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Carry-over of host nutrients during sampling enhances undesired growth of *Staphylococcus aureus* in liquid Amies transport medium

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ABSTRACT

Optimal transportation of bacteria is important for accurate clinical interpretation, quantitative assays, and microbiome studies. A transport medium should ideally keep the bacteria alive without supporting growth or altering the relative proportions of the constituent species. We investigated the effect of nasal mucus and mucin on the growth and survival of two *Staphylococcus aureus* strains in liquid Amies transport medium at room temperature and 4 °C for 14 days. The study showed that the presence of nasal mucus in microbiological samples stimulated undesired *S. aureus* growth at room temperature in a dose-dependent manner. These findings underscore that microbiological samples from humans and animals should be stored at 4 °C until analysis to avoid undesired *S. aureus* growth.

1. Introduction

Maintaining the viability of microorganisms during transport to the laboratory without altering the relative proportions of the constituent species is crucial for reliable identification of infectious agents from human and animal samples as well as for quantitative and semi-quantitative purposes (e.g., quantitative PCR and microbiome analyses).

In an ongoing study on livestock-associated methicillin-resistant *Staphylococcus aureus* clonal complex 398 (LA-MRSA CC398), we have noted that growth takes place during transport at room temperature. *S. aureus* survival in transport media at room temperature has been investigated previously with diverging results. Robinson et al. (2012) reported that the CFU counts in liquid Amies transport medium were stable at room temperature over a 2-week period. In contrast, Morosini et al. (2006) observed a one $\log_{10}$ CFU increase over a 1-week period in the same transport medium. Similarly, Smismans et al. (2009) showed that the CFU counts increased by 200% and 468% after 24 h and 48 h incubation at room temperature, respectively, and were also able to demonstrate pronounced growth of MRSA in clinical swabs kept at room temperature. None of these studies have evaluated the growth of bacteria in transport systems containing clinical samples from humans or animals, where host cells, secretions, and debris could act as nutritional supplement supporting bacterial growth. For example, it seems reasonable to assume that a considerable amount of mucus will be present in the transport medium following collection of nasal swabs.

The objective of the present study was to determine whether the presence of nasal mucus affects the growth characteristics of *S. aureus* during storage in ESwab Liquid Amies Collection and Transport System (Copan, Brescia, Italy). In an attempt to elucidate the possible mechanisms behind the observed growth, we further investigated whether there is a dose-dependent effect of mucin, which is the major constituent of mucus in mammals (Henderson et al., 2014).

2. Materials and methods

2.1. S. aureus strains and transport medium used in this study

S. aureus growth and survival were evaluated in the ESwab Liquid Amies Collection and Transport System, which consists of a FLOQSwab and an ESwab tube containing 1 ml of liquid Amies transport medium (Copan, Brescia, Italy). Two MRSA strains were included in the study: a Danish LA-MRSA CC398 strain isolated from a pig and the human MRSA strain ATCC 43300, which originates from a clinical sample and belongs to CC30. Prior to bacterial inoculation, ESwab tubes were supplemented with either nasal mucus (experiment 1) or mucin (experiment 2). ESwab tubes without nasal mucus or mucin were used as controls in both experiments. The swab elution method followed the CLSI guidelines (CLSI, 2014).

2.2. Testing the effect of human nasal mucous

In experiment 1, LA-MRSA CC398 was stored in liquid Amies transport medium in the presence of FLOQSwabs containing nasal mucus.

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Nasal mucus was obtained from a total of 13 human volunteers, who were previously found negative for MRSA (Angen et al., 2017), by inserting a FLOQSwab into the anterior nares and rotating 5 times. Individual FLOQSwabs were collected from each nostril at two different time points 1 week apart, resulting in a total of 13 sets of four swabs.

2.3. Testing the effect of porcine mucin

In experiment 2, LA-MRSA CC398 and *S. aureus* strain ATCC 43300 were stored in liquid Amies transport medium supplemented with 100 μl of porcine stomach mucin (Sigma-Aldrich, St. Louis, MO) at a concentration of either 2700 μg/ml or 4800 μg/ml, resulting in final concentrations of 270 μg/ml and 480 μg/ml, respectively.

2.4. Spiking and recovery of bacteria

ESwab tubes were inoculated with 100 μl of a 1:100,000 or a 1:10 dilution of a 0.5 McFarland suspension of either *S. aureus* strain, corresponding to approximately 1×10^2 or 1×10^6 CFUs per ESwab tube. The tubes were stored at room temperature or 4 °C, respectively, for 14 days. CFU counts were performed on days 0, 1, 2, 3, 7, and 14. Aliquots of 100 μl were used to make 10-fold dilutions in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and 100 μl was plated in duplicate onto Brilliance MRSA 2 agar plates (Oxoid, Basingstoke, United Kingdom). CFU counts were obtained after 18–24 h incubation at 35 °C.

2.5. Data analysis

The average CFU counts and corresponding standard errors were calculated (GraphPad Prism software, version 7, GraphPad, La Jolla, CA).

2.6. Ethics statements

The study was performed in accordance with principles of the Declaration of Helsinki and was approved by the National Committee on Health Research Ethics (Protocol H-15013814). Informed consent was obtained from the human volunteers.

3. Results

3.1. S. aureus growth in human nasal samples

In the first experiment, we evaluated the effect of nasal mucus on growth of an LA-MRSA CC398 strain in ESwab Liquid Amies Collection and Transport System at room temperature (Fig. 1A) and 4 °C (Fig. 1B) over a period of 14 days. Nasal mucus was collected from healthy human volunteers using FLOQSwabs, which were placed in 1 ml of liquid Amies transport medium containing either 1×10^2 or 1×10^6 CFUs (low and high bacterial starting concentrations). The CFU counts are illustrated in Fig. 1. In the presence of nasal mucus at room temperature, the CFU counts increased during the first 24 h for both concentrations. At low starting concentrations, the CFU counts increased during the first 48 h (from 1.8 to 3.7 $\log_{10}$ CFU), where after they declined slowly to 2.0 $\log_{10}$ CFU on day 14. In comparison, at high starting concentrations, the CFU counts increased only during the first 24 h (from 5.9 to 6.9 $\log_{10}$ CFU), where after they decreased slowly to 3.7 $\log_{10}$ CFU on day 14. In contrast, we observed a steady decline in the CFU counts when the bacteria were stored without nasal mucus, regardless of the bacterial starting concentration. The largest reduction was observed when the bacteria were stored in the absence of nasal mucus. At high starting concentrations, the CFU counts decreased from 5.8 to 3.7 $\log_{10}$ CFU after 48 h, whereas at low starting concentrations, the bacteria could not be cultivated after 24 h. At 4 °C, a steady decline in the CFU counts was observed, both in the presence and absence of nasal mucus and regardless of the bacterial starting concentration.

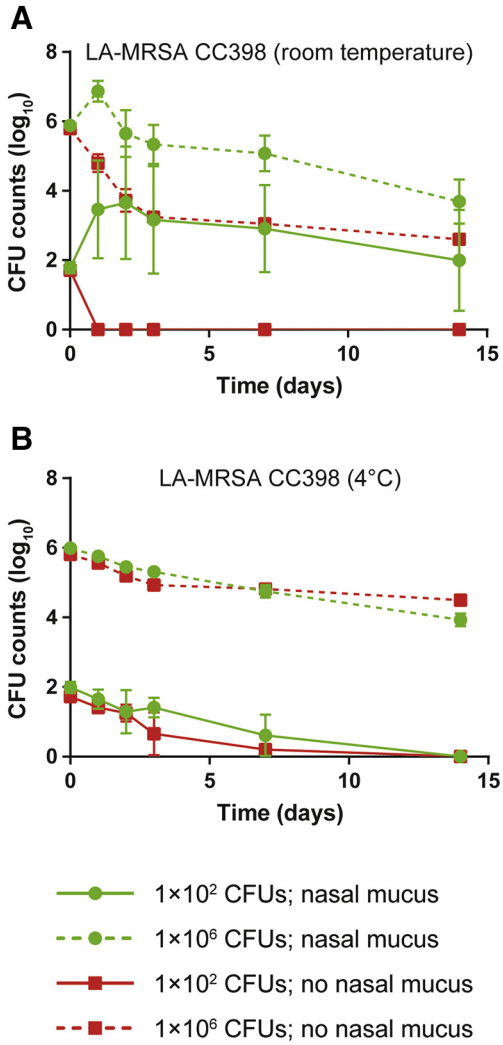

Fig. 1. Growth and survival of LA-MRSA CC398 in liquid Amies transport medium in the absence (red lines) or presence (green lines) of nasal mucus at room temperature (A) and 4 °C (B). The medium was either left untreated (red lines) or supplemented with nasal mucus (green lines) collected from healthy human volunteers, and were subsequently inoculated with 1×10^2 CFUs (solid lines) or 1×10^6 CFUs (broken lines). Each value represents the log-transformed CFU count ($\log_{10}$) at a given time point. Error bars illustrate the standard deviation of each CFU count from 5 to 13 replicate experiments.

3.2. Effect of porcine mucin on S. aureus growth

The observed bacterial growth at room temperature in the presence of nasal mucus led us to speculate whether the glycoprotein, mucin, could serve as a nutritional supplement. To test this hypothesis, we investigated the effect of different concentrations of mucin (0 μg/ml, 2700 μg/ml, and 4800 μg/ml) on bacterial growth, using the experimental procedure and the LA-MRSA CC398 strain described above. For comparative purposes, we repeated the analysis with a human MRSA CC30 strain. The CFU counts are illustrated in Fig. 2. At room temperature, the presence of mucin had a positive effect on bacterial growth in a dose-dependent manner, regardless of the bacterial starting concentration (Fig. 2A and B). In contrast, no growth was observed at 4 °C or when the bacteria were stored in the absence of mucin (Fig. 2C, D). The CFU counts for the human MRSA CC30 strain were generally higher than those for the LA-MRSA CC398 strain.

4. Discussion

The present study demonstrates that the level of *S. aureus* growth during storage in liquid Amies transport medium depends on the

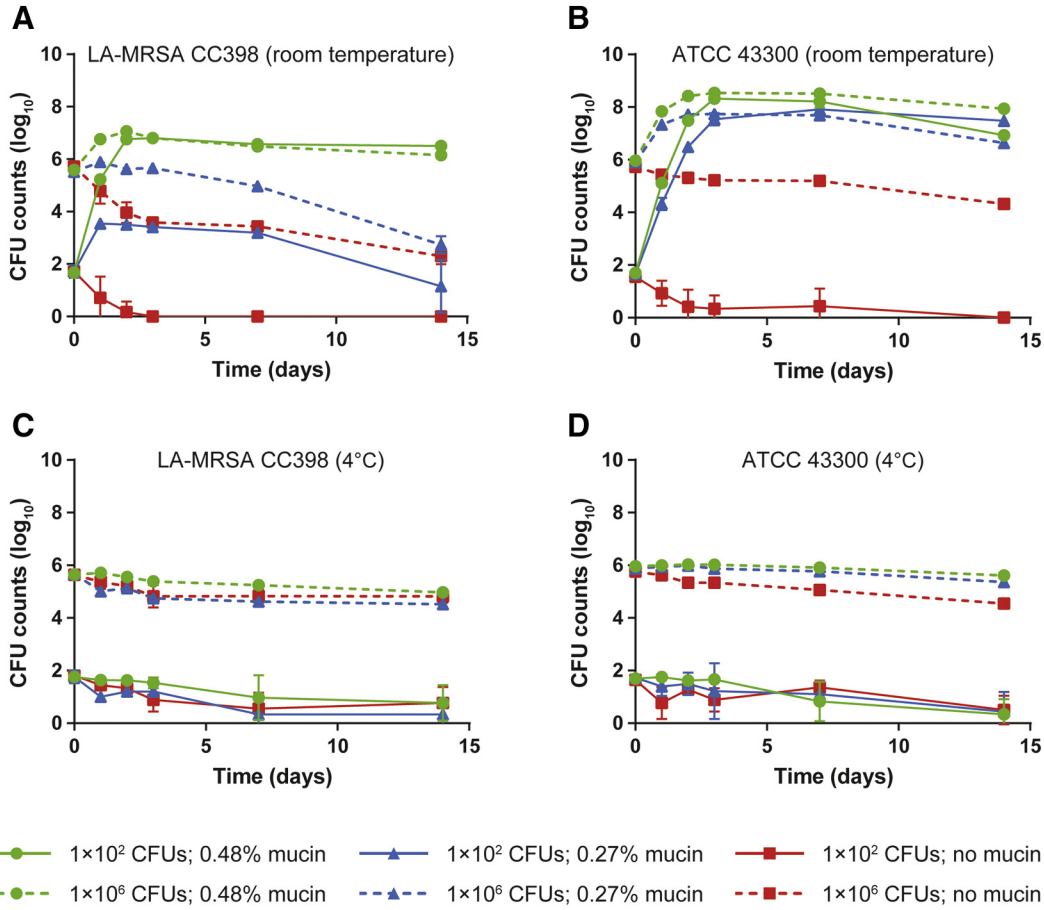

Fig. 2. Growth and survival of LA-MRSA CC398 (A, C) and MRSA CC30 (B, D) in liquid Amies transport at different concentrations of mucin at room temperature (A, B) and 4 °C (C, D). The medium was either left untreated (red lines) or supplemented with porcine stomach mucin at a concentration of 0.27% (blue lines) or 0.48% (green lines), and were subsequently inoculated with 1×10^2 CFUs (solid lines) or 1×10^6 CFUs (broken lines). Each value represents the log-transformed CFU count ($\log_{10}$) at a given time point. Error bars illustrate the standard deviation of each CFU count from 3 to 6 replicate experiments.

amount of nasal mucus (mucin), the number of bacterial cells in the collected sample, the storage temperature, and the bacterial strain.

From a diagnostic point of view, growth of bacteria at different rates in a transport medium is undesirable, as the microorganisms of interest may be overgrown and outcompeted by other microorganisms, resulting in a false-negative result. Although microbiological samples are generally processed within 24 h in most diagnostic laboratories, this is not always feasible in practice. Moreover, quantitative assays and microbiome studies will be prone to bias resulting from such changes in the absolute number or relative abundance of each microorganism. This is particularly true in studies where multiple samples are compared, because the concentration of mucus and thus the bacterial growth patterns will likely vary strongly between different samples. A number of studies have used liquid transport system for nasal samples and used this for bacterial quantification or microbiome analysis (Cheng et al., 2015; Espinosa-Gongora et al., 2016; McMurray et al., 2016). However, the specific transport time and the temperature are not reported by all studies, and this could have an influence on the results obtained. Thus, researchers should pay great attention to, and always report, the storage conditions, especially the temperature and time, when handling human or animal samples for diagnostic purposes or quantitative investigations, including microbiome studies.

Our results showed that *S. aureus* growth in transport media was facilitated by the presence of 2700 μg/ml mucin at a concentration of allows, which equals the average concentration of mucin in normal human sputum (Henderson et al., 2014). Mucin is the main component of mammalian nasal mucus (Callaghan Rose et al., 1987; Henderson et al., 2014) and is degradable by bacteria (Macfarlane et al., 2005). The dose-dependent effect was observed for both the LA-MRSA CC398 strain and the human MRSA CC30 strain, suggesting that the ability to utilize mucin is a general trait of *S. aureus*, regardless of the host association. In addition, mucin produced by the respiratory and intestinal epithelia are biochemically similar (Callaghan Rose et al., 1987, Phillips et al., 2006), and porcine stomach mucin is therefore used in respiratory models (Saceanu et al., 1993, Phillips et al., 2006). Thus, it seems reasonable to assume that nasal mucus has a similar stimulatory effect on *S. aureus* growth as stomach mucin.

The Clinical and Laboratory Standards Institute (CLSI) provides acceptance criteria and guidelines for assessing the performance of transport devices (CLSI, 2014). According to these criteria, there should be no more than a three $\log_{10}$ CFU decline after 48 h, both at room temperature (20–25 °C) and cold temperature (2–8 °C) and no more than a one $\log_{10}$ CFU increase after 48 h at cold temperature. The CLSI guidelines do not provide acceptance criteria for bacterial growth at room temperature, recognizing that none of the commercially available transport media can adequately inhibit such growth. Of note, the CLSI criteria are based on bacterial growth in the absence of nasal mucus or any other nutrients except those present in the transport media. Nonetheless, the growth characteristics observed in the present study conform to the CLSI acceptance criteria.

Previous studies have assessed the performance of different transport media without taking into account the presence of exogenous nutrients (Appelbaum et al., 1988; Delacour et al., 2009; Roelofsen et al., 1999). This may explain why most of these studies were able to show a steady decline in the recovery of *S. aureus* and other microorganisms at room temperature, whereas we observed rapid growth and elevated CFU counts for up to 14 days in the presence of nasal mucus and mucin. This discrepancy underscores the need to evaluate transport media under conditions that simulates their specific applications.

5. Conclusion

Our study shows that the presence of nasal mucus in microbiological samples may lead to undesired *S. aureus* growth in transport media at rates that depend on the concentration of mucin as well as the strain, confirming the importance of storing microbiological samples from humans and animals at 4 °C until analysis.

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Authors' contribution

All authors have contributed to the study. MZI, JL, RS and ØA designed the study. MZI and ØA prepared the initial manuscript. All authors contributed to the subsequent editorial revisions. MZI performed the laboratory analysis. MZI, JL and ØA conducted the statistical analysis. All authors proofread the article.

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