy, France).

Sequence analysis

The ccrA and ccrB genes amplicons were obtained using primers designed by Zong and Li (2010). PCR products were purified and sequenced in a 3100xl Genetic Analyzer (Applied Biosystems). The resulting sequences were deposited in GenBank with accession numbers KU523873 and
KU523874. These sequences were compared with ccr alleotypes, available in GenBank data: ccrA1 (S. aureus 45394 F, S. hominis GIFU12263), ccrA2 (S. aureus M06/0075, S. aureus JCSC6668, S. aureus N315), ccrA3 (S. aureus JK608, S. pseudintermedius KM1381), ccrA4-2 (S. aureus CHE482), ccrA4-1 (S. aureus CHE482), ccrA5 (S. pseudintermedius KM241), ccrASIP (S. haemolyticus strain H9), ccrB1 (S. aureus COL), ccrB2 (S. aureus JCSC1968, S. epidermidis CS8), ccrB3 (S. aureus TW20, S. pseudintermedius KM1381, S. cohnii WC28), ccrB4 (S. aureus BK20781, S. aureus HDE288, S. aureus strain CHE482), ccrB5 (S. pseudintermedius KM241), and ccrBSIP (S. haemolyticus H9). Similarity searches were carried out using BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/).

Results and discussion

The S. haemolyticus strains included in this study originated from hospitalized patients with nosocomial infections. All of these isolates expressed phenotype MR and carried a mecA gene. Moreover, all isolates possessed a multiresistant phenotype, i.e., exhibited resistance to at least three of the non-ß-lactam antibiotics tested. The majority of MRSH isolates were resistant to erythromycin (95%) and gentamicin (90%); fewer isolates were resistant to clindamycin (77%), trimethoprim-sulfamethoxazole (73%), ciprofloxacin (58%), tetracycline (34%), tobramycin (27%), and levofloxacin (18%). Only single strains were resistant to moxifloxacin, rifampin, fusidic acid, tigecycline, and fosfomycin. Resistance among S. haemolyticus strains isolated from clinical specimens underscores the importance of this species as a reservoir for mecA genes (Nunes et al. 2005; Tabe et al. 2001). The resistance to a great range of antibiotic agents is thought to be associated with the existence of many insertion sequences, which accumulate antibiotic resistance genes, in the S. haemolyticus genome (Takeuchi et al. 2005). It should be underlined that all isolates were susceptible to vancomycin and a relatively new antibiotic — linezolid. Recently, linezolid-resistant S. haemolyticus has been described in European countries, the USA, China, and India (Cai et al. 2012; Gupta et al. 2012; Mazzariol et al. 2012; Rodriguez-Aranda et al. 2009; Tewhey et al. 2014).

The SCCmec type V, containing ccrC and mec complex C, was detected in 11 out of 60 S. haemolyticus tested strains (18%) (Table 1). The results of this study are similar to those of Ruppe et al. (2009), who demonstrated that SCCmec type V is preferentially associated with S. haemolyticus strains isolated from distinct geographical areas, such as Cambodia, Algeria, Mali, and Moldova. Data obtained by Bouchami et al. (2012) indicated that ccrC and mec complex C are most prevalent among S. haemolyticus. It was also found that the ccrC and mec complex C may exist autonomously. In our study, the ccrC alone was observed in three S. haemolyticus strains. One strain was only positive for mec complex C. Forty-nine isolates (82%) were non-typeable for SCCmec using currently-available schemas based on the multiple PCR method. This can be explained by the presence of novel structures or frequent genomic rearrangements and recombination of the SCCmec element. It is noteworthy that the SCCmec types II, III and V have been detected in S. haemolyticus collected in China (Zong et al. 2011). It is believed that occurrence of different SCCmec types in China might reflect the genetic background of S. haemolyticus strains, connected with geographical locations. None of the studied isolates harboured the mec complex A. The four S. haemolyticus strains appeared to carry the class B mec. For two of these strains the crr gene could not be amplified. It should be noted that the class B mec complex, as well as the

Table 1 SCCmec typing results

| No. of isolates | SCCmec type | Origin | mec class | ccr type |
|-----------------|-------------|--------|-----------|---------|
| 11              | Type V      | blood, endotracheal aspirate, wound secretion, urine, fluid from the peritoneal cavity | class C | ccrC |
| 3               | NT          | blood | ND        | ND      |
| 1               | NT          | blood | class C   | ND      |
| 2               | NT          | blood, synovial fluid | class B | ccrAB<sub>ship</sub> |
| 2               | NT          | blood, endotracheal aspirate | ND | ccrAB<sub>ship</sub> |
| 39              | NT          | blood, endotracheal aspirate, wound secretion, urine, fluid from the peritoneal cavity, venous catheter | ND | ND |

ND, not detected
NT, untypeable
The analysis of \textit{ccrA} and \textit{ccrB} gene sequences of the studied \textit{S. haemolyticus} strains showed that they represent novel allotypes of recombinase. Therefore, newly-designed primers pairs were used to amplify \textit{ccrA} and \textit{ccrB} genes and the sequences of obtained amplicons were determined, deposited in GenBank with accession numbers KU523873 and KU523874, and compared to known \textit{ccr} sequences from GenBank database. The \textit{ccr} gene (KU523874) that was identified in a \textit{S. haemolyticus} isolate (MPU SH 68) displayed ≥96% sequence similarity to the \textit{ccrA} gene of the \textit{Staphylococcus aureus} aureus strains (M06/0075, JCSC6668 and N315). The \textit{ccrB} gene (KU523873) of MPU SH 68 shared the highest identity (97%) with the \textit{ccrB} gene of \textit{S. aureus} strain JCSC1968 and \textit{S. epidermidis} CS8. Thus, the nucleotide sequences of the studied \textit{ccr} genes showed that they can not be assigned to known allotypes based on the cut-off value of 85% identity. Our present data, together with previous reports, emphasize the great diversity of the SCCmec elements in \textit{S. haemolyticus} (Bouchami et al. 2012; Hansen and Ericson Solid 2007; Pi et al. 2009; Urushiibara et al. 2011; Zong and Liu 2010). 

In conclusion, \textit{S. haemolyticus} has been found to be an important source of the \textit{mec} complex C and the \textit{ccrC} complex, which are components of SCCmec type V. The analysis of \textit{ccrA} and \textit{ccrB} gene sequences of the \textit{S. haemolyticus} strain (MPU SH 68) showed their high nucleotide sequence similarity to those found in \textit{S. aureus} and \textit{S. epidermidis} strains. Moreover, the new combination, i.e., \textit{mec} complex B/\textit{ccrAB}_{\text{ship}}, was identified in coagulase-negative staphylococci. Our study underscores the great diversity of SCCmec structures in \textit{S. haemolyticus} strains, as well as the importance of these bacteria as a reservoir of \textit{mec} genes.

**Compliance with ethical standards** This study was funded by the Faculty of Biology, Adam Mickiewicz University in Poznań. We declare that there is no conflict of interests. This article does not rely on and does not report findings from clinical studies with human participants. Human specimens collected in order to perform diagnostic tests were obtained from hospitalized patients. The bacterial strains were isolated from human specimens in hospital laboratories, which are statutorily licensed by being entered into the national registry of The National Chamber of Laboratory Diagnosticians and must comply with its code of ethics.

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