Diagnostics of Staphylococci with Special Reference to MRSA

Diagnostik von Staphylokokken mit besonderer Berücksichtigung von MRSA

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Summary: Until now, 32 species of the genus *Staphylococcus* have been described, and most have been confirmed at DNA level (DNA-DNA hybridization kinetics, 16S rRNA sequences). Most clinical microbiological diagnostics use agglutination tests for the identification of *S. aureus*. Since these tests rely on the demonstration of the clumping factor and since this is problematic for epidemic methicillin-resistant *S. aureus* (MRSA), currently available tests are supplemented with IgG and antibodies against capsular polysaccharides. DNA-based tests have also been established with PCR (e.g., demonstration of a *S. aureus* specific sequence), some with reverse hybridization. Species diagnostics of coagulase-negative staphylococci is mainly performed by phenotypical biochemical reaction patterns. More recent approaches are based on 16S rRNA sequences, as well as polymorphisms of the *hsp60, gap,* and internal rRNA gene spacers.
Test substances used for phenotypical susceptibility testing of staphylococci should have an indicator function for certain important resistance mechanisms, and results should be interpreted with regard to cross resistance. The gold standard for the demonstration of oxacillin resistance is still PCR for *mecA,* which can be assessed by reverse hybridization. Phenotypically, penicillin-binding protein 2a can be reliably determined by an antibody-based assay. When using screening tests, the β-lactamase inhibitor sulbactam should be used for excluding borderline resistant isolates (BORSA). Identification of glycopeptide intermediate susceptible *S. aureus* (GISA) can still only be made phenotypically (screening test, E-test with high inoculum); reliable confirmation is possible by in vitro population assay.

Keywords: *Stapyhlococcus aureus;* epidemic methicillin-resistant *S. aureus* (MRSA).

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Zusammenfassung: Bisher wurden 32 Species der Gattung *Staphylococcus* beschrieben, die auch auf DNA-Ebene bestätigt wurden (DNA-DNA-Hybridisierung, 16S rRNA-Sequenzen). In der klinisch-mikrobiologischen Diagnostik werden zumeist Agglutinationstests für die Identifizierung von *S. aureus* benutzt. Da dies für Tests, die nur auf dem Nachweis des Verklumpungsfaktors beruhen, für die meisten epidemischen MRSA problematisch ist, enthalten die gegenwärtig kommerziell verfügbaren Testkits zusätzlich IgG und Antikörper gegen Kapselpolysaccharide. Es wurden auch Tests mittels PCR für *S. aureus* spezifische DNA-Sequenzen, zum Teil verbunden mit reverser Hybridisierung, eingeführt. Die Speciesdiagnostik von koagulasenegativen Staphylokokken erfolgt überwiegend mittels phänotypischer biochemischer Reaktionsmuster. Gegenwärtige Entwicklungen beruhen auf 16S rRNA-Sequenzen sowie auf Polymorphismen der Gene *hsp*60, *gap* sowie der internen rRNA-Genspacer.
Die Testpalette für die phänotypische Resistenzbestimmung sollte eine Indikatorfunktion für bestimmte, wichtige Resistenzmechanismen besitzen, die Ergebnisse dann im Hinblick auf Kreuzresistenzen interpretiert werden. Der „Goldstandard" für den Nachweis der Oxacillinresistenz ist nach wie vor die PCR für *mecA*, deren Ergebnis durch reverse Hybridisierung schnell detektiert werden kann. Der phänotypische Nachweis des Penicillinbindeproteins PBP2a kann verläßlich mit einem auf monoklonale Antikörper basierenden Agglutinationstest erfolgen. Bei der Verwendung von Screeningtests sollte den Kulturmedien zum Ausschluß der Borderline Oxacillin-resistanten *S. aureus* (BORSA) unbedingt der β-Laktamaseinhibitor Sulbactam zugefügt werden. Die Identifizierung von Glykopeptid intermediär empfindlichen *S. aureus* (GISA) kann nach wie vor nur phänotypisch erfolgen mittels Screeningtest oder E-Test mit hohem Inokulum; eine verläßliche Bestätigung der Ergebnisse ist durch in vitro Populationsanalyse möglich.

Schlüsselwörter: *Stapyhlococcus aureus*; MRSA.

Staphylococci are conditional pathogens. The genus *Staphylococcus* consists of at least 32 species of which *S. aureus* has special significance as a pathogen able to cause a variety of different kinds of infections.

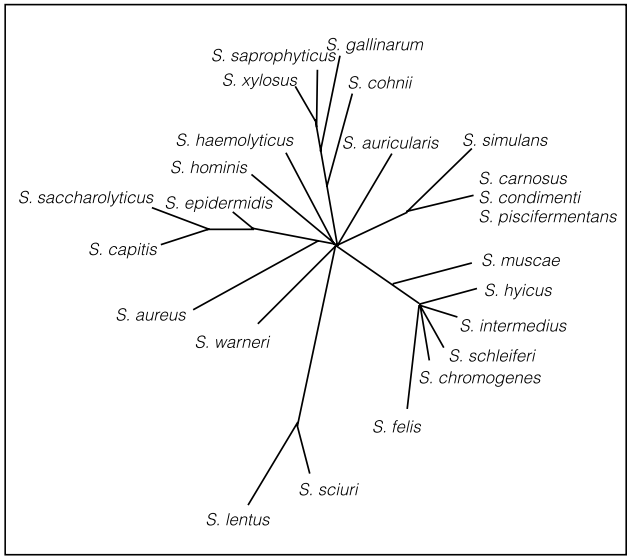

Figure 1 Phylogenetic relationship of staphylococcal species based on 16S rRNA sequences [3]

S. epidermidis and some other species of coagulase-negative staphylococci (CNS) are the major cause of catheter and foreign-body associated infections.

During the past years, especially hospital-associated strains have developed multiresistance to antibiotics. Clinical microbiological diagnostics must pay attention to correct identification of *S. aureus*, species differentiation of CNS, and reliable detection of resistance characteristics, in particular of those with heterogeneous phenotypical expression. Of further importance is the demonstration of particular toxins for verification of toxin-mediated staphylococcal infections and food poisoning.

Species diagnostics and identification of *S. aureus*

Speciation in bacterial systematics is based on a comparison of characteristics in order to arrange microorganisms in groups sharing common properties. The basic taxonomic unit, the species, is defined by the most characteristic properties necessary for reliable identification of a particular organism as belonging to this group. The classification of different species into groups leads to *genera* with a number of key characteristics. Besides the species characteristics, organisms exhibit a number of other properties that can be used for further differentiation into "types" below the species level. Ideally, typing should identify the clonal progeny of an organism within a species population. The purpose of speciation is identification of a microorganism as belonging to a basic *taxon* which, for example, has a particular ecological or clinical significance.

Bacterial taxonomy still joins gram-positive, catalase-positive cocci, such as staphylococci and micrococci, into the family of *Micrococcaceae*, although the *genera* of this family exhibit a number of very different molecular features (e.g., GC content, cell wall composition; Table 1). Further analysis at genomic level will reveal whether staphylococci form a quite separate taxonomic group.

Species described to date within the genus *Staphylococcus* are shown in Table 2. As with other microorganisms, speciation in staphylococci was first based on a data set of morphological characteristics, physiological properties, and chemical composition of the cell wall [1]. This scheme was not always unambiguous because some of the taxonomic characteristics, such as acid formation from carbohydrates and the formation of cell wall-associated proteins, extracellular enzymes, and toxins, were unstable, owing to genomic rearrange-

Table 1 Characteristics of the genus *Staphylococcus* in comparison with other genera classified as members of the family *Micrococcaceae*

Characteristic	Genus			
	Staphylococcus	*Micrococcus*	*Planococcus*	*Stomatococcus*
GC content of DNA (mol % G + C)	30–35	70–75	40–51	56–60
Cell wall composition (more than 2 mol of glycin per mol of glutaminic acid in peptidoglycan)	+	–	–	–
Type of fructose-1,6-diphosphate aldolase	I	II	ND*	II
Cytochrome c	–	+	ND	+
Characteristics used in bacteriological diagnostics				
Sensitivity to lysostaphin	+	–	–	–
Sensitivity to furazolidone	–	+	ND	ND

*ND = not determined

Table 2 Currently known species of the genus *Staphylococcus*

S. aureus
S. capitis subsp. capitis
S. capitis subsp. ureolyticus
S. caprae
S. saccharolyticus
S. warneri
S. pasteuri
S. haemolyticus
S. hominis
S. lugdunensis
S. schleiferi subsp. schleiferi
S. schleiferi subsp. coagulans
S. muscae
S. auricularis
S. saprophyticus
S. cohnii subsp. cohnii
S. cohnii subsp. urealyticum
S. xylosus
S. kloosii
S. equorum
S. arlettae
S. gallinarum
S. simulans
S. carnosus
S. piscifermentans
S. felis
S. intermedius
S. delphini
S. hyicus
S. chromogenes
S. caseolyticus
S. sciuri
S. lentus

ments or to acquisition or loss of accessory genetic elements.

Later DNA-DNA hybridization studies have shown that staphylococci form a well-defined genus, which can be subdivided into several species groups [2]. The results of DNA homology studies have been confirmed at the epigenetic level: comparative characterization of catalases, aldolases, L- and D-lactate dehydrogenases [2], and for a number of staphylococcal species, also by 16S and 23S rRNA sequence analysis [3, 4] (Table 3). Phylogenetic relationships based on 16S rRNA sequence analysis confirm the data from DNA-DNA hybridization for most staphylococcal species (Fig. 1). A more recent investigation revealed that the sequence polymorphism in a "hot-spot" stretch of the heat shock protein 60 gene (*hsp60*) is species specific in staphylococci [5]. In contrast to the variable parts of the 16S rRNA gene sequence, there are at least two, in some cases even more, mismatches. Therefore, *hsp60* is of special interest for DNA-based species diagnostics (Table 4). A more recent study revealed a species specific polymorphism of the glycerol aldehyde phosphatase (*gap*) gene which can be detected by PCR and *Alu*-I-digestion of the amplimers [6]. Another approach has used internal-transcribed-spacer-PCR (ITS-PCR) to identify staphylococcal species [7].

Identification of *S. aureus*

Coagulase-negative staphylococci differ phenotypically from *Staphylococcus aureus* by their lack of ability to form some extracellular products and cell-wall-associated proteins [8]. In routine clinical microbiology, the coagulase test-tube reaction has been used as the main species characteristic of *S. aureus* [8], as has DNase formation in food microbiology [9]. Although less sensitive and less specific than coagulase formation [10], the demonstration of clumping factor is often used in routine clinical microbiology. The clumping factor of *S. aureus* is a cell-wall-associated protein which reacts with fibrinogen monomers, thus leading to aggregation of *S. aureus* cells [11]. The recent emergence of epidemic methicillin-resistant *S. aureus* (MRSA) lacking this capability [12] has rendered tests based only on the demonstration of protein agglutination unreliable [13].

Therefore, currently available agglutination tests have been supplemented with antibodies against capsular polysaccharides and with IgG (which reacts specifically with protein A of *S. aureus* [14]) and therefore possess sufficient sensitivity and specificity as described recently [15]. In this study, fibrinogen solution and the Staphylslide-Test (based only on fibrinogen) were used for comparison. The specificity of these test kits is only slightly reduced with regard to a positive reaction of *S. schleiferi* (Dry Spot Staphytect Plus) and *S. schleiferi* and *S. cohnii* (Pastorex Staph-Plus, Staphaurex Plus). In a few cases, a shared noncapsular antigen has been shown to be responsible for false positive reactions by *S. epidermidis* in commercial slide agglutination tests [16]. For *S. schleiferi,* a positive clumping reaction has been described when suspended in human blood plasma [17]. The absence of clumping in fibrinogen solution suggests that *S. schleiferi* probably possesses another surface component besides the clumping factor, reacting with plasma constituents other than fibrinogen.

In clinical bacteriology, the coagulase tube test should be performed for strains which are intermediate in agglutination tests. For the demonstration of free coagulase using the test-tube method, five colonies are

Table 3 Examples for species specific sequences of the 16S rRNA gene (nucleotides 66–95) in coagulase-negative staphylocci (according to [3])

Strain	Sequences
S. aureus	CGAA CGG ACG AGA AGC TTG CTT CTC T GAT
S. epidermidis	CGAA CAG ACG AGG AGC TTG CTC CTC T GAC
S. capitis	CGAA CAG ACG AGG AGC TTG CTC CTC T GAC
S. warneri	CGAA CAG ATA AGG AGC TTG CTC CTT T GAC
S. haemolyticus	CGAA CAG ATA AGG AGC TTG CTC CTT T GAC
S. hominis	CGAA CAG ACG AGG AGC TTG CTC CTT T GAC
S. saprophyticus	CGAA CAG ATA AGG AGC TTG CTC CTT T GAC

Table 4 Hot spot region of *hsp*60 of staphylococci with significance of species identification (according to [5])

Species	Nucleotides
	91 100 110 120
S. aureus	CAAAGCAGT–-TAAAGTTGCTGTTGAAGCG-TTACA
S. epidermidis	CAAAGCAG–GC- AACTGGCTATAGAAGCG-CTCCA
S. lugdunensis	TAAAGCAGT–-TAAAGTAGCTATCGAAGCA-TTACA
S. saprophyticus	TAAAGCTGT–-AGAAGTAGCAATTGAGGCA-TTACA

suspended in 0.5 mL of OXOID nutrient broth. After incubation for 2 h at 37 °C, 0.5 mL of human blood plasma is added. The tubes are inspected for formation of clots after 3 h at 37 °C and again after 24 h at room temperature.

Identification of *S. aureus* by PCR-based tests in bacteriological routine

Until now, PCRs have been established for the following characteristics specific for *S. aureus*:

- *nuc*, the gene for heat stable deoxyribonuclease [18] with primers f:5'-GCG ATT GAT GGT GAT ACG GTT, r:5'-AGC CAA GCC TTG ACG AAC TAA AGC
- specific sequences in r-RNA [19] primers SA V:5'-CAT ATT GTA TTC AGT TTT GA, SA, VI:5'-TCC ACC ATT TTT ATA AGTC, annealing temperature: 55 °C
- DNA sequence specific for *S. aureus* as identified by subtractive hybridization [20] with primers Sau-f:5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG, Sau-r:5'-CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA

Also, PCR-demonstration of *fem* genes, such as *fem*A has been recommended [21]; there are, however, doubts that a sufficient number of isolates from different CNS species have been checked for this characteristic.

More rapid identification of *S. aureus* by use of reverse hybridization tests

This methodology is based on PCR amplification of the DNA sequence specific for *S. aureus* and hybridization to a capture probe. PCR-based methods still require agarose gel electrophoresis which needs additional time, and when applied in routine, further confirmation of the specifity of the amplimer is usually not performed. Two tests are current commercially available:

- The Genotype MRSA® test offered by HAIN-Diagnostika, Nehren, Germany, is based on amplification of a part of *mec*A gene and of a sequence specific for *S. aureus* ("sau", see above) and subsequent hybridization of the denatured amplicons to their complementary sequences coated to membrane strips. The PCR performance is controlled by simultaneous amplification of an universal 16S rRNA sequence. This test has revealed as 100 % specific and sensitive for the identification of MRSA (22).
- Ampliwell StaphyloTox from Mikrogen uses amplification of *mec*A and, for species diagnostic, of the "hot spot" sequence of *hsp60*; the corresponding capture probes are fixed to wells of polystyrene microliter pla-

Table 5 Antibiotics used for susceptibility testing of staphylococci and cross resistance (interpretative reading)

Antibiotic tested	Relevant resistance mechanism(s)	Cross resistance
Benzylpenicillin	β-Lactamase	β-Lactamase susceptible penicillines (aminoacylureidopenicillines)
Oxacillin	Low-affinity penicillin-binding protein PBP 2a	All β-lactams (cephalosporins and carbapenems included)
Gentamicin	Aminoglycoside acetyltransferase – aminoglydoside phosphotransferase (*aac6'-aph2")*	Potentially all kanamycin-derived aminoglycosides
Erythromycin	23S rRNA methylases	When constitutively expressed all macrolides, lincosamidines, and streptogramin B
Clindamycin	23S rRNA methylases	Constitutively expressed 23S rRNA methylase
Ciprofloxacin	Target mutation in topoisomerase IV (*grl*A) and gyrase (*gyr*A)	All fluoroquinolones of group II
Moxifloxacin	Target mutation in topoisomerase IV (*grl*A) and gyrase (*gyr*A)	All currently available fluoroquinolones
Rifampicin	Target mutation in β-subunit of RNA polymerase (*rpo*B)	All rifamycins
Trimethoprim	Insensitive dihydrofolate reductase (*dfr*A)	–
Fusidic acid	Not investigated in sufficient detail	–
Quinupristin/dalfopristin	Additional to 23S rRNA methylase mechanisms for streptogramin A compounds: acetyltransferase [*vat*(A),(B),(C)] efflux [*vga*(A), *vga*(B)]	All streptogramin combinations
Linezolid	Point mutations in the V-loop of 23S rRNA	Not to other classes of antibiotics
Vancomycin/ Teicoplanin	Thicker cell wall (trapping effect of non cross linked N-acetylmuraminic acid) in case of glycopeptide intermediate susceptible *S. aureus* (GISA)	Potentially to other glycopeptides

tes. This allows the use of lab equipment for washing of these plates when working with large sample numbers.

Diagnostics of coagulase-negative staphylococci

In conventional clinical bacteriology, identification of coagulase-negative staphylococcal species is primarily based on a set of biochemical characteristics [1]. Commercially available panels in microliter scale (e.g. the well-established Staph-ID-32 panel from bioMerieux) include between 30 and 40 metabolic characteristics as well as resistance to novobiocin as a characteristic of the *S. saprophyticus* group and resistance to furazolidone for separation from micrococci. The frequency distribution of metabolic characteristics used for speciation of staphylococci is based on an empirically established data bank. Attribution of particular reaction profiles to a species can be performed by a code book, or, more quickly and precisely, by computerized probability analysis. There are also automated or semiautomated systems, such as VITEK from bioMerieux and PHOENIX from Becton Dickinson which are based on biochemical reaction profiles. For routine purposes, these systems guarantee sufficient specificity [23]. A PCR-based rapid identification of coagulase-negative staphylococci by length polymorphism has been described recently [24]. Further developments will use microarrays based on rRNA-sequences or other species specific genomic polymorphisms, e.g. *hsp*60 [5].

Susceptibility testing

Multiresistance in *S. aureus* can reach the limits of currently available antibiotics for treatment of staphylococcal infections. Most of the resistance mechanisms of *S. aureus* confer cross resistance to most substances of particular classes of antibiotics; therefore susceptibility testing should be performed with a limited number of substances for which the test procedure is well defined, this allows reading of the results in an interpretative manner. For staphylococci, this is summarized in Table 5.

Testing of oxacillin and of glycopeptides need special attention

During the last 15 years, there has been a consistently high prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA; synonym ORSA – oxacillin-resistant

Table 6 Primers used for PCR demonstration of enterotoxin and exfoliative toxin genes of *S. aureus* (according to [46])

Toxin gene	Primer sequences	Annealing temperature	Molecular mass of amplimers
sea	sea-f: TTG GAA ACG GTT AAA ACG AA sea-r: GAA CCT TCC CAT CAA AAA CA	55 °C	120
seb	seb-f: TCG CAT CAA ACT GAC AAA CG seb-r: GCA GGT ACT CTA TAA GTG CC	55 °C	478
sec	sec-1-f: GAC ATA AAA GCT AGG AAT TT sec-1-r: AAA TCG GAT TAA CAT TAT CC	50 °C	257
sed	sed-f: CTA GTT TGG TAA TAT CTC CT sed-r: TAA TGC TAT ATC TTA TAG GG	55 °C	317
see	see-f: TAG ATA AAG TTA AAA CAA GC see-r: TAA CTT ACC GTG GAC CCT TC	45 °C	170
tst	tst-f: ATG GCA GCA TCA GCT TGA TA tst-r: TTT CCA ATA ACC ACC CGT TT	45 °C	350
eta	eta-f: CTA GTG CAT TTG TTA TTC AA eta-r: TGC ATT GAC ACC ATA GTA CT	55 °C	119
etb	etb-f: ACG GCT ATA TAC ATT CAA TT etb-r: TCC ATC GAT AAT ATA CCT AA	55 °C	200

Staphylococcus aureus – because oxacillin is used as the test substance) in many parts of the world [25, 26]. Methicillin resistance is mainly due to the elaboration of penicillin-binding protein PBP 2a coded by the *mec*A gene; isolates with reduced affinity for PBP 2 due to mutations are more rare [27]. The phenotypic expression of PBP 2a-based methicillin resistance is dependent upon a number of other factors [28], and most of the recently disseminated MRSA strains express this phenotype heterogeneously (heteroresistant MRSA [29]). Particular attention has recently been focused on *Staphylococcus aureus* with borderline levels of susceptibility to oxacillin and related β-lactam agents (borderline oxacillin-resistant *Staphylococcus aureus*, BORSA). BORSA strains belong to the widely disseminated clonal group exhibiting phage pattern 94, 96 [30]. Hyperproduction of a β-lactamase has been described for BORSA, but this β-lactamase is unable to hydrolyze methicillin [31]. It is likely that there is a second β-lactamase in BORSA, with some capacity to hydrolyze methicillin and oxacillin [31, 32]. The MRSA genotype can be easily assessed by the polymerase chain reaction for *mecA*, which allows the detection of intrinsic methicillin resistance in strains with low-level resistance to oxacillin, according to criteria of routine susceptibility testing [33]. Because of heteroresistance, phenotypic methods for the detection of MRSA require a higher inoculum than that generally used for the agar diffusion assay or MIC determination, e.g., the agar screening test [34]. Furthermore, current guidelines for antibiotic susceptibility testing recommend testing *Staphylococcus aureus* under conditions designed to elicit expression in *mec*A-positive isolates. These conditions include incubation at 35 °C and supplementation of media with 2–4 % NaCl (e.g., agar screening test performed according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) [35]. This, however, bears the risk that BORSA isolates will appear resistant (inoculum effect, effect of NaCl). As already described, the susceptibility of BORSA to amoxicilin/sulbactam or, better, to ampicillin/sulbactam has been regarded as a criterion for differentiating BORSA from truely resistant MRSA. When, however, routine conditions for susceptibility testing were used, a fraction of BORSA isolates were still identified incorrectly [36]. This could be overcome by combining oxacillin and sulbactam in screening tests [37].

ORSA agar from OXOID, containing oxacillin, NaCl, polymyxin B, and aniline blue as indicator for mannitol fermentation, detects heteroresistant MRSA with exclusion of BORSA, however, it needs 48 hours of incubation at 37 °C (own unpublished data). More rapid detection of MRSA is possible by agglutination tests based on monoclonal antibodies against PBP 2a, the low affinity penicillin-binding protein being the major mechanism of oxacillin resistance. For this method, a few colonies from a fresh culture on agar are used, the test has been shown to be highly specific and sensitive [37].

The "gold standard" for oxacillin susceptibility testing is still PCR for *mec*A [38], recently commercially available diagnostic kits are based on reverse hybridization as described above in connection with species identification. Methodological details: PCR for *mec*A with primers mecAf:5'-AAA ATC GAT GGT AAA GGT TGG and mecAr:5'-AGT TCT GCA GTA CCG GAT TTGC, annealing temperature 55 °C.

Oxacillin resistance can be easily detected by PCR, a method for quick isolation of highly pure template DNA has been described previously [37].

In parallel to standard methods of phenotypical susceptibility testing, the following borderline concentration tests are in use for screening for MRSA:

- Oxacillin screening plates according to NCCLS M7–A3: with the addition of sulbactam Mueller Hinton agar or Isosensitest agar containing 4 % NaCl and 6 mg/l oxacillin and 8 mg/l sulbactam. Inoculum: colonies from a fresh original culture on blood agar are suspended in 0.9 % NaCl to give an inoculum with a turbidity corresponding to McFarland standard 0.5 (~ 1×10^8 cfu/ml). Inoculation as a spot or streak ("Christmas tree"). The plate is incubated at 35 °C; first reading after 24 h, second reading after 48 h.
- Mannitol salt agar is a selective medium for NaCl-tolerant bacterial species such as staphylococci and enterococci. Mannitol fermentation is a species characteristic of *S. aureus* in contrast to *S. epidermidis*. However, a number of other coagulase-negative species, e.g., *S. haemolyticus,* are also able to form acid from mannitol, this biases the use of mannitol salt agar for MRSA screening.
- Oxacillin broth screening test: 1 ml Isosensitest broth containing 2 mg/l oxacillin, 8 mg/l of sulbactam and 2 % NaCl. The prepared test tubes can be stored at -20 °C for 2 months without loss of antibiotic activity. Inoculum: 10 µl of a suspension of the corresponding strain in 0.9 % NaCl or nutrient broth with a turbidity that corresponds to McFarland standard 0.5. Tubes are incubated at 30 °C for 24 h, reading after a short shaking of the tube.

Rapid detection of oxacillin resistance by automated systems

The BBL crystal system in PHOENIX and VITEK II allows a computer assisted reading of results already after 3–4 hours of incubation (growth indicated by cleaving fluorogenic groups from suitable substrates, slope of growth curve). In comparison to PCR for *mec*A, the GPS106 card of the VITEK II system had a sensitivity of 95.7 % and a specificity of 85.7 % [37] when testing coagulase-negative staphylococci.

BBL crystal MRSA ID had a sensitivity of 98.5 % and a specificity of 98.0 % when *S. aureus* was tested with PCR for *mec*A as reference [39].

Detection of glycopeptide intermediate susceptible *S. aureus* GISA

GISA were first been described in Japan with a few infections and numerous cases of colonization [40]. In the meantime, there are reports from many parts of the world, of special concern are outbreaks of infections in French hospitals: in one hospital, the outbreak strain had also acquired quinupristin/dalfopristin resistance and was only susceptible to phosphomycin and to linezolid [41]. In Germany to date, one small nosocomial outbreak has been recorded [42], and a few sporadic cases of infections. The reduced susceptibility of GISA is obviously due to an elevated synthesis of peptidoglycan resulting in a thicker cell wall with non cross-linked D-ala-D-ala-muraminic acid molecules, the target of glycopeptides. This results in a trapping effect which reduces the number of glycopeptide molecules reaching their targets at the site of cell wall synthesis [40]. The genetic (most probably regulatory) basis of the GISA phenotype is largely unknown, susceptibility testing is still based on phenotypical methods.

Breakpoints for vancomycin resistance according to DIN 58 940 from year 2000 are: ≤ 4 mg/l susceptible, 8 mg/l intermediate, ≥ 16 mg/l resistant. Because the large glycopeptide molecules diffuse only slowly in agar media, the agar diffusion assay is not useful for vancomycin susceptibility testing. The GISA phenotype can be expressed homogeneously or heterogeneously. Vancomycin MICs ≥ 4 mg/l are an indicator of homo-GISA, 1–2 mg/l of hetero-VISA [42].

GISA can be easily detected by use of a plate test: Isosensitest agar or brain heart infusion agar containing 6 mg/l of vancomycin; spot inoculation with ≈ 10 µl of an inoculum prepared from a suspension in 0.9 % NaCl or broth culture, both with a turbidity corresponding to McFarland standard 0.5; incubation at 35 °C for 24 h. Growth from 10 colonies to confluence indicates GISA [43]. As already the practice for *mec*A coded heteroresistance to oxacillin, in case of GISA higher inocula are used on screening plates.

As an alternative method, the E-test for vancomycin with use of a higher inoculum (corresponding to McFarland standard 2) has been proposed. Here, MICs from 8 mg/l are suspicious for GISA!

Interpretation of the GISA phenotype: a clinical treatment success is uncertain in case of unfavourable location of the infection, e.g., pneumonia or bone infections.

Confirmation of heterogeneously expressed intermediate susceptibility to glycopeptides in staphylococci can be problematic [43]; although laborious, in vitro population analysis is still required [44].

Detection of the toxin-formation capacity of *S. aureus*

Enterotoxins formed by *S. aureus* have a special clinical significance: such as enterotoxins (A, B, C, D) for food intoxications, enterotoxins B and C as superantigens for septic shock, toxic shock syndrome toxin TSST-1, and exfoliative toxins A and B for dermatitis exfoliativa (staphylococcal scalded skin syndrome). Besides those mentioned above, additional proteins with superantigen activity have been described (enterotoxins [45]) and their clinical role needs to be established.

In particular, certain *S. aureus* strains from East Asia are able to form an inhibitor of Rho-GTPases named EDIN which is able to cause epidermolysis [46]. In vitro enterotoxin formation (A, B, C, D) can be easily de-

tected by the slide agglutination test provided by OXOID which is based on monoclonal antibodies. PCR for the relevant toxin genes is also well established (Table 6; [47]), this PCR is also possible as a multiplex assay [48].

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