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Validation of a New PCR-Based Screening Method for Prevention of *Serratia marcescens* Outbreaks in the Neonatal Intensive Care Unit

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Keywords
Cohorting · Barrier nursing · Colonization screening · *Serratia marcescens* · Very low birth weight infant

Abstract
Background: *Serratia marcescens* may cause severe nosocomial infections, mostly in very low birth weight infants. Since *S. marcescens* exhibits by far the highest adjusted incidence rate for horizontal transmission, it can cause complex outbreak situations in neonatal intensive care units. ***Objective:*** The aim of this study was to establish a fast and highly sensitive colonization screening for prompt cohorting and barrier nursing strategies. ***Methods:*** A probe-based duplex PCR assay targeting the 16S *rRNA* gene of *S. marcescens* was developed and validated by using 36 reference strains, 14 *S. marcescens* outbreak- and nonoutbreak isolates, defined by epidemiological linkage and molecular typing, and applied in 1,347 clinical specimens from 505 patients. ***Results and Conclusions:*** The novel PCR assay proved to be highly specific and had an in vitro sensitivity of 100 gene copies per reaction (~15 bacteria). It showed a similar (in laryngeal/tracheal specimens) or even higher (in rectal/stoma swabs) in vivo sensitivity in comparison to routine microbial culture and was much quicker (<24 h vs. 2 days). By combining different oligonucleotide primers, there was robust detection of genetic variants of *S. marcescens* strains. PCR inhibition was low (1.6%) and observed with rectal swabs only. Cohort analysis illustrated applicability of the PCR assay as a quick tool to prevent outbreak scenarios by allowing rapid decisions on cohorting and barrier nursing. In summary, this novel molecular screening for colonization by *S. marcescens* is specific, highly sensitive, and substantially accelerates detection.

Introduction

Querying the public data base www.outbreak-database.com revealed that *Serratia marcescens* is among the top pathogens of nosocomial outbreaks in neonatal intensive care units (NICUs), second only to *Klebsiella* spp. Although *S. marcescens* generally displays rather low virulence, it may cause severe infections, particularly in very low birth weight infants (<1,500 g) or term neonates with inherited disorders [1]. In NICUs and pediatric intensive care units (PICUs), *S. marcescens* contributes to 5–16% of all nosocomial infections with positive blood culture [2, 3] (https://www.nrz-hygiene.de/surveillance/kiss/neo-kiss). Data from outbreaks indicate invasive infection in one out of six neonates colonized with *S. marcescens*, which is very high compared to the risk in case

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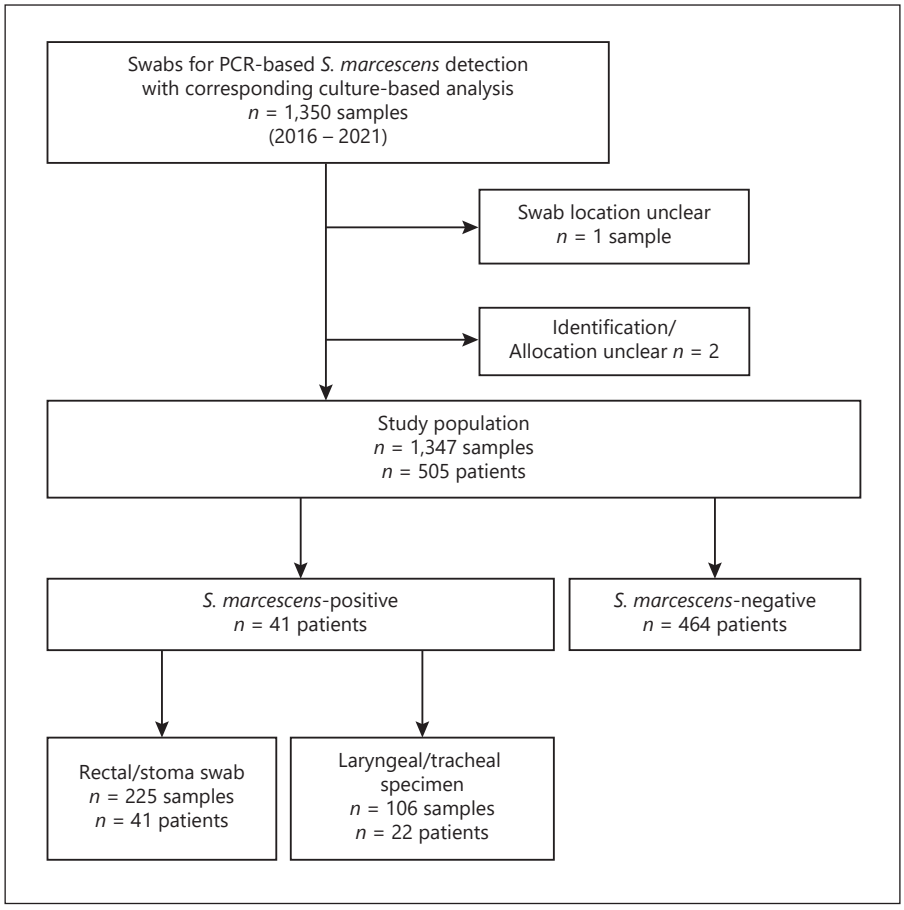

Fig. 1. Study population and sample materials.

Table 1. PCR-based detection of *S. marcescens* strain variants defined by epidemiological linkage and molecular typing that were isolated at Charité – Universitätsmedizin Berlin between 2008 and 2013

Strain	Origin	Year	Strain variant	PCR result
S. marcescens AND0213	Outbreak (colonization)	2008	I	+
S. marcescens AND2161	Nonoutbreak (infection)	2012	I	+
S. marcescens AND2211	Nonoutbreak (colonization)	2012	I	+
S. marcescens AND2261	Nonoutbreak (colonization)	2012	I	+
S. marcescens AND2290	Nonoutbreak (colonization)	2012	I	+
S. marcescens AND3279	Nonoutbreak (colonization)	2013	I	+
S. marcescens AND1088	Nonoutbreak (colonization)	2010	II	+
S. marcescens AND1234	Nonoutbreak (colonization)	2010	II	+
S. marcescens AND2229	Nonoutbreak (infection)	2012	II	+
S. marcescens AND3171	Nonoutbreak (colonization)	2013	II	+
S. marcescens AND0214	Nonoutbreak (colonization)	2008	III	+
S. marcescens AND2088	Outbreak (infection)	2012	III	+
S. marcescens AND2147	Nonoutbreak (colonization)	2012	III	+
S. marcescens AND2885	Nonoutbreak (colonization)	2013	III	+

of other pathogenic *Enterobacteriacae* [4]. Confirmed risk factors for infections are very low birth weight, prematurity, and mechanical ventilation [5, 6]. Nosocomial infection manifests most frequently as blood culture-positive sepsis (47%), pneumonia (13%), and meningitis (7%), causing significant long-term morbidity and very high mortality rates

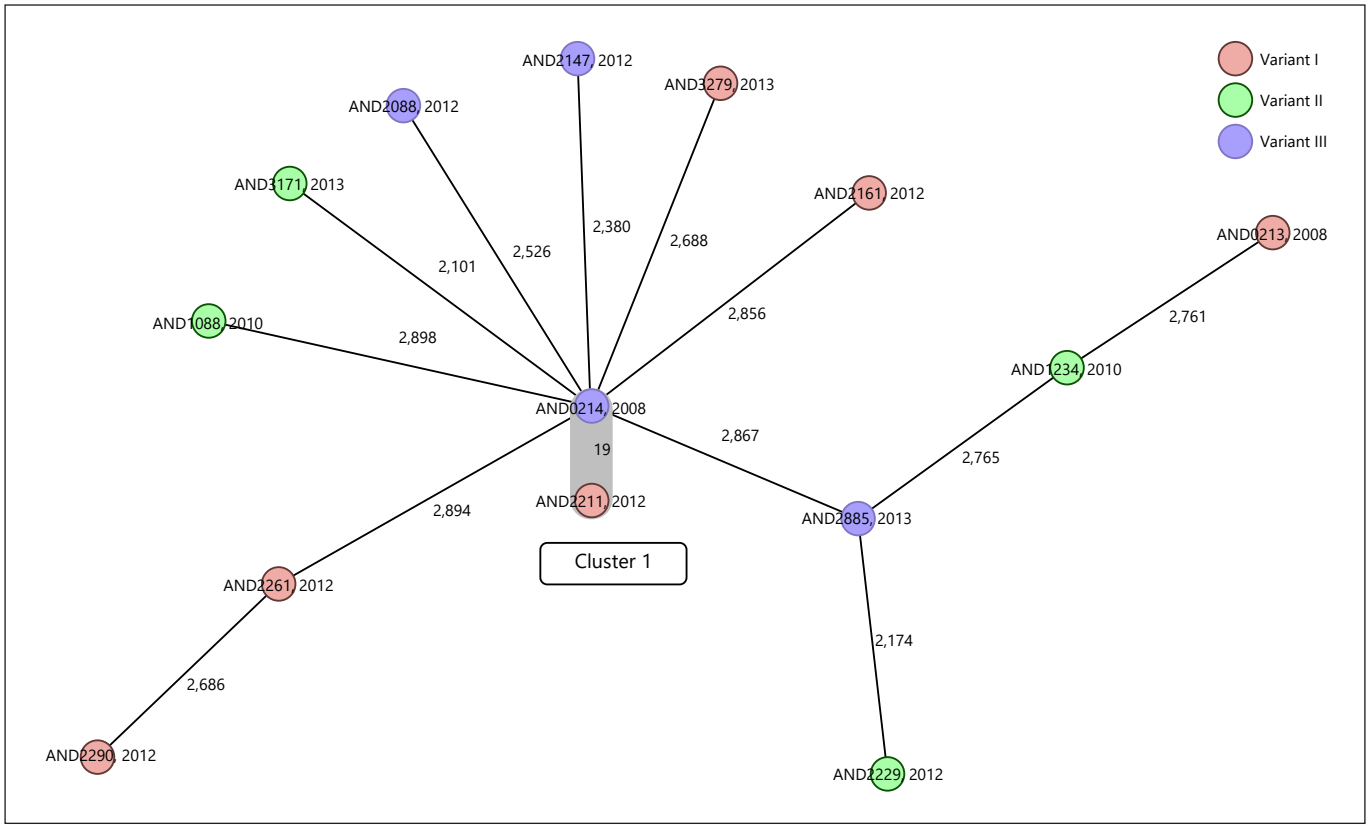

Fig. 2. Molecular typing of *S. marcescens* isolates used for PCR assay validation. Minimum spanning tree based on cgMLST allelic profiles for tested strains. The circles denote the specific *S. marcescens* isolates and are colored by the nucleotide variants I–III (online suppl. Fig. S1). The connecting lines illustrate the numbers of differing alleles in the target genes. The figure shows that the tested strains were not closely related.

(30–55% of cases with positive blood culture) [7–9]. *S. marcescens* exhibits an extraordinarily high adjusted incidence rate ratio (IRR 164), a measure for the likelihood of a second positive blood culture with the same pathogen in a second patient of the same NICU within a 30-day time period, as compared to *Klebsiella* spp. (IRR 12) or *Staphylococcus aureus* (IRR 10). This rate underlines its predominance in causing horizontal transmission or outbreak scenarios [10, 11].

After an outbreak situation causing headlines in national TV channels and newspapers in 2013, the German national commission for hospital hygiene and infection prevention (KRINKO) recommended a bacterial colonization screening program for neonates during intensive care that included a dynamic (weekly) screening for *S. marcescens* [12]. The KRINKO also emphasized the importance of developing further diagnostic tools [13]. Current colonization screening is commonly based on microbial culture using Columbia blood-containing agar and requires 48 h. In the NICU, however, such time interval is too long for decisions on cohorting and barrier nursing. Therefore, we established a specific, quick, and highly sensitive PCR-based assay for detection of *S. marcescens* colonization that should be tested concerning its putative implication on decision-making for infection prevention and control in Neonatology.

Materials and Methods

For development of PCR screening method, 36 bacterial reference strains were obtained representing the neonatal feces microbiome (online suppl. Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000526836) as well as 14 *S. marcescens* outbreak and nonoutbreak isolates (Table 1). Furthermore, 1,347 rectal/stoma swabs or laryngeal/tracheal specimens from 505 patients were analyzed (Fig. 1). With admission to the NICU, the patients' parents provided written consent for bacterial colonization screening. Genomic DNA (gDNA) was collected in eSwab regular or minitip vials (Mast Diagnostica, Germany) and then released by cooking a 500 µL aliquot for 10 min at 98°C and collecting the supernatant after centrifugation for 3 min at 16,100 g.

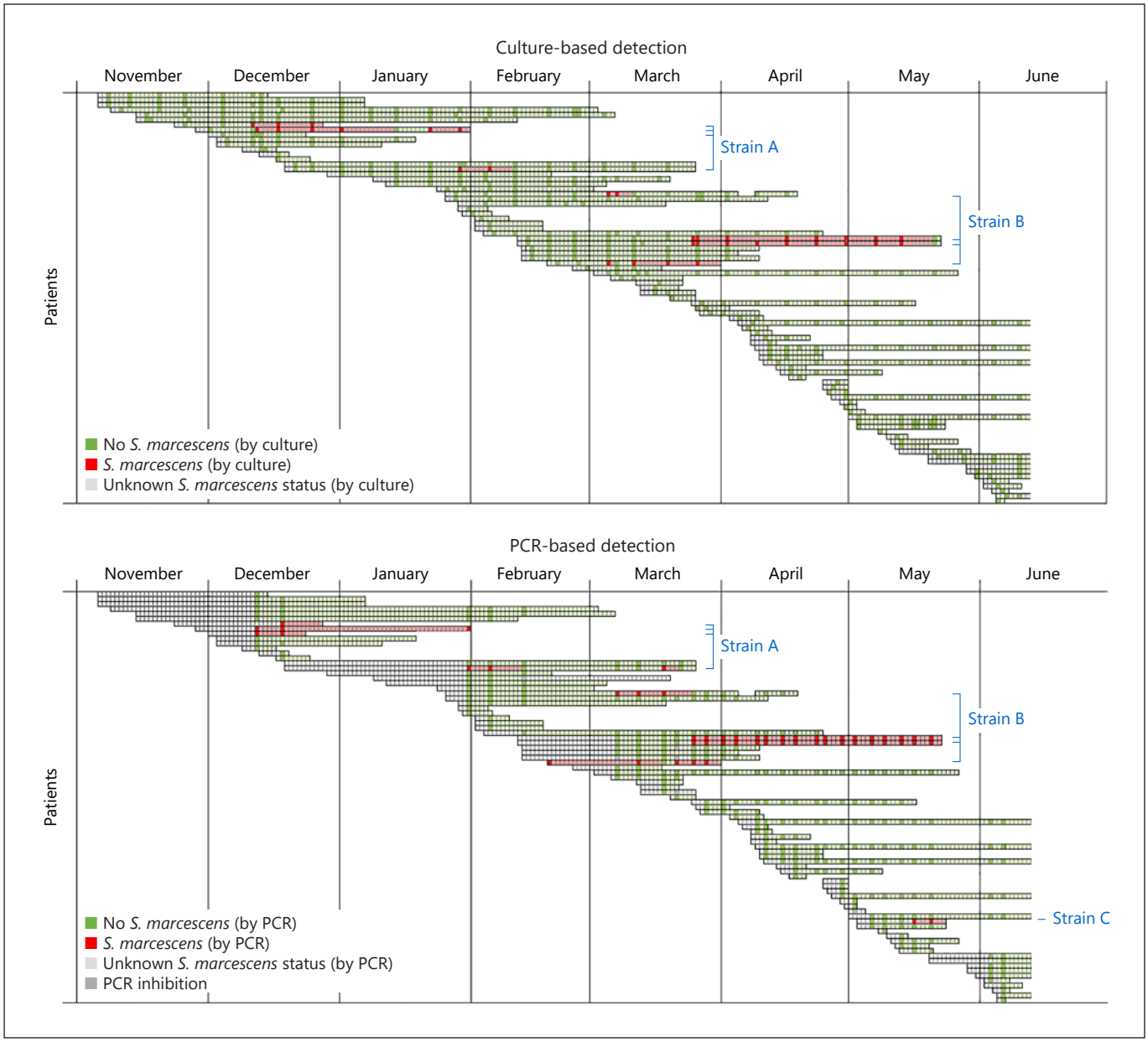

Fig. 3. Case vignette of the culture- and PCR-based *S. marcescens* colonization screening in a level 3 NICU during an 8-month study period. Each box represents 1 patient treatment day. The color of the box indicates the screening result (all rectal swabs). The colonization status is marked as follows: unknown (gray), *S. marcescens* positive (red), *S. marcescens* negative (green). Darker color boxes indicate the day of sampling. The colonization status coloring is retained until the next sampling result. Genetically different strains (A, B, C) of *S. marcescens* are indicated.

The sequence of the PCR-targeting region in the *S. marcescens* strains was determined by Sanger sequencing (LGC Genomics, Germany) of a 16S *rRNA* PCR product obtained with the primers 5′-AGAGTTTGATCMTGGCTCAG-3′ (forward) and 5′-TACGGYTACCTTGTTACGACTT-3′ (reverse). Whole genome sequencing was performed at the partner site of the German Center for Infection Research, University of Giessen, Germany.

Molecular typing was performed by core-genome multilocus sequence typing with Ridom SeqSphere + software version 7.7.5 (Ridom, Germany). For the gene-by-gene comparison, an ad hoc task template scheme was established following the SeqSphere + software guide with *S. marcescens* reference genome NZ_CP026050.1 (GenBank accession: GCA_000783915.2) and resulted in 3,283 core-genome multilocus sequence typing targets.

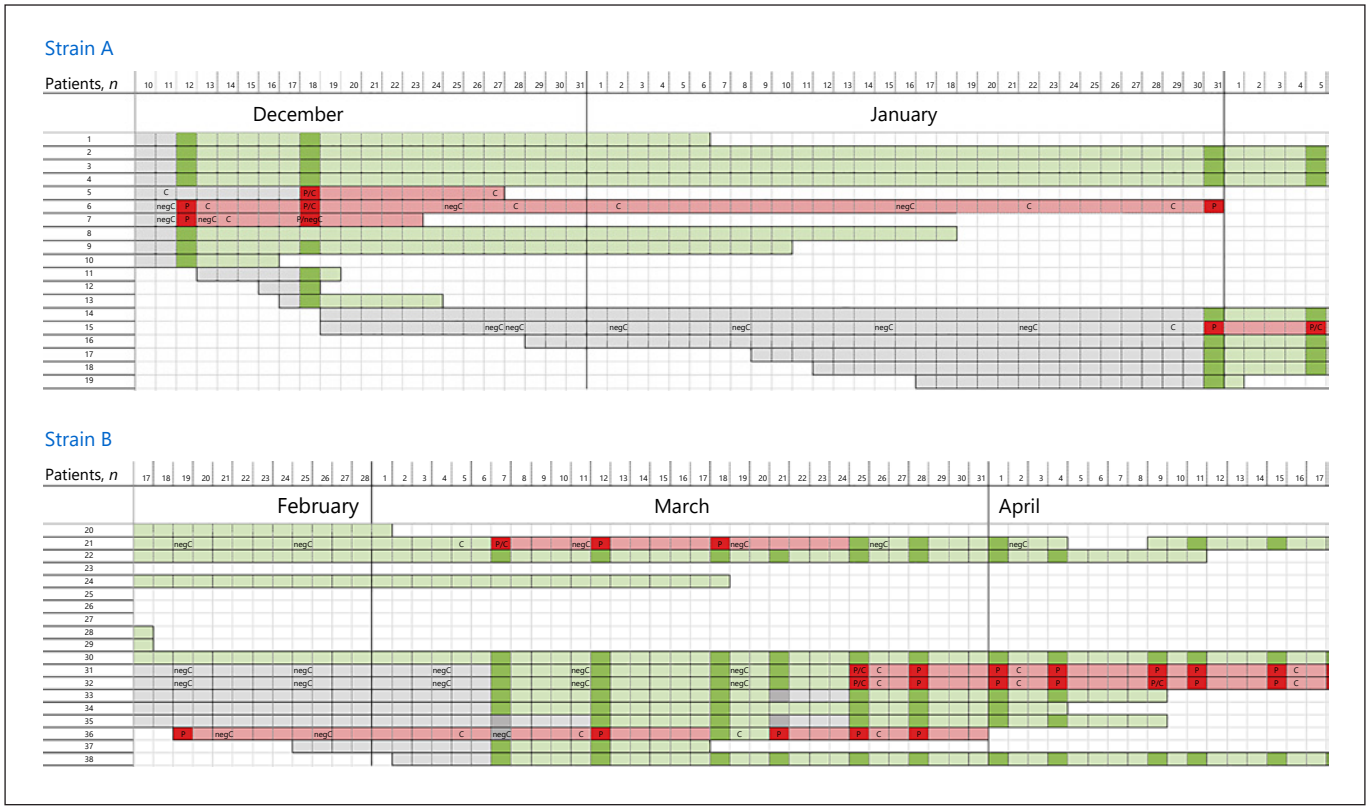

Fig. 4. Temporal correlation of culture (C)- and PCR (P)-based *S. marcescens* screening results. Two situations are illustrated in detail: strain clusters A (top), B (bottom). Each box represents 1 patient treatment day. The color of the box indicates the PCR-based screening result from rectal swabs while the text indicates the culture-based Sm screening result: *S. marcescens* colonization status unknown due to PCR inhibition or lack of sample (gray), no *S. marcescens* detected (green), *S. marcescens* detected (red). Darker color boxes indicate the day of sampling. The colonization status coloring is retained until the next sampling result.

The probe-based duplex PCR assay was established, accounting for the genetic diversity within the *S.marcescens* isolate strains. Primers and probes (online suppl. Table S2) were synthesized (Eurofins Genomics, Germany). The PCR mixture contained TaqMan Universal PCR Mastermix (#4304437, Thermo Fisher Scientific), 0.4 µM*S. marcescens* primer mix, 0.4 µM*Lactobacillus* spp. primer mix, 0.1 µM of each TaqMan probe, 1 pg gDNA from *Lactobacillus spec.* and 1/10 vol of the processed patient sample. PCR was performed on a StepOnePlus cycler (Applied Biosystems, USA) with the following protocol: 95°C for 10 min, 40 cycles of 95°C for 15 s, 62°C for 10 s, and 72°C for 30 s. Positive and negative controls were performed with each assay.

PCR specificity was evaluated with 20 ng pure gDNA of each bacterial strain (Table 1, online suppl. Table S1). PCR sensitivity in vitro was determined by serial dilutions of sequence-verified, double-stranded DNA fragments (500 bp gBlocks) covering the 16S *rRNA* PCR product region from *S. marcescens* and *L.* spp., synthesized by Integrated DNA Technologies (Coralville, IA, USA). Corresponding samples to the ones used for the PCR-based screening were analyzed by the standard microbial culture followed by strain identification by VITEK®2 GN ID card (bioMérieux, France) or MALDI-TOF MS (Labor Berlin, Germany).

Results

Assay Design and Validation of PCR Analysis

The PCR assay was developed based on previously published, strain-discriminating regions of the 16S *rRNA* genes of *S. marcescens* [14] and *Lactobacillus* ssp. [15], respectively, but detection of three different *S. marcescens* strain variants I–III required multiplex forward primers (online suppl. Fig. S1).

PCR specificity was evaluated using gDNA from strains representing the common microbiome in neonatal feces [16–18] and 11 nonpathogenic *Serratia* strains (online suppl. Table S1). Only the 16S *rRNA* genes of *S. nematodiphila* and *S. ureilytica* are so closely related to *S. marcescens* genetically that their amplification could not be excluded. As those strains are neither pathogenic to humans nor to be expected in the neonatal microbiome, we accepted this cross-reactivity. Concerning the outbreak and nonoutbreak isolates (online suppl. Table S1, Fig. 2), 16S *rRNA* sequencing identified three different nucleotide

Table 2. Demographic data and clinical characteristics of neonates with *S. marcescens* colonization, based on rectal/stoma-positive swabs

Clinical parameter	*S. marcescens*-positive-tested infants (*n* = 40)
Female	15 (37.5)
Birth weight, among them	
<500 g	4 (10.0)
500–999 g	11 (27.5)
1,000–1,499 g	9 (22.5)
1,500–2,499 g	6 (15.0)
>2,500 g	10 (25.0)
Gestational age at birth, weeks	29.2 (24.0–40.6)
Term infants	9 (22.5)
Preterm infants, among them	31 (77.5)
<28+0 weeks	14 (45.2)
>28+0 to <32+0 weeks p.m.	12 (38.7)
>32+0 to <37+0 weeks p.m.	5 (16.1)
Mechanical ventilation	25 (62.5)
Congenital malformation	6 (15.0)
Immunological disorders	5 (12.2)
Major surgery	
Intestinal	6 (15.0)
Cranial	1 (2.5)
Cardiac	1 (2.5)
Initial *S. marcescens* screening, day of life	3 (2–16)

Categorical data are given as *n* (%), continuous data as median (range).

variants of *S. marcescens* in the forward primer binding region: variants I–III, which were all detected by the PCR assay. To determine the genetic variability of the PCR product region in a wider variety of strains in silico, a sequence similarity search was performed (BLASTn) with 3,680 sequences from the taxid *S. marcescens*. The search retrieved 3,115 sequences from 2,362 different strains. In the consensus sequence, the variants I–III were confirmed in an unbiased array of isolates, suggesting a wide applicability of the PCR assay (online suppl. Fig. S1).

Since the neonatal microbiome may significantly vary upon age or antimicrobial treatment, we added 1 pg gDNA from *Lactobacillus spec*. as external inhibition control which roughly equals endogenous *Lactobacillus* amounts during the early neonatal period (≤7 days). Hereby, Ct values ≥35 and <40 were considered "partially inhibited," and samples were remeasured in a higher dilution. Samples with Ct values of ≥40 were considered "inhibited."

The in vitro sensitivity was determined by dilution series of the PCR product to be approx. 100 gene copies per reaction (online suppl. Fig. S2). As the *S. marcescens* genome contains 7 copies of the 16S *rRNA* gene, the in vitro detection limit of the PCR assay was approx. 15 bacteria per reaction.

Among all clinical specimens, PCR inhibitions were exclusively found in swabs of the rectum or abdominal stoma, mostly containing a high amount of feces. Inhibition occurred (101/1,347 samples = 7.5%), but mostly in *S. marcescens*-positive samples. In such cases, PCR for *S. marcescens* might have used up all reagents, thereby compromising the (*Lactobacillus spec* based) inhibition control PCR–leaving only 1.6% (22/1,347 samples) to be truly inhibited. This effect was also observed in the in vitro sensitivity analysis where the *Lactobacillus* PCR was the more often inhibited, the more *S. marcescens* DNA was present (online suppl. Fig. S2).

Application of the PCR-Based Screening

To determine how much quicker information on *S. marcescens* colonization was accessible by PCR diagnostics in comparison to the routine culture-based approach, 1,137 noninhibited samples were evaluated. The median time from the sampling to the laboratory findings report was <24 h for PCR-based and 2 days for microbial culture-based diagnostics. Of note, in case of *S. marcescens* detection, the initial culture-based report usually included antimicrobial susceptibility testing results.

In the patients' cohort, *S. marcescens* colonization by either PCR- or culture-based screening was detected in rectal/stoma swabs of 40 neonates (1 patient was only positive in tracheal specimens, *n* = 41 in Fig. 1). The clinical characteristics of this cohort (Table 2) showed that colonization with *S. marcescens* mainly occurred in infants with a gestational age <32 weeks, a high frequency of mechanical ventilation and with congenital malformations, immunological disorders, and/or intestinal major surgery.

In total, 231 samples were found to be *S. marcescens*-positive by PCR, in comparison to 178 samples identified by microbial culture. This difference mainly occurred in the analysis of rectal/stoma swabs with 151 positive PCR results versus 101 positive culture results (+50%). In laryngeal/tracheal specimens, the culture-to-PCR detection ratio was nearly identical (80 vs. 77; +4%). This indicates a significantly higher sensitivity of the PCR-based screening, especially for rectal/stoma swabs. As the discrepancy between culture- and PCR-based analysis could result from detecting DNA of dead bacteria during or after antibiotic treatment, the PCR-positive rectal specimens were analyzed in depth. Among the 55 specimens (30 patients) exclusively positive by PCR diagnostics, 7 out of 30 patients (23%, 11 specimens)

received antibiotic treatment at the time point or latest 2 days before the specimens were drawn. Antibiotic treatment included 2nd line treatment with a carbapenem in 4 out of 7 of those patients. However, 22 out of the 40 patients (55%) PCR-positive in rectal swabs were identified in parallel by culture-based screening, but 18 of those 40 patients (45%) would not have been identified as colonized by *S. marcescens* at the earliest time point by only applying microbiological screening. On the opposite, among all 178 specimens found to be *S. marcesens*-positive in microbial culture, 5 rectal/stoma swabs (5/101 = 5%) and 10 laryngeal/tracheal specimens (10/77 = 13%) were not identified by PCR technique. The contribution of pre-analytic issues remains unclear. The combined data indicate that the PCR-based *S. marcescens* colonization screening in rectal/stoma swabs is superior in terms of sensitivity and speed.

To elucidate the value of the novel screening method in managing an outbreak risk, a case vignette from our level 3 NICU (Campus Charité Mitte) is illustrated in Figure 3. This ward consists of 20 (plus 4 reserve) beds in 11 rooms (3 quadruple, 4 double, 4 single). During an 8-month period, 83 neonates were nursed. Among them, nine infants were tested positive for *S. marcescens*. In-depth analysis revealed three genetically different strains of *S. marcescens;* strains A and B within a cluster, strain C a single finding. From a distance, the detection dynamics did not hugely differ between both screening methods. However, the detailed analysis (Fig. 4) indicated a beneficial effect of the PCR-based screening. For cluster A, when the routine culture-based screening was still negative in patient 5 (December 11th), the quick PCR-based *S. marcescens* screening of the whole NICU revealed 2 other colonized patients (6 and 7). Patient 6 remained negative in microbial culture for additional 2 days, so barrier precautions would not have been performed without the information from the PCR-based screening. Later on, culture and PCR concordantly showed horizontal transmission to a fourth patient (14, January 31st). In the other *S. marcescens* cluster B, the index patient (36) was already identified by PCR-based screening at admission while microbiological culture remained negative for another 14 days. At this time point, horizontal transmission could already be detected in another patient (21), who was negatively tested in microbiological culture for another 4 days. A third and fourth transmission (31 and 32) were then detected simultaneously by both approaches. This illustrates the potential of the quick PCR-based screening to prevent clusters or even outbreak situations by timely applying cohorting and barrier nursing.

Discussion

This study describes a novel approach for the fast and reliable detection of *S. marcescens*. Due to the extremely high risk of horizontal transmission, the high rates of mortality, and the severe long-term morbidities in neonates infected with *S. marcescens*, integrating molecular methods into the colonization screening is an important progress which may extend or eventually substitute the standard screening approach by microbial culture.

In comparison to published primer sequences [14], our qPCR assay was modified in order to detect all 16S *rRNA* sequence variants of *S. marcescens* ssp., found in vivo and in silico. Considering significant homologies between other *Serratia* ssp. and their common presence in the human (adult) microbiome (*S. fonticola*, *S. liquefaciens*, etc.), the primer set also discriminates *S. marcescens* from these Enterobacteriaceae. Moreover, the qPCR assay exhibits high in vitro sensitivity with a lower limit of detection of approximately 100 gene copies per reaction, corresponding to approximately 15 bacteria. The concept of using a duplex assay qPCR, detecting *Lactobacillus spec.* as external control, allowed identification of assay inhibition, which was found in case of too much fecal material in the PCR. In most cases, this issue was easily overcome by subsequent analysis of a diluted specimen. One other limitation remains: due to sequence similarities, our qPCR detects non-pathogenic *S. nematodiphila* and *S. ureilytica*–but this might even be an advantage as NICU outbreaks with other *Serratia* ssp., including *S. ureilytica*, have been reported [19, 20].

Most importantly for application in hospital hygiene, the PCR-based screening was superior to microbial culture-based screening regarding both speed and sensitivity. While the difference in sensitivity was marginal in the analysis of laryngeal/tracheal specimens, PCR detected 50% more *S. marcescens*-positive rectal swabs. This translated into a 45% higher detection rate in patients colonized with *S. marcescens*. One major reason for this discrepancy could be the fact that the qPCR technique cannot distinguish between DNA from living versus nonviable bacteria. However, 5 out of 16 patients, whose colonization with *S. marcescens* was uniquely detected by PCR, were not treated with antimicrobial substances, which argues for a truly higher sensitivity of the PCR- versus culture-based screening. In addition, a potential detection of DNA from nonviable bacteria is negligible in a screening program to prevent horizontal transmission in the NICU or PICU because sustained decolonization of *S. marcescens* by antibiotic treatment is rather unlikely [21, 22]. The higher speed in diagnostics may be the major advantage of the PCR technique. In comparison to the microbial culture,

the same-day result of the PCR diagnostics is extremely valuable for cohorting and barrier nursing as recommended in the German guidelines for infection prevention and control in neonates [12]; even if a quicker approach using *Serratia*-selective agar plates has recently become available [23]. Comparing the costs (consumables + personnel) for the initial culture-based strain identification with antibiotic resistance testing versus that for PCR-based screening in the entire cohort of 505 patients enrolled in this study, the novel method was about 3-fold more expensive than the routine culture. This raises the question of whether the novel approach has its significance primarily in outbreak scenarios or after identification of an index case as major risk of horizontal transmission. Since *S. marcescens* usually do not exhibit multidrug resistance, the antibiotic testing is not a prerequisite of such screening program. For a rational antibiotic treatment, however, antibiotic resistance testing can be performed from the asservated patient sample. Current pre-analytic use of eSwabs or minitip vials provides enough material for such testing that can be initiated on the same day as obtaining the PCR results, thus without delay in the gain of information. Although the case vignettes indicated an advantage of the rapid colonization screening by PCR in single cases, the general efficacy in preventing clusters or outbreaks with *S. marcescens* cannot be estimated yet. The efficacy of the colonization screening program recommended by the KRINKO is suggested by a retrospective analysis [24]. Currently, it is impossible to conclude on the reduction of nosocomial sepsis or invasive infection by faster decision on cohorting and barrier nursing. To overcome this limitation, a broad, multicenter application of the PCR-based colonization screening is required. At its best, this would be combined with PCR-based detection of other bacteria that are crucial for preterm and sick term infants (such as *Klebsiella* spp. or *Acinetobacter* spp.). Such PCR-based screening could be implemented as point-of-care method as currently developed in adult intensive care [25]. Independent of the screening method, it is also unclear yet, whether the results affect the physician's choice of the antimicrobial substance in case of infection, but there is evidence that public reports on outbreak scenarios lead to an increased prescription rate of third-line antibiotics in the community of neonatal care [26].

Conclusion

We present a novel duplex PCR-based strategy for *S. marcescens* colonization screening, which is far quicker and more sensitive than the standard microbial culture-based approach. This could be an integral part of a molecular screening panel for bacteria known to cause severe infections in patients admitted to the NICU or PICU.

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Statement of Ethics

With admission to the NICU, the patients' parents provided written consent for bacterial colonization screening. Additional institutional approval was not required according to German legal regulations. This has been confirmed by the Institutional Review Board of the Charité – Universitätsmedizin Berlin.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Lina K. Sciesielski conceptualized and designed the study, collected data, carried out the analyses, and drafted and finalized the manuscript. Luisa K.M. Osang collected data, performed demographic data analyses, and contributed to the manuscript. Nicole Dinse collected data, carried out analyses, and reviewed the manuscript. Anna Weber performed bioinformatic analyses and reviewed the manuscript. Christoph Bührer contributed

to the manuscript for important intellectual content. Axel Kola coordinated and supervised data collection, proofed the microbial analysis, and reviewed the manuscript for important intellectual content. Christof Dame conceptualized and designed the study, collected specimens, drafted, and finalized the manuscript.

Data Availability Statement

The data are available from the corresponding author on request. All data generated or analysed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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