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Evaluation of isolation procedures and chromogenic agar media for the detection of MRSA in nasal swabs from pigs and veal calves

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ABSTRACT

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

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

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1. Introduction

The prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA) is increasing world-wide, especially since the emergence of community-acquired and animal-related MRSA (Khanna et al., 2008; Nahimana et al., 2006; Tiemersma et al., 2004). Recently, a specific MRSA clone has been reported at unexpected high prevalence among pig farmers and veterinarians in different geographical areas (Voss et al., 2005; Weese and van Duijkeren, 2009). Strains belonging to this clone are resistant to SmaI macrorestriction and therefore referred to as non-typable (NT-MRSA). They all belong to Multi Locus Sequence Type 398 (ST398) and show closely related *spa*-types (mainly

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t011, t108 and t1254) (De Neeling et al., 2007). A case control study showed that pig and cattle farmers have an increased risk for being positive for ST398 (Van Loo et al., 2007). The source of these human infections can be found in the pig population and veal calves.

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

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

2. Materials and methods

2.1. Survey on the farms

Between April and May 2007, nasal swabs (Cultiplast®) were collected in duplicate from 70 pigs at seven different swine farms (10 pigs each farm) and 100 nasal swabs from veal calves were collected at three different veal farms (approximately 30 calves each barn) in The Netherlands. On each farm the animals were selected and sampled of convenience. From each animal, two nasal swabs were taken each from both nares. Collecting animal samples was in accordance with the animal welfare law.

2.2. Bacterial procedures

A total of 70 pig samples and 100 veal calf samples were analysed using 3 different procedures and 3 different agars. In total 630 plates (70 samples $\times$ 3 procedures $\times$ 3 plating agars) were read for the pig samples and 900 plates (100 samples $\times$ 3 procedures $\times$ 3 plating agars) were read for the veal calf samples.

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

2.2.1. Procedures 1 and 2

Swabs tested for procedures 1 and 2 were individually inoculated into tubes containing a pre-enrichment with 5 ml Mueller Hinton broth (MH^+ broth) (Becton Dickenson), containing 6.5% NaCl. This broth was incubated at 37 °C, overnight. Thereafter, the pre-enrichment was split into 2 procedures (procedures 1 and 2).

2.2.2. Procedure 1

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

2.2.3. Procedure 2

1 ml of the pre-enrichment was transferred into tubes containing 9 ml phenyl mannitol broth ($PHMB/ca^+$) (bioMérieux) with 5 μg/ml L ceftizoxime and 75 μg/ml aztreonam. After overnight incubation 10 μl of this $PHMB/ca^+$ broth was plated onto the agars mentioned below.

2.2.4. Procedure 3

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

2.2.5. Chromogenic agars

Three different chromogenic agars were applied: (i) MRSA Screen (Oxoid), (ii) MRSA*Select*™ (Bio-Rad) and (iii) MRSA ID (bioMérieux). Since Oxoid has optimised the MRSA Screen plate recently, also a selection of the calve samples was streaked out onto the Brilliance™ MRSA agar.

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

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

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

Furthermore, the detection limit of procedures 1 and 2 was determined by spiking MRSA-negative pig and calf samples with MRSA (clinical isolate *spa*-type t011). This was done using serial dilutions from a suspension with an optical density of 0.1 Å with parallel plating onto non-selective agar to determine the CFUs.

2.3. Typing

In a study to identify the optimal procedure it is important to know what MRSA types are analysed.

Therefore the isolates were *spa*-typed by sequencing the repetitive region of the protein A gene *spa* (Harmsen et al., 2003). Data were analysed by using the Ridom Staphtype software version 1.4 (www.ridom.de/staphtype).

2.4. Statistical analysis

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

3. Results

3.1. Pigs

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

3.2. Calves

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

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

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

3.3. Detection limit

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

4. Discussion

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

Spa-typing showed that all isolates were of the previously reported animal-related spa-types (*spa*-types mainly t011, t034, t108) belonging to clone ST398 (data

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

Pigs ($n = 70$)			
	MRSA Screen (Oxoid)	MRSA*Select*™ (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%)

*$P < 0.005$.

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

Calves ($n = 100$)			
	MRSA Screen (Oxoid)	MRSA*Select*™ (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%)

* $P < 0.005$.

not shown). NaCl-containing pre-enrichment media were used because of the inhibitory activity to many non-staphylococcal organisms and the fact that staphylococci can multiply in the presence of salt. For human samples an enhanced sensitivity and an additional yield of MRSA in human clinical specimens was also reported, using salt-containing pre-enrichment before plating (Gardam et al., 2001; Safdar et al., 2003). The concentrations of salt in the broth varied widely between different studies but recommendations of using a broth with 6.5% or 7.5% NaCl are common (Brown et al., 2005). However, salt tolerance of MRSA seems to vary between strains. Jones et al. (1997) found that salt enrichment broth inhibited the growth of epidemic MRSA-16, when NaCl concentrations higher than 2.5% were used. In our study, a higher yield of MRSA was found when a high salt pre-enrichment was used, compared to the yield after enrichment without NaCl. We did not systematically analyse what step(s) made procedures 1 and 2 superior to procedure 3. As animal samples may contain far more competing flora with another composition compared to human clinical samples, the pre-enrichment with salt-containing broth might have played a role in the additional yield of MRSA-positive samples in these animal specimens. Procedure 3 contains 4% NaCl in the selective enrichment. This is far less than the 6.5% NaCl used in the procedures 1 and 2. Van Enk and Thompson (1992) have shown that media containing 4.5% NaCl were not considered to be sufficiently selective, since the growth of non-MRSA flora is not adequately reduced. This in contrast with media containing 6.5% NaCl. The addition of a 6.5% NaCl in the selective enrichment step could potentially avoid the use of a non-selective pre-enrichment and thereby save time and cost of the isolation protocol. However, combining high salt concentrations and antimicrobials in the same broth could potentially inhibit growth of certain MRSA strains. This should be evaluated in more detail.

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

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

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

In conclusion, out of the three commonly used procedures, for MRSA screening of nasal swabs from pigs or veal calves, the procedures 1 and 2, both using pre-enrichment containing Mueller Hinton and 6.5% NaCl prior selective enrichment, should be recommended. No significant differences were found between the procedures using either oxacillin or ceftizoxime in the selective broth. MRSA Screen is the plate of choice in this study taking into account practical reasons and performance.

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