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# Assessment of the suitability of mannitol salt agar for growing bovine-associated coagulase-negative staphylococci and its use under field conditions

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ABSTRACT

This study aimed at testing the applicability of mannitol salt agar (MSA), a medium generally used in human medicine for differentiating *Staphylococcus aureus* from coagulase-negative staphylococci (CNS), for culturing bovine-associated CNS species. All test isolates from a comprehensive collection of well-identified CNS species, including both reference strains and field isolates, were able to grow. Subsequently, bulk milk samples and teat apex swabs were used to examine the capability of MSA for yielding CNS under field conditions. Sixty-nine and 47 phenotypically different colonies were retrieved from bulk milk and teat apices, respectively. The majority of isolates from teat apices were staphylococci, whereas in bulk milk, staphylococci formed a minority. After 24 h of growth, recovery of separate colonies of CNS was much more convenient on MSA compared to a non-selective blood agar. The results of this study indicate that MSA is a suitable medium for both growth and recovery of bovine-associated CNS.


## 1. Introduction

In many countries, coagulase-negative staphylococci (CNS) have become the most common cause of subclinical mastitis in dairy cows (e.g. Piepers et al., 2007; Sampimon et al., 2009a; Schukken et al., 2009). In addition, CNS are abundantly present both in the cows' environment (Taponen et al., 2006; Piessens et al., 2011) and on their teat apices (De Vliegher et al., 2003; Braem et al., 2013). Increasing evidence exists that the different CNS species vary in virulence and epidemiological behavior (Taponen et al., 2006; Park et al., 2011; Piessens et al., 2011, 2012a; Sampimon et al., 2011). More research is needed to understand the herd-specific distribution of CNS species, to identify species-specific infection sources and transmission routes and to picture differences in virulence characteristics (Pyörälä and Taponen, 2009; Supré et al., 2011).

Current microbiological techniques in mastitis research mostly rely on differential non-selective (blood) isolation media, also for recovery of CNS (Rajala-Schultz et al., 2009; Persson Waller et al., 2011). Studies focusing on CNS might benefit from adequate selective isolation media as a range of bacteria other than CNS could be cultured from different bovine-related niches (Piessens et al., 2011; Braem et al., 2013). Recent studies have made use of mannitol salt agar (MSA) to grow CNS (Piessens et al., 2011; Quirk et al., 2012; Braem et al., 2013), although it has not been shown that all bovine-associated CNS grow on this medium. MSA was developed in 1945 (Chapman, 1945). Most bacteria other than staphylococci are not able to grow on this agar due to the high sodium chloride concentration (7.5%) (Chapman, 1945; Finegold and Sweeney, 1961; Shittu et al., 2006). Currently, MSA is commercially available and recommended for the recovery of staphylococci as the mannitol fermentation offers advantages in the differentiation of *Staphylococcus aureus* and CNS species (Kateete et al., 2010). Mannitol salt agar has so far mainly been used for selective growth of human *S. aureus* and human CNS (Shittu et al., 2004, 2006; Han et al., 2007), but also to follow desirable growth of CNS during meat fermentations (Janssens et al., 2012). Obviously, a prerequisite for any kind of selective medium is that the ability to grow the desired species is proven both from storage (e.g. −80 °C) and from the source material. As phenotypic identification techniques developed for human CNS isolates have been shown to lack accuracy for

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bovine- (Sampimon et al., 2009b; Zadoks and Watts, 2009) and caprine- (Koop et al., 2012) associated CNS, differences in growth capabilities between human and the most common bovine-associated CNS on MSA should be anticipated. This study aimed at evaluating the growth capabilities of the most common CNS species, using both reference strains and field isolates, originating from dairy cows and their environment on MSA. In addition, the applicability of MSA for routine recovery of CNS species was assessed, using bulk milk samples and swabs of teat apices.

## 2. Materials and methods

### 2.1. Assessment of growth of CNS species on MSA

In a first experiment, the 25 most frequently isolated CNS species from bovine intra- and extra-mammary sites were assembled (Table 1). Of each species, both a reference strain and a field isolate were included ($n_{total} = 50$). All field isolates were previously described in published studies and were identified to the species level using either transfer RNA intergenic spacer PCR (tDNA-PCR) and/or *rpoB* sequencing ($n = 18$) (Supré et al., 2009, 2011 and unpublished data), amplified fragment length polymorphism (AFLP) genotyping ($n = 5$) (Piessens et al., 2010, 2011), $(GTG)_5$-PCR fingerprinting ($n = 1$) (Braem et al., 2011, 2013) or *rpoB*, *tuf* and 16S rRNA sequencing ($n = 1$) (Taponen et al., 2006, 2012). All 50 isolates were stored at −80 °C in either Microbanks (Microbank™, Pro-lab diagnostics, Bromborough, UK) or brain heart infusion with 15% (w/v) glycerol (Oxoid, Basingstoke, UK). For performing of the test, the isolates were plated directly (one quadrant per isolate) on MSA (Chapman medium, Oxoid, Basingstoke, UK) and on Columbia agar with sheep blood (Oxoid), the latter being used as a non-selective medium. Plates were aerobically incubated at 37 °C. Growth was examined after 24 h and 48 h, after which incubation for another 24 h at room temperature was initiated. If no growth could be detected on MSA after this first attempt, the procedure was repeated on both agars.

Subsequently, in a second experiment, the same procedure was performed for 10 isolates of the six and four CNS species that have most frequently been isolated at our laboratory from milk and teat apices, respectively ($n_{total} = 100$) (Table 2). All these isolates were previously collected as part of different field studies (Piessens et al., 2011; Supré et al., 2011; Braem et al., 2013) and were identified to the species level using tDNA-PCR (all *Staphylococcus chromogenes*, *Staphylococcus cohnii*, *Staphylococcus equorum*, *Staphylococcus haemolyticus*, *Staphylococcus simulans* and *Staphylococcus xylosus*), AFLP genotyping (all *Staphylococcus epidermidis*) or $(GTG)_5$-PCR fingerprinting (all *Staphylococcus saprophyticus*). All 100 isolates were stored at −80 °C in either Microbanks or brain heart infusion with 15% (w/v) glycerol. The test was performed as described above. If no growth could be detected on MSA the procedure was repeated once on both agars.

### 2.2. Suitability of MSA for CNS recovery in field applications

A bulk milk sample was collected from 20 randomly selected Flemish dairy herds by the Milk Control Centre (MCC, Lier, Flanders). Each milk sample (10 µl) was plated on MSA and Columbia agar with sheep blood (Oxoid). The resulting 40 plates were aerobically incubated at 37 °C and read after 24 h and 48 h. All colonies were phenotypically assessed by size, shape, smoothness, opacity, color, butyrous consistency, mannitol fermentation, haemolysis and lustre of the colonies. From all colony types represented by

**Table 1**
Origin and growth of reference strains and field isolates, respectively, of 25 coagulase-negative *Staphylococcus* species frequently isolated from cows' milk or environment, on mannitol salt agar (aerobic incubation at 37 °C).

| CNS species | Reference strain | | | Field isolate | | |
|---|---|---|---|---|---|---|
| | Origin | Strain number[a] | Growth | Origin | Strain number[c] | Growth |
| *S. agnetis* | Bovine milk | CCUG 59809$^T$ | 24 h | Bovine milk | ST1 | 24 h |
| *S. arlettae* | Poultry skin | LMG 19114$^T$ | 24 h | Sawdust | VP 0428 | 24 h |
| *S. auricularis* | Human ear | ATCC 33753$^T$ | 24 h | Milker's hand | KS 536 | 24 h |
| *S. capitis* | Human skin | ATCC 49326$^T$ | 24 h | Milker's hand | KS 547 | 24 h |
| *S. chromogenes* | Bovine skin | NCTC 10530$^T$ | 24 h | Bovine teat apex | KS 81 | 24 h |
| *S. cohnii* | Human skin | DSM 20260$^T$ | 24 h | Bovine teat apex | KS 567 | 24 h |
| *S. devriesei* | Bovine teat apex | CIP 110234$^T$ | 24 h[b] | Teat cup | KS 447 | 24 h |
| *S. epidermidis* | Human nose | LMG 10474$^T$ | 24 h | Milker's hand | KS 414 | 24 h |
| *S. equorum* | Surface of cheese | DSM 15097$^T$ | 24h-48 h | Teat cup | KS 402 | 24 h |
| *S. fleurettii* | Caprine cheese | CCM 4922$^T$ | 24 h | Teat cup | KS 339 | 24 h |
| *S. gallinarum* | Poultry skin | CCM 3572$^T$ | 24 h | Stable floor | VP 0303 | 24 h |
| *S. haemolyticus* | Human skin | CCM 2737$^T$ | 24 h | Bovine teat apex | KS 516 | 24 h |
| *S. hominis* | Human skin | CCM 2732 | 24 h | Teat cup | KS 338 | 24 h-48 h |
| *S. hyicus* | Porcine skin | NCTC 10350$^T$ | 24 h | Bovine milk | KS 167 | 24 h |
| *S. kloossii* | Skin squirrel | DSM 20676$^T$ | 24 h | Bovine teat apex | GB G057 | 24 h |
| *S. lentus* | Caprine udder | ATCC 29070$^T$ | 24 h | Stable floor | VP 273 | 24 h |
| *S. lugdunensis* | Human axillary lymph node | ATCC 43809$^T$ | 24 h | Teat cup | KS 699 | 24 h–48 h |
| *S. nepalensis* | Caprine nasal mucosa | DSM 15150$^T$ | 24 h | Milker's hand | KS 774 | 24 h |
| *S. pasteuri* | Human vomit | ATCC 51129$^T$ | 24 h | Bovine milk | KS 219 | 24 h |
| *S. sciuri* | Skin eastern gray squirrel | ATCC 29062$^T$ | 24 h | Teat cup | KS 491 | 24 h |
| *S. simulans* | Human skin | CCM 2705$^T$ | 24 h | Bovine teat apex | KS 129 | 24 h |
| *S. succinus* | Dominican amber | LMG 22185$^T$ | 24 h | Stable floor | VP 613 | 24 h |
| *S. vitulinus* | Ground lamb | CCM 4511$^T$ | 24 h | Stable floor | VP 105 | 24 h |
| *S. warneri* | Human skin | ATCC 27836$^T$ | 24 h | Milker's hand | KS 412 | 24 h |
| *S. xylosus* | Human skin | CNRS N850267 | 24 h | Bovine milk | KS 13 | 24 h |

[a] CCUG = Culture Collection, University of Göteborg, Göteborg, Sweden; LMG = Laboratory of Microbiology, Ghent University, Ghent, Belgium; ATCC = American Type Culture Collection, Rockville, MD, USA; NCTC = National Collection of Type Cultures, London, UK; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Weringerode, Germany; CIP = Collection de l' Institut Pasteur, Paris, France; CCM = Czech Collection of Microorganisms, Prague, Czech republic; CNRS = Centre National de Référence des Staphylocoques, Lyon, France; $^T$ = type strain.

[b] Growth within 24 h (37 °C) at second attempt.

[c] ST: Taponen et al., 2006; VP = Piessens et al., 2011; KS = Supré et al., 2011 (bovine milk) and Supré et al., unpublished results (milkers's hand, bovine teat apex, teat cup); GB = Braem et al., 2013.