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1 **Evaluation of isolation procedures and chromogenic agar media for detection**  
2 **of MRSA in nasal swabs from pigs and veal calves.**

3  
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## 31 Abstract

32 Since the emergence of MRSA in livestock, screening of animals for the detection of MRSA is widely  
33 practised. Different procedures are published for animal samples but a systematic comparison of  
34 methods has not been performed. The objective of this study was to compare three available  
35 commonly used procedures and three chromogenic agars for detecting MRSA in nasal swabs from  
36 pigs (n=70) and veal calves (n=100). Procedures 1 and 2 used a pre-enrichment comprising Mueller  
37 Hinton broth with 6.5% NaCl followed by selective enrichment with 4 µg/ml oxacillin + 75 µg/ml  
38 aztreonam (Procedure 1) and 5 µg/ml ceftizoxime + 75 µg/ml aztreonam (Procedure 2) respectively.  
39 Procedure 3 used a selective enrichment broth only, containing 4% NaCl, 5 µg/ml ceftizoxime + 50  
40 µg/ml aztreonam. After selective enrichment, media were streaked on to three different chromogenic  
41 agars. Significantly more MRSA were found for pig as well as for veal calf samples with procedures 1  
42 and 2. No significant differences were found between procedures 1 and 2. For nasal swabs from pigs  
43 significantly more MRSA positive samples were found when MRSA Screen (Oxoid) or MRSASelect™  
44 (Bio-Rad) agars were used compared to MSRA ID (bioMérieux). For calf samples no significant  
45 differences between the different agars were found.

46 In conclusion, the results of this study show that procedures 1 and 2, both using additional high salt  
47 pre-enrichment are superior and should be recommended for MRSA detection in nasal swabs from  
48 pigs and veal calves. The preferred choice of chromogenic agar depends on the sample matrix.

49  
50 *Keywords:* Chromogenic media, Methicillin Resistant *Staphylococcus aureus*; MRSA, enrichment, pig,  
51 veal calves

52

## 53 Introduction

54 The prevalence of Methicillin Resistant *Staphylococcus aureus* (MRSA) is increasing world-wide,  
55 especially since the emergence of community-acquired and animal related MRSA (Khanna et al.,  
56 2008; Nahimana et al., 2006; Tiemersma et al., 2004). Recently, a specific MRSA clone has been  
57 reported at unexpected high prevalence among pig farmers and veterinarians in different geographical  
58 areas (Voss et al., 2005; Weese and van Duijkeren 2009). Strains belonging to this clone are resistant  
59 to *Sma*I macrorestriction and therefore referred to as non-typable (NT-MRSA). They all belong to Multi  
60 Locus Sequence Type 398 (ST398) and show closely related *spa* types (mainly t011, t108 and t1254)  
61 (De Neeling et al., 2007). A case control study showed that pig and cattle farmers have an increased  
62 risk for being positive for ST398 (Van Loo et al., 2007). The source of these human infections can be  
63 found in the pig population and veal calves.

64 Screening for MRSA among various human populations with increased risk has become important for  
65 control of nosocomial infections. In human health care settings, studies have shown that different  
66 procedures employed for the detection of MRSA from clinical specimens have varying results  
67 depending on the isolation methods used (Brown et al., 2005). For animal samples less is known  
68 about differences between MRSA detection procedures, in particular on the detection of ST398 in pig  
69 and veal calf samples.

70 Three existing commonly used procedures are applied for MRSA screening in pig samples (De  
71 Neeling et al., 2007 (procedure 1)) and human samples (Wertheim et al., 2001; with additional pre-  
72 enrichment (procedure 2)), (Van Duijkeren et al., 2008 (procedure 3)). To ascertain the performance of  
73 these MRSA detection methods, we conducted a study to compare three different procedures for the  
74 isolation of ST398 and the usefulness of three different chromogenic agar media. Nasal swabs of pigs  
75 and veal calves were used as matrix.

76

## 77 Materials and Methods

### 78 Survey on the farms

79 Between April and May 2007, nasal swabs (Cultiplast®) were collected in duplicate from 70 pigs at  
80 seven different swine farms (10 pigs each farm) and 100 nasal swabs from veal calves were collected  
81 at three different veal farms (approximately 30 calves each barn) in The Netherlands. On each farm  
82 the animals were selected and sampled of convenience. From each animal, two nasal swabs were

83 taken each from both nares. Collecting animal samples was in accordance with the animal welfare  
84 law.

85

86 Bacterial procedures

87 A total of 70 pig samples and 100 veal calf samples were analysed using 3 different procedures and 3  
88 different agars. In total 630 plates (70 samples x 3 procedures x 3 plating agars) were read for the pig  
89 samples and 900 plates (100 samples x 3 procedures x 3 plating agars) were read for the veal calf  
90 samples.

91 Swabs were transported to the laboratory and processed within 4 hours after collection. Because  
92 procedures 1 and 2 used the same pre-enrichment step, one of the duplicate nasal swabs of each  
93 animal was used for analysis in procedures 1 and 2, and the other nasal swab for analysis by  
94 procedure 3 (Figure 1). Assignment of the first and second swab of each animal over the procedures  
95 was of convenience.

96 **Procedures 1 and 2:** Swabs tested for procedures 1 and 2 were individually inoculated into tubes  
97 containing a pre-enrichment with 5 ml Mueller Hinton Broth (MH<sup>+</sup> broth) (Becton Dickenson),  
98 containing 6.5% NaCl. This broth was incubated at 37°C, overnight. Thereafter, the pre-enrichment  
99 was split into 2 procedures (procedures 1 and 2).

100 **Procedure 1:** 1 ml of the pre-enrichment was transferred into 9 ml phenyl mannitol broth (PHMB/oa<sup>+</sup>)  
101 (Brunschwig Chemie, Amsterdam) with 4 µg/ml oxacillin (Sigma) and 75 µg/ml aztreonam (ICN). This  
102 broth was freshly prepared daily. This broth was incubated overnight at 37°C and then 10 µl of the  
103 PHMB/oa<sup>+</sup> broth was plated onto the agars mentioned below.

104 **Procedure 2:** 1 ml of the pre-enrichment was transferred into tubes containing 9 ml phenyl mannitol  
105 broth (PHMB/ca<sup>+</sup>) (bioMérieux) with 5 µg/ml L ceftizoxime and 75 µg/ml aztreonam. After overnight  
106 incubation 10 µl of this PHMB/ca<sup>+</sup> broth was plated onto the agars mentioned below.

107 **Procedure 3:** the duplicate swab was inoculated into a tube with 5 ml MRSA broth containing, tryptic  
108 soy broth, 4% NaCl, 1% mannitol, phenol red (16 µg/ml), aztreonam (50 µg/ml) and ceftizoxime  
109 (5 µg/ml). After incubation 48 hours at 37°C, 10 µl of the MRSA broth was plated onto the agars  
110 mentioned below.

111 **Chromogenic agars:** Three different chromogenic agars were applied: (i) MRSA Screen (Oxoid), (ii)  
112 MRSASelect<sup>TM</sup> (Bio-Rad) and (iii) MRSA ID (bioMérieux). Since Oxoid has optimised the MRSA

113 Screen plate recently, also a selection of the calve samples was streaked out onto the Brilliance™  
114 MRSA agar .

115 After 24 hours and 48 hours incubation 37°C plates were read according to the recommendations of  
116 the respective manufactures (technical files). Characteristic MRSA colonies are blue on MRSA  
117 Screen, large and green on MRSA ID, and small and pink on MRSASelect™.

118 Suspected colonies were subcultured on blood agar and subsequently identified using standard  
119 techniques, colony morphology and slide coagulase test. A selection of the coagulase-positive  
120 colonies were tested by PCR for the presence of the *S. aureus* specific DNA fragment (Martineau et  
121 al., 1998). All coagulase-positive colonies were tested by PCR for the presence of the *mecA* gene (De  
122 Neeling et al., 1998

123 Additionally, to investigate the effect of selective enrichment after pre-enrichment in MH<sup>+</sup> broth, all  
124 non-selective pre-enrichment calf samples were also streaked out directly onto plates.

125 Furthermore, the detection limit of procedures 1 and 2 was determined by spiking MRSA-negative pig  
126 and calf samples with MRSA (clinical isolate spa type t011). This was done using serial dilutions from  
127 a suspension with a optical density of 0,1 Å with parallel plating onto non-selective agar to determine  
128 the CFUs.

129

130 Typing

131 In a study to indentify the optimal procedure it is important to know what MRSA types are analysed.  
132 Therefore the isolates were *spa*-typed by sequencing the repetitive region of the protein A gene *spa*  
133 (Harmsen et al., 2003). Data were analyzed by using the Ridom Staphtype software version 1.4  
134 ([www.ridom.de/staphtype](http://www.ridom.de/staphtype)).

135

136 Statistical analysis

137 We tested differences for statistical significance by a logistic regression on the outcome of the  
138 analyses on procedure and agar using the GENMOD Procedure, of SAS software 9.1. A P value of <  
139 0.05 was considered statistically significant. In all analyses correlations between repeated  
140 measurements within one animal were taken into account.

141

142 Results

## 143 Pigs

144 Out of 70 samples we detected 46 (66%) MRSA-positive swabs with procedure 1, 46 (66 %) with  
145 procedure 2, and 32 (46%) with procedure 3. We detected statistically significant less MRSA-positive  
146 samples with procedure 3 compared to the procedures 1 and 2 ( $P=0.0002$ ). Furthermore there was a  
147 statistically significant effect of the type of agar used. Statistically significant less MRSA-positive  
148 samples ( $P=0.0016$ ) were found using MRSA ID. No statistically significant differences between  
149 procedures 1 and 2, and between MRSA Screen and MRSASelect™ were found. We detected most  
150 MRSA positive samples from pigs with procedure 1 combined with the MRSA Screen agar and with  
151 procedure 2 and the MRSASelect™ agar (both 46 (66 %)) (Table 1).

## 152 Calves

153 Out of 100 samples we found 24 (24%) positive samples with procedure 1, 31 (31%) with procedure 2  
154 and 15 (15%) with procedure 3. Statistically significant less positive samples were detected using  
155 procedure 3 ( $P=0.0014$ ). No significant differences between agars were found. Although not  
156 statistically significant, we detected most MRSA-positive samples with procedure 2 combined with the  
157 MRSA ID agar (Table 2).

158

159 Streaking out the pre-enrichment (MH<sup>+</sup> broth) of the calves samples directly onto plates resulted in  
160 lower yield compared to both procedures 1 and 2. On average 9% more positive samples were found  
161 after an additional selective enrichment. However, a few positive (2%) samples were detected after  
162 MH<sup>+</sup> enrichment, which were not detected after selective enrichment (data not shown).

163 No differences were observed with respect to the MRSA Screen plate and Brilliance™ (both Oxoid)  
164 when analysing veal calve samples (data not shown).

165

## 166 Detection limit

167 The detection limit of procedures 1 and 2 was determined by spiking MRSA-negative pig and calf  
168 nasal swabs. Both in pig as well as in calf samples, MRSA was recovered with a detection limit of 1-10  
169 CFU per sample.

170

## 171 Discussion



172 This study shows that out of the three commonly used MRSA screening procedures, the procedures 1  
173 and 2, both using an additional pre-enrichment containing Mueller Hinton with 6.5% NaCl in  
174 combination with a selective enrichment, resulted in statistically significant additional yield of MRSA in  
175 pig as well as veal calf nasal swab samples compared to the screening procedure in which the sample  
176 is directly inoculated in a selective enrichment broth. In pig samples, a higher rate of positive samples  
177 was found using MRSA Screen or MRSASelect™ agar plates compared to MRSA ID agar. No  
178 statistically significant differences between plates were obtained for veal calf nasal swabs. A  
179 comparison was made between MRSA Screen plate and Brilliance™ (both Oxoid) for veal calve  
180 samples only. The results showed that the optimized Brilliance™ plate is comparable to the Screen  
181 plate for this matrix.

182 Spa-typing showed that all isolates were of the previously reported animal-related spa-types (*spa*-  
183 types mainly t011, t034, t108) belonging to clone ST398 (data not shown). NaCl-containing pre-  
184 enrichment media were used because of the inhibitory activity to many non-staphylococcal organisms  
185 and the fact that staphylococci can multiply in the presence of salt. For human samples an enhanced  
186 sensitivity and an additional yield of MRSA in human clinical specimens was also reported, using salt-  
187 containing pre-enrichment before plating (Gardam et al., 2001; Safdar et al., 2003). The  
188 concentrations of salt in the broth varied widely between different studies but recommendations of  
189 using a broth with 6.5% or 7.5% NaCl are common (Brown et al., 2005). However, salt tolerance of  
190 MRSA seems to vary between strains. Jones et al., (1997) found that salt enrichment broth inhibited  
191 the growth of epidemic MRSA-16, when NaCl concentrations higher than 2.5% were used. In our  
192 study, a higher yield of MRSA was found when a high salt pre-enrichment was used, compared to the  
193 yield after enrichment without NaCl. We did not systematically analyse what step(s) made procedures  
194 1 and 2 superior to procedure 3. As animal samples may contain far more competing flora with  
195 another composition compared to human clinical samples, the pre-enrichment with salt containing  
196 broth might have played a role in the additional yield of MRSA positive samples in these animal  
197 specimens. Procedure 3 contains 4% NaCl in the selective enrichment. This is far less than the 6.5%  
198 NaCl used in the procedure 1 and 2. Van Enk and Thompson (1992) have shown that media  
199 containing 4.5% NaCl were not considered to be sufficiently selective, since the growth of non-MRSA  
200 flora is not adequately reduced. This in contrast with media containing 6.5% NaCl. The addition of a  
201 6,5% NaCl in the selective enrichment step could potentially avoid the use of a non selective pre-

202 enrichment and thereby save time and cost of the isolation protocol. However, combining high salt  
203 concentrations and antimicrobials in the same broth could potentially inhibit growth of certain MRSA  
204 strains. This should be evaluated in more detail.

205 The detection limit of the procedures with spiked nasal samples in high-salt pre-enrichment showed a  
206 high sensitivity of the procedures confirming the salt-tolerance of clone ST398.

207 Because of the heterogeneity of MRSA strains in general and its behaviour under particular test  
208 conditions, there is no single media that recovers all MRSA strains (Brown et al., 2005). In pig  
209 husbandry one specific clone (ST398) comprising closely related spa types (t011, t108 and t1254) is  
210 present (De Neeling et al., 2007). This high-salt tolerant clone is also widely spread in veal calf  
211 samples (unpublished data). For use in MRSA-screening programs for pigs and veal calves,  
212 procedures 1 and 2 are recommended realising that salt-sensitive strains may be missed. It should be  
213 noted that selective enrichment increases the sensitivity of the procedure. This was also recently  
214 found by Van Loo et al., (2007) who found that the use of an enrichment broth prior to plating  
215 increased the number of MRSA strains detected by 12% in human clinical samples compared to the  
216 absence of selective enrichment. The difference in antimicrobials used in the selective broths  
217 potentially influenced the MRSA yield. However, since no differences were found between procedure  
218 1 and 2 this is not likely. A more plausible explanation could be the difference in antimicrobial  
219 concentrations used. Procedure 3 used just 50 µg/ml aztreonam compared to 75 µg/ml aztreonam in  
220 the other procedures. It is possible that the lower aztreonam concentration is not able to reduce the  
221 other competing flora and therefore results in lower MRSA yield. This has to be evaluated in more  
222 detail.

223 With regard to plating, a significant higher yield was found in pig samples when MRSA Screen or  
224 MRSASelect™ plates were used after selective enrichment. This is in accordance with the results with  
225 human clinical samples as reported by Cherkaoui et al., (2007). In our study, the MRSASelect™ plates  
226 resulted in more false positive colonies (suspected based upon colony morphology, but *mecA*  
227 negative). The light sensitivity of the MRSA ID plates makes them less practical for use.

228 In conclusion, out of the three commonly used procedures, for MRSA screening of nasal swabs from  
229 pigs or veal calves, the procedures 1 and 2, both using pre-enrichment containing Mueller Hinton and  
230 6.5% NaCl prior selective enrichment, should be recommended. No significant differences were found

231 between the procedures using either oxacillin or ceftizoxime in the selective broth. MRSA Screen is  
232 the plate of choice in this study taking into account practical reasons and performance.

233

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311 detection of methicillin-resistant *Staphylococcus aureus* using phenyl mannitol broth  
312 containing aztreonam and ceftizoxime. *J. Clin. Microbiol.* 39, 2660-2662.

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- 1 Table 1: MRSA-positive samples detected by the different detection procedures in combination with  
2 different agar plates in pig nasal swabs.

<b>Pigs (N = 70)</b>			
	MRSA Screen (Oxoid)	MRSASelect™ (Bio-Rad)	MRSA ID* (bioMérieux)
Procedure 1	46 (66%)	40 (57%)	36 (51%)
Procedure 2	44 (63%)	46 (66%)	32 (46%)
Procedure 3*	32 (46%)	27 (39%)	21 (30%)

- 3 \* P < 0.005

4

5 Table 2: MRSA-positive samples detected by the different detection procedures in combination with  
 6 different agar plates in veal calf nasal swabs.

<b>Calves (N = 100)</b>			
	MRSA Screen (Oxoid)	MRSASelect™ (Bio-Rad)	MRSA ID (bioMérieux)
Procedure 1	21 (21%)	22 (22%)	23 (23%)
Procedure 2	29 (29%)	27 (27%)	31 (31%)
Procedure 3*	15 (15%)	14 (14%)	12 (12%)

7 \* P < 0.005

8