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Citation for published version:
Kriegeskorte, A, Idelevich, EA, Schlattmann, A, Layer, F, Strommenger, B, Denis, O, Paterson, GK, Holmes, MA, Werner, G & Becker, K 2017, 'Comparison of Different Phenotypic Approaches to Screen and Detect mecC-Harboring Methicillin-Resistant Staphylococcus aureus', *Journal of Clinical Microbiology*. https://doi.org/10.1128/JCM.00826-17

Digital Object Identifier (DOI):
10.1128/JCM.00826-17

Published In:
Journal of Clinical Microbiology

JCM Accepted Manuscript Posted Online 4 October 2017
J. Clin. Microbiol. doi:10.1128/JCM.00826-17

Comparison of Different Phenotypic Approaches to Screen and Detect *mecC*-Harboring Methicillin-Resistant S*taphylococcus aureus*

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Running Head: Phenotypic Approaches to Detect *mecC* MRSA

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Journal of Clinical Microbiology
JCM
Journal of Clinical Microbiology

Abstract

Similar to *mecA*, *mecC* confers resistance against beta-lactams, leading to the phenotype of a methicillin-resistant *Staphylococcus aureus* (MRSA). However, *mecC*-harboring MRSA pose special difficulties in their detection. The aim of this study was to assess and compare different phenotypic systems for screening, identification, and susceptibility testing of *mecC*-positive MRSA isolates. A well-characterized collection of *mecC*-positive *S. aureus* isolates (n = 111) was used for evaluation. Routinely used approaches were studied to determine their suitability to correctly identify *mecC*-harboring MRSA including three (semi-)automated antimicrobial susceptibility testing (AST) systems and five selective chromogenic agar plates. Additionally, a cefoxitin disk diffusion test and an oxacillin broth microdilution assay were examined. All *mecC*-harboring MRSA isolates were able to grow on all chromogenic MRSA screening plates tested. Detection of these isolates in AST systems based on cefoxitin and/or oxacillin testing yielded overall positive agreement with the *mecC* genotype of 97.3 % (MicroScan WalkAway™, Siemens), 91.9 % (Vitek 2®, bioMérieux), and 64.9 % (Phoenix™, BD). The phenotypic resistance pattern most frequently observed by AST devices was "cefoxitin resistance/oxacillin susceptibility", ranging from 54.1 % (Phoenix) over 83.8 % (Vitek 2) to 92.8 % (WalkAway). The cefoxitin disk diffusion and oxacillin broth microdilution assays categorized 100 % and 61.3 % of isolates to be MRSA, respectively. The chromogenic media tested confirmed their suitability to reliably screen for *mecC*-harboring MRSA. The AST systems showed false-negative results with varying numbers, misidentifying *mecC* MRSA as methicillin susceptible *S. aureus*. This study underlines cefoxitin's status as the superior surrogate *mecC* MRSA marker.

Introduction

The still worrying occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) in many parts of the world poses a major challenge to health care systems by increasing the burden of disease. Rapid and effective MRSA identification and susceptibility testing is paramount to prevent further dissemination and to adapt antimicrobial treatment. In 2011, a novel PBP2a-encoding *mecA* homologue designated *mecC* (originally $mecA_{LGA251}$) has been reported with homologies on the nucleotide and protein level of only 70 % and 63 %, respectively [1, 2]. Later on, *mecC* has been confirmed as the genetic determinant that confers methicillin resistance in *S. aureus* for those isolates [3]. Farm and wildlife animals have been revealed as reservoirs for *mecC* MRSA [4, 5], and the zoonotic potential of these livestock-associated MRSA has been shown [6, 7, 8].

The limited homology of *mecC* to *mecA* and their respective proteins led to major diagnostic challenges in identification and susceptibility testing of *mecC*-harboring MRSA [9]. In addition to obvious but easily resolved difficulties in targeting the divergent *mecC* nucleotide sequence by DNA-based diagnostic tests [10, 11], phenotypic approaches exhibited considerable difficulties due to comparatively low oxacillin MICs [1, 7, 8] which may be caused by differences in the *mecA* and *mecC* promoters [3]. Moreover, low homology between the encoded PBP2a proteins is the reason for the failure of existing PBP2a agglutination tests to detect *mecC*-positive isolates [5, 7, 8]

In this study, we compared several routinely applied diagnostic approaches in their capability to identify *mecC*-harboring MRSA from a comprehensive, heterogeneous, and representative collection. In detail, we compared (i) three (semi-)automated susceptibility testing (AST) systems, (ii) five selective chromogenic agar plates (MRSA screening plates), (iii) a cefoxitin disk diffusion test, and (iv) an oxacillin broth microdilution.

Results

Applicability of AST systems to detect *mecC*-positive isolates

Analyzing resistance towards cefoxitin and oxacillin by AST systems, different susceptibility patterns were observed. For all systems, the most frequently detected pattern was the combination of the categorization "cefoxitin-resistant, but oxacillin-susceptible", ranging from 54.1 % (Phoenix) over 83.8 % (Vitek 2) to 92.8 % (WalkAway) of all tested isolates (Table 1). In the WalkAway system, three isolates (2.7 %) were categorized cefoxitin- and oxacillin-susceptible, whereas in the Vitek 2 and the Phoenix system, 9 isolates (8.1 %) and 39 isolates (35.1 %), respectively, were categorized susceptible to both. One isolate was categorized as cefoxitin-susceptible and oxacillin-resistant by the Phoenix system.

The MIC_{90} values for oxacillin were ≥2 µg/ml (Phoenix), 2 µg/ml (MicroScan), and 2 µg/ml (Vitek 2). The MIC_{90} values for cefoxitin were >8 µg/ml (Phoenix) and >4 µg/ml (WalkAway); the Vitek 2 detected 91.9 % of isolates as resistant to cefoxitin without reporting an MIC value. Less than 10 % of isolates were tested resistant to both cefoxitin and oxacillin (Phoenix: 9.9 %; MicroScan: 4.5 %; Vitek 2: 8.1 %).

Applicability of chromogenic MRSA screening plates for detection of *mecC*-positive isolates

The vast majority of isolates showed typical growth on all tested cefoxitin-containing chromogenic MRSA screening plates. Reduced growth, i.e. smaller colonies, but with characteristic MRSA-indicating color, was observed for a small fraction of isolates (Table 2). Oxoid Brilliance™ MRSA 2 plates showed a mixed phenotypic appearance with blue (presumptive for MRSA) and white colonies for all isolates.

Additionally, a subset of nine isolates and positive control *S. aureus* USA 300, tested in triplicate, showed growth on screening plates from four manufacturers using an inoculum of 100 µl from of a 10^{-5} dilution of a 0.5 McFarland standard suspension (approximately 100 cfu/plate). MRSA Select™ agar plates (Bio-Rad) were not tested in this additional experiment due to supply unavailability.Negative control *S. aureus* ATCC 29213 exhibited no growth on chromogenic agar plates.

Applicability of cefoxitin disk diffusion and oxacillin broth microdilution test for detection of *mecC*-positive isolates

The cefoxitin disk diffusion test detected *mecC*-encoded methicillin resistance in 111/111 isolates, i.e. 100 %. The oxacillin broth microdilution resulted in a categorization of 43 susceptible (38.7 %) and 68 resistant (61.3 %) isolates.

Discussion

The occurrence of *mecC*-harboring MRSA has been described in several European countries in humans, companion animals, and livestock [14]. While the overall prevalence of these isolates seems to be low, it has been suspected that *mecC* prevalence might be underestimated because of its misidentification as methicillin-susceptible *S. aureus* (MSSA) due to its borderline resistant phenotype. Additionally, negative results in MRSA PCR and agglutination assays if only the *mecA* gene, i.e. PBP2a is targeted, hamper *mecC* MRSA detection efforts. Furthermore, it has been shown that the prevalence of *mecC*-positive *S. aureus* isolates increased at least in Denmark and that *mecC* MRSA isolates are also capable to cause infections in humans [4]. A reliable detection of these isolates is important to ensure both an adequate treatment of *mecC* MRSA infections and the use of the same prevention measures as already established for *mecA* MRSA. This study revealed that all chromogenic media and the cefoxitin disk diffusion test were able to categorize all *mecC*-positive MRSA properly. Additionally, we were able to show for a subset of strains that inocula as low as approximately 100 cfu per plate result in growth on chromogenic media, indicating that a recovery from clinical swab samples with low MRSA loads can likely be achieved. However, these findings are limited because they could mimic the usual clinical specimen as encountered in the laboratory only partially. To varying degrees, all three AST systems displayed limitations in the ability to detect *mecC* MRSA. While the detection rate of WalkAway (97.3 %) was also high, the Vitek 2 (91.9 %) and particularly the Phoenix system (64.9 %) showed considerably lower rates. A study by Cartwright *et al.* showed a detection rate of 88.7 % (n = 62 *mecC* MRSA) for the cefoxitin-resistant/oxacillin-susceptible pattern using the Vitek 2 [15]; similarly, this AST device detected this pattern in 83.8 % of the tested isolates in our study. The oxacillin broth microdilution performed poorly, showing a detection rate of only 61.3 %. This is in accordance with previous studies [16].

In conclusion, automated systems may fail to detect *mecC*-encoded methicillin resistance, while all chromogenic screening media displayed colonies presumptive for MRSA growth. In comparison to oxacillin, cefoxitin was confirmed as superior surrogate marker to detect *mecC*-harboring MRSA isolates. Discrepancies between positive screening results based on the use of chromogenic media and categorization as methicillin-susceptible by AST systems should be verified by molecular assays or disk diffusion.

Material and Methods

A large set of *mecC*-harboring MRSA isolates (n = 111) from human and animal specimens isolated in Germany, the United Kingdom, and Belgium were included in the study. All isolates were confirmed as *mecC*-positive by PCR [12] and characterized by *spa*-typing (t843, n = 51; t6292, n = 13; t1736, n = 6; t1535, n = 4; t3391, n = 3; t978, t9165, t742, t6902, t6521, t6220, t5930, t1773, t11706, n = 2 each; t9910, t9738, t9280, t9123, t8842, t7914, t7603, t7189, t6300, t524, t13233, t1207, t11702, t11290, t11120 and not typeable, n = 1 each). Isolates were of human (n = 80), unknown (n = 24), bovine/bulk milk (n = 4), sheep (n = 2), and environmental (n = 1) origin. No copy isolates were included.

Selective chromogenic agar plates (1. Oxoid: Brilliance™ MRSA 2; 2. bioMérieux: chromID® MRSA; 3. BD: BBL™ CHROMagar® MRSA II; 4. Bio-Rad: MRSA Select™; 5. MAST Diagnostica: CHROMagar™ MRSA) were inoculated with a single colony from overnight blood agar plate cultures. To simulate potentially low inocula of clinical specimens, nine isolates with different *spa*-types (t843, t978, t1207, t1535, t1736, t391, t5930, t6292 and t6902) were each adjusted to 0.5 McFarland standard turbidity and serial dilutions with the final dilution factor of 10^{5} were prepared. Subsequently, 100 µl of the final dilutions were used to inoculate all chromogenic media (except MRSA Select™ from Bio-Rad due to supply constraints) and blood agar plates for growth control in triplicate. *S. aureus* strains USA300 and ATCC29213 were used as positive and negative controls, respectively. Growth was evaluated after 24 h and 48 h. Automated systems were inoculated from the same plates as chromogenic media. Automated systems for susceptibility testing were used according to the manufacturers' recommendations, i.e. the BD Phoenix™ (Becton Dickinson, Heidelberg, Germany) was executed with the test panel PMIC-72, the Vitek 2® (bioMérieux, Marcy l'Etoile, France) with the test panel AST P580, and the MicroScan WalkAway® 96 plus (Siemens Healthcare Diagnostics, Eschborn, Germany) with the test panel Pos MIC 28.

Cefoxitin disk diffusion assays (Cefoxitin discs, 30 µg, bestbion dx, Cologne, Germany) were performed according to EUCAST and using *S. aureus* ATCC 29213 as control. The EUCAST guidelines (version 7.0, valid from 01.01.2017: Inhibition zone of <22 mm, resistant) and CLSI criteria (M100-S27, Twenty-seventh Edition, January 2017: inhibition zone of ≤21 mm, resistant) were followed in the interpretation of the results.

Oxacillin (Sigma-Aldrich, Taufkirchen, Germany) susceptibility was determined by broth microdilution, using a final inoculum of approximately 5×10^5 CFU/ml and *S. aureus* ATCC 29213 as quality control. MICs were interpreted according to EUCAST guidelines (version 7.0, valid from 01.01.2017: MIC >2 µg/ml) and CLSI criteria (M100-S27, Twenty-seventh Edition, January 2017: MIC ≥4 µg/ml).

Acknowledgements

We are grateful to B. Grünastel and F. Erdmann for expert technical assistance. This work was supported in part by grants from the German Ministry for Education and Research (BMBF 03ZZ0802H (K.B.) and 03ZZ0805B (K.B.) and performed under the auspices of the Paul Ehrlich Gesellschaft für Chemotherapie, Section "Basics" and its working groups "Susceptibility testing and resistance" (G.W.) and "Staphylococcal infections" (K.B.). The Instruments Phoenix™ (BD) and MicroScan WalkAway® (Siemens) were provided free of charge during the study. The authors declare no conflict of interest.

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Table 1: Susceptibility pattern testing cefoxitin and oxacillin for *mecC*-positive *S. aureus* isolates (n = 111)

Cefoxitin/oxacillin susceptibility pattern[a]	Number and (% agreement) of isolates tested by[b]		
	Phoenix	MicroScan WalkAway	Vitek 2
R/R	11 (9.9 %)	5 (4.5 %)	9 (8.1 %)
R/S	60 (54.1 %)	103 (92.8 %)	93 (83.8 %)
S/R	1 (0.9 %)	0 (0.0 %)	0 (0.0 %)
Total R[c]	72 (64.9 %)	108 (97.3 %)	102 (91.9 %)
S/S	39 (35.1 %)	3 (2.7 %)	9 (8.1 %)

[a] R, resistant; S, susceptible;

[b] *S. aureus* ATCC 29213 (MSSA) and *S. aureus* ATCC 43300 (MRSA) were used as quality control strains. Both were correctly categorized by all three systems;

[c] Positive agreement based on resistance to at least one of the compounds tested (cefoxitin or oxacillin).

256 Table 2: Growth on selective chromogenic agar media

Chromogenic agar[a]	Number of isolates (n) and (% agreement) with		
	Normal growth[b]	Reduced growth[c]	No growth
Brilliance™ MRSA 2	111 (100 %)	0 (0.0 %)	0 (0.0 %)
chromID® MRSA	111 (100 %)	0 (0.0 %)	0 (0.0 %)
BBL™ CHROMagar® MRSA II	101 (91.0 %)	10 (9.0 %)	0 (0.0 %)
MRSA Select™	105 (94.6 %)	6 (5.4 %)	0 (0.0 %)
CHROMagar™ MRSA	99 (89.2 %)	12 (10.8 %)	0 (0.0 %)

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258 [a] *S. aureus* ATCC 29213 (MSSA) and *S. aureus* ATCC 43300 (MRSA) were used as quality
259 control strains;

260 [b] According to the respective manufacturer's instructions;

261 [c] Colonies with smaller size, but with color change as indicated for MRSA.