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1 **Evaluation of a Fourth Generation Latex Agglutination Test for**
2 **Identification of *Staphylococcus aureus*.**

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23

1 ***Introduction***

2 Infections with *Staphylococcus aureus* are common, both inside and outside the hospital, and
3 can have serious medical consequences if not diagnosed in a timely manner. Therefore, in
4 clinical microbiology laboratories quick and reliable identification of *S. aureus* is a major
5 quality characteristic. Differentiating *S. aureus* from other staphylococci has traditionally
6 been done using a coagulase tube test. Although this test is reliable, it takes up to 24 hours to
7 provide results. Since the late 70's several alternative procedures have been developed with
8 variable success. In 1980 the first latex agglutination assay with accurate identification of *S.*
9 *aureus* was introduced by Esser and Radebold [1]. This assay is based on detection of both
10 coagulase activity (clumping factor) and Protein A, both being specific determinants of *S.*
11 *aureus*. In the last 30 years several commercial assays based on this principle have been
12 launched. However, certain methicillin resistant *S. aureus* (MRSA) strains produced false
13 negative results in some agglutination assays. In attempt to overcome this problem 'third
14 generation' latex agglutination assays have been developed that also detect capsular
15 polysaccharide 5 and 8, specific antigens present on the surface of up to 80% of *S. aureus*
16 strains [2]. In effect detection of capsular polysaccharide 5 and 8 has demonstrated to improve
17 the detection of MRSA strains [3].

18 Recently the Staph Plus Latex Kit (DiaMondiaL (DML), Sees, France), a new latex
19 agglutination assay for identification of *S. aureus* has been marketed. In order to further
20 reduce the number of non-specific reactions and hence false negative test results, in this
21 fourth generation assay newly developed blue carboxylated microparticles were used in
22 combination with detection of coagulase, Protein A and capsular polysaccharide 5 and 8.
23 In this study we compared the performance of the Staph Plus Latex Kit to three third-
24 generation latex assays (A) on a well-defined collection of staphylococci and (B) in daily
25 practice in a routine microbiology laboratory.

1 ***Materials and Methods***

2 In order to analyze both agglutination performance on different individual strains and
3 staphylococcal subtypes versus practical assay performance during daily routine, evaluation
4 of the DiaMondiaL Staph PLUS Latex KitTM (DML Staph) was performed in two separate
5 sub-studies: (A) evaluation of the DML Staph performance on a predefined staphylococcal
6 strain collection ('strain study'), comparing DML Staph results to historical data of three
7 third-generation latex agglutination assays, and (B) comparing DML Staph with Slidex Staph
8 Plus on consecutive clinical staphylococcal strains in a routine microbiology laboratory
9 ('daily practice study').

10

11 *Strain study*

12 The collection of staphylococcal strains used in the 'strain study' was derived from two
13 previously published studies by Van Griethuysen et al [4;5]. From these studies historical data
14 were available on the performance of three third-generation latex agglutination assays: (I)
15 Slidex Staph Plus (bioMerieux), (II) Staphaurex Plus (Murex Diagnostics) and (III) Pastorex
16 Staph-Plus (Sanofi Diagnostics Pasteur). In total 265 Coagulase Negative Staphylococci
17 (CNS) and 528 *S. aureus* strains (266 methicillin-resistant (MRSA) and 262 methicillin-
18 susceptible *S. aureus* (MSSA)) were conserved and available for use in this study. All isolates
19 were derived from human clinical isolates. Amongst the CNS strains were a wide variety of
20 subspecies was available (table 1).

21 All strains were defined in the Van Griethuysen studies by the same principle [4;5]: If the
22 tube coagulase test and all three above-mentioned latex agglutination tests were positive, the
23 isolate was considered to be *S. aureus*. If all tests were negative, the isolate was considered to
24 be a CNS and further identification to the species level was determined with the ID32 Staph
25 (bioMerieux). If the results of the tube coagulase test and the latex agglutination tests were

1 discordant, these tests were repeated from a new subculture and an ID32 Staph test, and either
2 an Accuprobe culture identification test in one study [5] (Gen-Probe, San Diego, Calif.) or
3 coagulase gene PCR in the other study [4] (see further for specifications) were performed.
4 The result of either the AccuProbe or coagulase gene PCR was considered to be the “gold
5 standard.” MRSA strains were defined by the presence of the *MecA* gene and the coagulase
6 gene by multiplex PCR [4;5].
7 The collection of strains was stored by -70°C since the year 2000. Shortly before testing, all
8 isolates were subcultured from the -70°C freezer onto Columbia agar +5% sheep blood
9 (bioTrading, Mijdrecht, The Netherlands). Subsequently all strains were subcultured one
10 more time on the same media to obtain fresh growth.
11 The freshly cultured strains were tested using the DML Staph according to the manufacturer’s
12 instructions in a random fashion: the performing laboratory physician was not aware of the
13 strain type during testing. The assay consists of a negative control reagent and a test reagent
14 containing blue carboxylated microparticles sensitized with human fibrinogen and
15 monoclonal antibodies for simultaneous detection of coagulase activity, staphylococcal
16 Protein A and capsular polysaccharides 5 and 8. Every strain was tested using a negative
17 control latex reagent to exclude non-specific agglutination. A test was considered positive if
18 there was visible agglutination and clearing of the background and no agglutination in the
19 control reagent. One person performed all tests on ten separate days during a three week
20 period and results were recorded in a spreadsheet (Excel).
21 All isolates with discordant test results of the DML Staph were retested for deoxyribonuclease
22 (DNase) activity (in house product) [6], coagulase activity (Coagulase Plasma Rabbit, Becton
23 Dickinson (Bedford, MA, USA)), the presence of the *MecA* gene and *S. aureus* specific
24 genomic DNA (PCR) to confirm identification of the specific strain. The *MecA/S. aureus*-
25 PCR was developed ‘in-house’ and consisted of simultaneous detection (LightCycler 2.0,

1 Roche) of the *MecA* gene [7] and *S. aureus* specific genomic DNA [8] Primers and probes
2 were designed as follows: *MecA* gene (forward primer 5'-GAT-CGC-AAC-GGT-CAA-TTT-
3 AAT-TTT-G-3', reversed primer 3'-GCT-TTG-GTC-TTT-CTG-CAT-TCC-T-5' and
4 fluorescent (FAM) Taqman probe FAM-GGT-ATG-TGG-AAG-TTA-GAT-TGG-GAT-CAT-
5 AGC-GTC-BHQ1) and *S. aureus* specific genomic DNA (forward primer 5'-CAT-CGG-
6 AAA-CAT-TGT-GTT-CTG-TAT-G-3', reversed primer 3'-TTT-GGC-TGG-AAA-ATA-
7 TAA-CTC-TCG-TA-3' and Yakima Yellow labelled Taqman probe YY-AAG-CCG-TCT-
8 TGA-TAA-TCT-TTA-GTA-GTA-CCG-AAG-CTG-GT-BHQ1).

9 For all four latex agglutination assays the interpretative reading comprised of either a positive,
10 negative or indeterminate test result. If the agglutination was indeterminate, the strain would
11 be excluded from statistical analysis. Statistical analysis was performed using the McNemar
12 test (two-tailed, GraphPad Inc. Software 2005) and all p-values were calculated versus the
13 DML Staph.

14

15 *Daily practice study*

16 During a three month period in our clinical microbiology laboratory, presumptive
17 identification of staphylococcal strains was performed by using both the Slidex Staph Plus
18 and the DML Staph. All staphylococcal strains from cultures of consecutive clinical
19 specimens were handled according to the laboratories' routine procedures. Both agglutination
20 assays were performed by any of the 30 laboratory physicians during daily practice. All
21 isolates were included based upon inspection of colony morphology: if a staphylococcal
22 isolate was suspected, both agglutination tests were performed in conjunction with the DNase
23 test, coagulase test and a cefoxitin disc susceptibility test on Mueller-Hinton agar plate (30µ,
24 cut-off zone 22 mm, Oxoid Ltd., Basingstoke, United Kingdom) or automated susceptibility
25 testing (bioMerieux Vitek, Inc., Hazelwood, Mo). Strains with a negative Slidex Staph Plus,

1 DML Staph, DNase and coagulase test were determined as CNS; Strains with a positive
2 result in all tests were determined as *S. aureus*: determination of MSSA or MRSA was
3 defined by cefoxitin susceptibility or resistance respectively. All isolates with discordant test
4 results of either agglutination assay, DNase or coagulase result were retested for DNase
5 activity, coagulase activity, the presence of the *MecA* gene and *S. aureus* specific genomic
6 DNA (PCR) to confirm identification of the specific strain, analogous to the 'strain study'.
7 Interpretation and statistical analysis was performed analogous to the 'strain study'.
8

1 **Results**

2 *Strain study*

3 In all isolates tested with the DML Staph a clear interpretational reading of the agglutination
4 could be made (see Tabel 1). In contrast, in 0.6, 0.4 and 1.5% of all the strains tested using the
5 Slidex Staph Plus, Staphaurex Plus or Pastorex Staph-Plus, respectively, the result was
6 doubtful and no final interpretation could be made. After excluding the strains with
7 indeterminate test results, the results of all four assays were statistically analyzed. The overall
8 sensitivity was highest for the DML Staph with 99.2%. Within the MRSA strains subset
9 sensitivity of the DML Staph was also highest (sensitivity 98.8%) compared to the other
10 assays. However, sensitivity of the Slidex Staph Plus was highest (100%) in MSSA strains,
11 followed closely by the DML Staph sensitivity (99.6%). In the statistical analysis only the
12 Staphaurex Plus sensitivity was significantly lower ($p=0.0001$). In the sub analysis this
13 significantly lower sensitivity could be attributed to a lesser performance in the MRSA strain
14 subset especially.

15 Specificity of the DML Staph was unremarkable (100%) for all strains altogether and for
16 MSSA and MRSA strains specifically. Similar specificity was calculated for Slidex Staph
17 Plus and Staphaurex Plus, however the Pastorex Staph-Plus showed a less than perfect
18 specificity (98.8%).

19 The DML Staph failed to display agglutination in five *S. aureus* strains (false negatives: 1
20 MSSA and 4 MRSA). Subsequent testing of these strains confirmed the original identification
21 as either MSSA (DNase positive, coagulase positive, Meca gene negative, *S. aureus*
22 genomic DNA positive) or MRSA (DNase positive, coagulase positive, Meca gene positive,
23 *S. aureus* genomic DNA positive). Between all four latex assays only the Pastorex Staph Plus
24 had shown false-positive reactions: two *S. lugdunensis* and one *S. capitis* strain.

25

1 *Daily practice study*

2 In total 1865 staphylococcal strains (883 CNS, 847 MSSA and 135 MRSA) were tested with
3 both the DML Staph and the Slidex Staph Plus. Forty-five strains (41 CNS, 2 MSSA, 2
4 MRSA) were excluded from statistical analyses because one or both agglutination tests were
5 indeterminate. The DML Staph was indeterminate in 20 strains (1.1%): 17 CNS, one MSSA
6 and two MRSA strains. The Slidex Staph Plus was indeterminate in 36 strains (1.9%): 34
7 CNS, one MSSA and one MRSA. In 11 CNS strains both the DML Staph and the Slidex
8 Staph Plus were indeterminate.

9 Overall sensitivity of the DML Staph and Slidex Staph Plus was similar compared to the
10 'strain study' results. Similarly, the NPV was comparable for both tests in both studies.
11 However, in the MRSA strain subset sensitivity of the DML Staph was less than of the Slidex
12 Staph Plus, whereas in the strain study this was the other way around. Specificity of the
13 Slidex Staph Plus was significantly higher than the DML Staph ($p < 0.0005$). Similarly, the
14 PPV of the DML Staph was significantly lower than the Slidex Staph Plus.

15 The false positive results in both the DML Staph and the Slidex Staph Plus were mostly found
16 in MRSA strains: 5/1 (MRSA/MSSA) and 3/0 (MRSA/MSSA), respectively. In two MRSA
17 strains both test produced false negative results. The DML Staph and the Slidex Staph Plus
18 produced false positive results in 36 and 14 CNS strains, respectively, where all 14 CNS
19 strains were false positive in both tests.

20 Remarkably, in 30 CNS strains DNase and coagulase were false positive (negative *S.*
21 *aureus*/MecA PCR), where both the DML Staph and the Slidex Staph Plus gave a true
22 negative result. In 29 cases this CNS was derived from cultures taken from veterinarians as
23 part of MRSA surveillance.

24

1 ***Discussion***

2 In 2001 an international multicenter evaluation of latex agglutination tests for identification of
3 *S. aureus* was published by Van Griethuysen et al [5]. In that study the Slidex Staph Plus
4 (bioMerieux) was compared to Staphaurex Plus (Murex Diagnostics) and Pastorex Staph-Plus
5 (Sanofi Diagnostics Pasteur). In addition to clumping factor and Protein A, all three assays
6 also detect certain surface antigens: Slidex Staph Plus and Staphaurex Plus detect group
7 specific antigens on the *S. aureus* cell surface, and Pastorex Staph Plus detects capsular
8 polysaccharides 5 and 8. In the ‘strain study’ the DML Staph assay (DiaMondiaL)
9 performance was superior compared to the other three latex agglutination assays in
10 differentiating *S. aureus* from CNS, in the same strain collection as was used by Van
11 Griethuysen et al. Overall sensitivity and specificity of Slidex Staph Plus was comparable,
12 however, in contrast to the DML Staph, it failed to produce a definite result in 0.6% of all
13 isolates. This leads to additional confirmatory testing and costs.

14 Twenty-nine CNS strains gave false positive results in the van Griethuysen study [5]. It was
15 suggested that especially *S. lugdunensis*, *S. schleiferi* and *S. haemolyticus* were prone for false
16 positive test results. However, in this study only three false positives were found and only
17 when using the Pastorex Staph Plus. Since the van Griethuysen study was an international
18 collaboration, only the Dutch subset of the strain collection originally used was available for
19 this study and consequently the number of false positives can not be compared. Local
20 epidemiology may affect the performance of diagnostic tests and this should be borne in mind
21 when translating the results of studies into routine diagnostics.

22 In the ‘strain study’ detection of MSSA strains was very accurate in all four assays. The DML
23 Staph performed particularly well in MRSA strains: sensitivity and specificity was superior
24 compared to the other three assays. In literature several reports have suggested that additional
25 detection of capsular polysaccharide 5 and 8, similar to the DML Staph assay, could explain

1 better performance of such assays in MRSA strains [2;9]. However, the Pastorex Staph-Plus
2 assay also detects capsular polysaccharide 5 and 8, but appeared to be only slightly less
3 effective than the DML Staph: sensitivity in the MRSA strain subset was 97.3% versus
4 98.8%, respectively (not significant). The Slidex Staph Plus and Staphaurex Plus assays have
5 also been constructed to detect additional surface antigens of *S. aureus*. In the ‘strain study’
6 both assays performed less well (sensitivity 96.1% and 91.3%) than the DML Staph (98.8%)
7 in detecting MRSA, however only for Staphaurex Plus this reached statistical significance.
8 The microparticles in the DML Staph assay were especially engineered to enhance antibody-
9 antigen complex formation. This may explain the increase in sensitivity compared to the third
10 generation assays.

11 In order to rule out observational bias by performing DML Staph by a single person, a
12 secondary study was performed. In this ‘daily practice study’ the results of the DML Staph
13 were different than in the ‘strain study’. In the former the DML Staph appeared superior,
14 whether in the latter the Slidex Staph Plus performed significantly better. In the daily practice
15 study differences between the DML Staph and Slidex Staph Plus were almost exclusively
16 attributable to a significant difference in the number of false positives: the DML Staph
17 displayed positive agglutination in 36 CNS strains versus 14 in the Slidex Staph Plus. This is
18 why these agglutination assays should always be used for presumptive identification only and
19 in combination with other tests like DNase and coagulase tests for definite results. Although
20 performance of the Slidex Staph Plus appeared superior in the daily practice study, in both
21 sub-studies the number of indeterminate results was considerably higher for the Slidex Staph
22 Plus. Taking this in account, in daily practice the DML Staph and Slidex Staph plus have
23 equal performance characteristics.

24 A remarkable subset of thirty CNS strains was found in the ‘daily practice study’, where both
25 agglutination assays gave true negative results in contrast to the DNase and coagulase test.

1 All but one of these strains was isolated from veterinarians during a targeted MRSA
2 surveillance project. It is known that *S. intermedius* and *S. hyicus* strains can also secrete
3 coagulase during bacterial growth [10]. Furthermore, *S. intermedius* and *S. hyicus* are well
4 known for their pathogenicity in animals but not in humans [11].
5 Overall, the results of the fourth generation latex agglutination assay proved to be comparable
6 to third generation assays, both in sensitivity/specificity and ease to identify *S. aureus* strains.

7

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1 **Table 1**

2 Table 1 displays the number of different Staphylococcal subspecies among the coagulase
3 negative strains.

4

Staphylococcal species	No. tested
S. epidermidis	136
S. hominis	36
S. lugdunensis	24
S. capitis	20
S. warneri	8
S. xylosus	5
S. haemolyticus	5
S. saprophyticus	4
S. simulans	2
S. sciuri	2
S. caprae	2
S. cohnii	3
S. auricularis	1
CNS undetermined	17
Total	265

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1 **Table 2**

2 Table 2 displays the results of the DML Staph in the ‘strain study’, comparing the results with
 3 three third-generation latex assays. The golden standard determination (CNS, MSSA, MRSA)
 4 of the non-interpretable strains is shown; these strains were excluded from further statistical
 5 analysis. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value
 6 (NPV) are shown. The p-value was calculated two-tailed using the McNemar’s test for any of
 7 the third-generation assays versus the DML Staph.

8

n=793	DML Staph			Slidex Staph Plus			Staphaurex Plus	
Non-interpretable strains (%)	0 (0)			3x CNS; 2x MRSA (0.6)			3x MRSA (0.4)	
n=782	All	MSSA	MRSA	All	MSSA	MRSA	All	MSSA
Sensitivity (%)	99.2	99.6	98.8	98.1	100	96.1	95.2	99.2
Specificity (%)	100	100	100	100	100	100	100	100
PPV (%)	100	100	100	100	100	100	100	100
NPV (%)	98.4	99.6	98.8	96.1	100	96.1	91.3	99.2
*p-value	-	-	-	0.1489	0.2482	0.1489	0.0001	0.4795

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1 **Table 3**

2 Table 3 displays the results of the DML Staph versus the Slidex Staph Plus in the ‘dialy
 3 practice study’. The golden standard determination (CNS, MSSA, MRSA) of the non-
 4 interpretable strains is shown; these strains were excluded from further statistical analysis.
 5 Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)
 6 are shown. The p-value was calculated two-tailed using the McNemar’s test for the Slidex
 7 Staph Plus versus the DML Staph.

8

N=1865	DML Staph			Slidex Staph Plus		
Non-interpretable strains (%)	17x CNS; 1x MSSA; 2x MRSA (1.1)			34x CNS; 1x MSSA; 1x MRSA (1.9)		
n=1820	All	MSSA	MRSA	All	MSSA	MRSA
Sensitivity (%)	99.4	99.9	96.2	99.7	100	97.7
Specificity (%)	95.7	95.7	95.7	98.3	98.3	98.3
PPV (%)	96.4	95.9	78.1	98.6	98.4	90.3
NPV (%)	99.3	99.9	99.4	99.6	100	99.6
*p-value	-	-	-	0.0005	0.0000	0.0002

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