The development of a real-time quantitative polymerase chain reaction (qPCR) method for the detection of *Staphylococcus aureus* in peripherally inserted central catheter (PICC) colonisation

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Keywords Central line-associated blood stream infection, CLABSI, peripherally inserted central catheter, PICC, *nuc gene, Staphylococcus aureus, S. aureus,* quantitative real-time polymerase chain reaction, qPCR.

For referencing Higgins M *et al.* The development of a real-time quantitative polymerase chain reaction (qPCR) method for the detection of *Staphylococcus aureus* peripherally inserted central catheter (PICC) colonisation. Vascular Access 2019; 5(2):23-28.

DOI https://doi.org/10.33235/va.5.2.23-28

ABSTRACT

Background Peripherally inserted central catheters (PICCs) are susceptible to *Staphylococcus aureus (S. aureus)* colonisation and subsequent dissemination into the bloodstream, leading to central line-associated bloodstream infections (CLABSI). Current detection for *S. aureus* PICC colonisation relies on the use of traditional culture-dependent methods, including the semi-quantitative roll-plate culture method. However, the minimum time to detection is between 24–48 hours. Furthermore, a definitive diagnosis may take up to 7 days and is therefore not useful in guiding appropriate and timely patient management. A quantitative real-time polymerase chain reaction (qPCR) assay has the potential to overcome these limitations.

Methods A qPCR assay, targeting the nuclease *(nuc)* gene, was developed to detect *S. aureus* PICC colonisation. The sensitivity threshold of the assay was determined using purified *S. aureus* genomic DNA (gDNA) and validated using 41 clinical PICC samples which were compared to results from the roll-plate culture method.

Results The sensitivity threshold of the qPCR assay was 10^2 CFU/mL^{-1}. From a total of 41 clinical PICC samples, *S. aureus* colonisation was detected from one PICC by both qPCR (10^3 CFU/mL^{-1}) and the roll-plate culture method (10^3 CFU/mL^{-1}). The qPCR assay processing time was less than 2 hours after bacterial gDNA isolation compared with 24–48 hours for the roll-plate culture method.

Conclusion This developed qPCR assay is an accurate and rapid method to detect *S. aureus* PICC colonisation. With further research, this method has the potential to be used in a clinical setting.

INTRODUCTION

Peripherally inserted central catheters (PICCs) are increasingly used for intermediate and long-term access in patients for the delivery of vital fluids, including blood products, medication and nutrition[1,2]. However, PICCs are susceptible to bacterial colonisation and subsequent dissemination into the bloodstream, leading to central line-associated bloodstream infections (CLABSI) which can occur with prolonged insertion time, patient immunosuppression, and the administration of parenteral nutrition[3]. Furthermore, microbial attachment on PICCs may occur within 24 hours of insertion, with microbial biofilm formation evident within 48–72 hours[4].

A well-recognised complication of PICC usage, CLABSI results in increased morbidity, mortality, and the length and cost of hospital stays[5-7]. An estimated 250,000 bloodstream infections occur annually, with 80,000 CLABSI occurring in intensive care units each year[8]. These infections contribute towards mortality rates ranging between 12–25% in critically ill patients, with an estimated cost between $4,000–56,000 per episode[9-11]. Common causal pathogens of PICC colonisation include *Staphylococcus aureus (S. aureus)*, Enterococci and *Candida* spp.[12]. In Australia, an estimated 7,000 *S. aureus* bloodstream infections occur annually, and are largely associated with CLABSI[13]. The infection is difficult to treat, may require removal of the colonised PICC, and often involves prolonged antibiotic therapy[8]. Furthermore,

S. *aureus* is widely known for its disease-causing potential, including vascular, cardiac and pulmonary complications[14], and is associated with 25–35% mortality despite strict efforts to minimise infection[15]. Consequently, there is a need for an accurate and rapid method to detect *S. aureus* PICC colonisation to guide appropriate and timely patient management and to reduce the potential for CLABSI[16].

Routine detection of *S. aureus* PICC colonisation heavily relies on the use of traditional culture-dependent methods, including the semi-quantitative roll-plate culture method, employing selective media for direct enumeration or recovery of isolates after enrichment in selective broth[17]. A count of 15 colony-forming units (CFU) or more per plate indicates PICC colonisation, while a definitive diagnosis of CLABSI requires that the same organism, associated with PICC colonisation, grows from at least one percutaneous blood culture[7,8]. However, the semi-quantitative roll-plate culture method has several disadvantages. First, the minimum time to detection and identification of the causal organism is between 24–48 hours. Second, the sensitivity of this phenotypic method is limited to approximately 70% and even lower for fastidious and slow-growing bacteria[18]. Third, prior administration of antibiotic therapy may significantly impact the sensitivity of cultures[19-21]. These disadvantages can be overcome with the use of quantitative real-time polymerase chain reaction (qPCR), a time- and cost-effective high-throughput method that can be performed directly on isolated genomic DNA (gDNA) with a processing time of less than 2 hours[22].

Previously, rapid, sensitive and specific qPCR assays for the detection of *S. aureus* have been developed targeting the nuclease *(nuc)* gene[23]. The main reason for targeting the *nuc* gene is that it is well conserved in *S. aureus* at the nucleotide level and hence evolutionary stable[24]. Studies have used the *nuc* gene to accurately detect *S. aureus* from blood cultures[25,26], wound swabs, bodily fluids, tissue samples, urine, vascular access sites, and sputum[27]. In particular, the *nuc* gene has been used to successfully detect *S. aureus* gDNA in blood to diagnose bacteraemia[22]. However, this sequence has not previously been used to detect *S. aureus* colonisation directly from PICCs, after being withdrawn from patients, using qPCR.

There is a significant requirement for an accurate and rapid method to detect *S. aureus* PICC colonisation, allowing for more informed decision-making, with the potential for early intervention in the diagnosis of *S. aureus* CLABSI[3]. A qPCR assay using primers specific to the *nuc* gene has the potential to meet these requirements. Therefore, the aim of this study was to assess and validate a qPCR assay using primers specific to the *nuc* gene to detect *S. aureus* PICC colonisation. This was achieved by:

a) Assessing existing published literature to identify an appropriate target gene sequence, and to confirm the target amplification size of the gene sequence using conventional PCR;
b) Converting the target sequence into a qPCR assay;
c) Determining the sensitivity of the optimised qPCR assay to detect *S. aureus* in an environment that simulates a clinical setting; and
d) Validating the sensitivity of the qPCR assay by determining the detection level of *S. aureus* on PICCs derived from clinical samples.

MATERIALS AND METHODS

Clinical PICC samples were supplied from 41 in-patients at the Queensland Children's Hospital, Queensland, including traditional polyurethane PICCs (Turbo-Ject Power-Injectable PICCs [Cook Medical; Bloomington, IN]) and hydrophobic PICCs (Bioflo® with Endexo® PICCs [Angiodynamics Inc; Queensbury, NY]). Ethical approval for the study was granted by Queensland Health Human Research Ethics Committee (HREC/15/QRCH/164) and Griffith University Human Research Ethics Committee (HREC/2016/077), and the trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12615001290583).

STUDY INCLUSION CRITERIA

Patients were eligible to participate in the study if they were less than 18 years of age, required a PICC inserted for treatment for more than 24 hours, and did not have a bloodstream infection at time of recruitment. We needed to have written informed consent from legal guardians and to be able to safely collect PICC materials at the time of the PICC removal. All aspects of PICC insertion, use, management and removal were completed by clinical staff in accordance with local clinical practice guidelines, including the use of 2% chlorhexidine gluconate in 70% ethanol for skin antisepsis[28].

SAMPLE COLLECTION

All PICC samples were collected under aseptic conditions by a qualified research nurse with experience in preparation of specimens for culture. The distal 2–3cm of the PICC was collected using sterile scissors, deposited in a sterile container, and immediately transported to the laboratory for culture using the semi-quantitative roll-plate culture method[29]. Descriptive information – including patient demographics, PICC utilisation, clinical characteristics and PICC complications – were collected from patients by a research nurse and stored on a secure online database[30].

Identification of *S.aureus nuc* gene

Existing published literature was reviewed to identify the 16S *nuc* gene primers F (5'-GCGATTGATGGTGATACGGTT-3') and R (5'-AGCCAAGCCTTGACGAACTAAAGC-3') used in this study. The primers consisted of 21 and 24 bases, respectively, and were located within the 270-bp *nuc* gene, encoding the *nuc* gene. The nucleotide positions were 48–70 and 303–328, respectively[24].

S.aureus AU-19 reference strain

One clinical isolate *Staphylococcus* spp. (*S. aureus* AU-19), previously sequenced using 16s rRNA analysis, was included in this study as a reference strain [Li Zhang, 2011, Nathan Campus, Griffith University] to evaluate the diagnostic performance of the qPCR assay.

S.aureus AU-19 gDNA extraction

A single colony of purified *S. aureus* AU-19 was suspended in 10mL of Luria broth and incubated overnight at 37˚C in preparation for gDNA extraction. Bacterial gDNA was extracted using the PowerSoil DNA Isolation Kit [Mo Bio; California, USA].

Conventional PCR: amplification of the *nuc* gene

Conventional PCR was used to confirm the target amplification of the expected size and sequence of the *nuc* gene with *S. aureus* AU-19 using the MyCycler™ Thermal Cycler System [BioRad; California, USA]. All reactions were performed in triplicate and included both positive and negative controls. The PCR amplification was performed as per the manufacturer's instructions[31], using primers specific to the *nuc* gene[24], and the PCR product was visualised using 2% agarose gel electrophoresis [Thermo Fisher Scientific; Victoria, AUS].

Sensitivity of the optimised qPCR assay

The sensitivity threshold of the qPCR assay was evaluated using a 10-fold serial dilution of *S. aureus* AU-19 with a starting gDNA concentration of 10ng/µl determined by a Nanodrop Spectrophotometer [Thermo Fisher Scientific; Victoria, AUS]. The qPCR assay was performed with the Takara SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) Detection Kit [Scientifix; Victoria, AUS] using the Real-Time CFX Connect PCR System [BioRad; California, USA]. All reactions were performed in triplicate and included both positive and negative controls. The qPCR assay was performed as per the manufacturer's instructions[32] using primers specific to the *nuc* gene[24].

Clinical PICC sample gDNA extraction

Following the semi-quantitative roll-plate culture, clinical PICC samples were individually suspended overnight in 200µl of lysis buffer which contained 20mg/mL of lysozyme, 20mm Tris-HCl (pH 8.0), 2mm EDTA, 1.2% Triton, and Proteinase K at 37˚C. Bacterial gDNA was extracted from each PICC using the PowerSoil DNA Isolation Kit [Mo Bio; California, USA].

Validating the sensitivity of the qPCR assay using clinical PICC samples

The qPCR assay was performed directly on extracted bacterial gDNA from each clinical PICC sample. The qPCR assay was performed with the Takara SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) Detection Kit [Scientifix; Victoria, AUS] using the Real-Time CFX Connect PCR System [BioRad; California, USA]. All reactions were performed in triplicate and included both positive and negative controls according to the manufacturer's instructions[32], using primers specific to the *nuc* gene[24].

RESULTS

Conventional PCR: amplification of the nuc gene

The *nuc* gene primers[24] successfully amplified *S. aureus* AU-19 as the target amplicon with the PCR product amplified as a 270-bp fragment visualised using 2% agarose gel electrophoresis [Thermo Fisher Scientific; Victoria, AUS] (Figure 1).

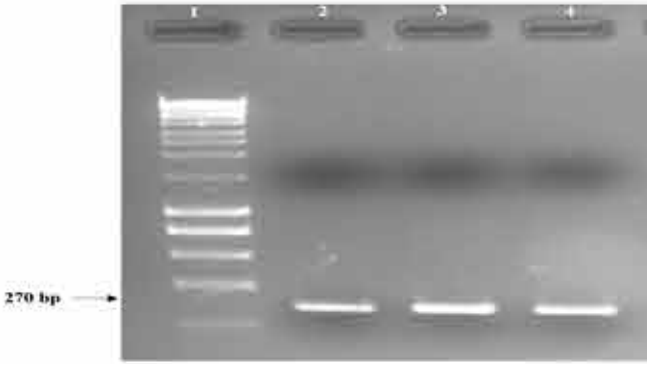

Figure 1. S. aureus *AU-19 conventional PCR product showing amplification of the expected size fragment obtained with nuc1 – nuc2 primers, analysed under UV light using 2% agarose gel.*
Lane 1: 1 Kbp molecular weight marker;
Lane 2: S. aureus *AU-19 PCR product replicate 1;*
Lane 3: S. aureus *AU-19 PCR product replicate 2;*
Lane 4: S. aureus *AU-19 PCR product replicate 3.*

SENSITIVITY OF THE OPTIMISED QPCR ASSAY

The qPCR assay detection limit was 10^2 CFU/mL^{-1} (Cq 33.63 ± 0.58) using a 10-fold serial dilution of *S. aureus* AU-19 gDNA. The Cq values of the 10-fold serial dilution (10^6 to 10^1 CFU/mL^{-1}) ranged from Cq 15.01 ± 0.13 to Cq 37.46 ± 1.18, respectively. Non-specific amplification was observed after 35 cycles. The Real-Time CFX Connect PCR System [BioRad; California, USA] analysis software indicated an efficiency of 98.8%, correlation coefficient (R^2)=0.974 and slope of the graph (y)=-3.352 (Figure 2).

VALIDATION OF THE QPCR ASSAY USING CLINICAL PICC SAMPLES

The qPCR assay was validated using a total of 41 clinical PICC samples. One PICC produced positive amplification (Cq 29.82 ± 0.18) equivalent to 10^3 CFU/mL^{-1} using the *nuc* gene primers[22]. Non-specific amplification was observed after 35 cycles. The Real-Time CFX Connect PCR System [BioRad; California, USA] analysis software indicated an efficiency of 96.7%, correlation coefficient (R^2)=0.989 and slope of the graph (y)=-3.403 (Figure 3). The results of the qPCR assay correlated (100%) with the results from the semi-quantitative roll-plate culture method (10^3 CFU/mL^{-1}).

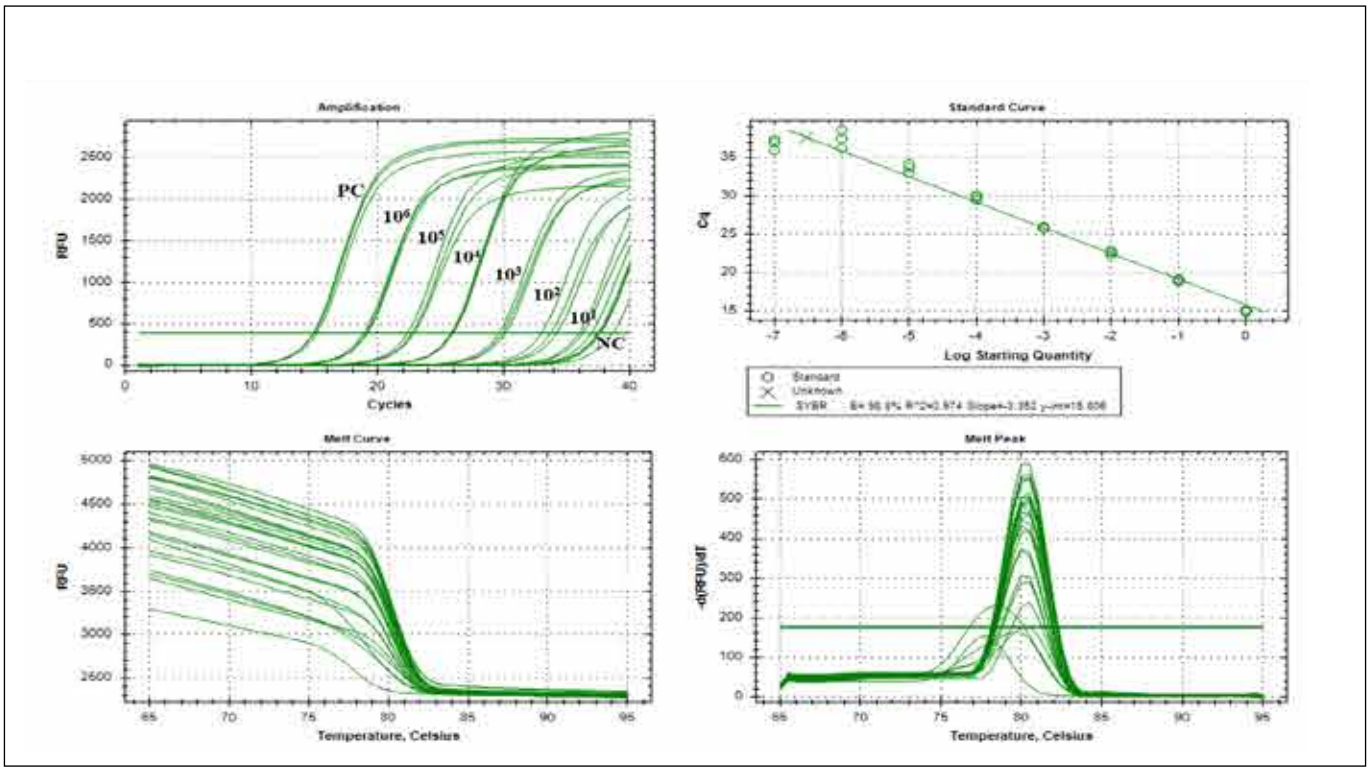

Figure 2. Real-time qPCR assay. Amplification of the nuc *gene (106–101* nuc *gene copy) using gDNA isolated from an overnight culture of* S. aureus *AU-19. The standard curve plotted using the log starting quantity against the mean Cq. Data from the straight lines calculated by linear regression yielded an efficiency of 98.8%, correlation coefficient (R2)=0.974 and slope of the graph (y)=-3.352. Error bars indicate the standard deviations based on three replications.*

DISCUSSION

Molecular diagnostic methods including qPCR are becoming widely accepted as methods of choice for accurate and rapid detection of bacteria in clinical samples[33]. The qPCR assay developed in this study has proved to be a time- and cost-effective high-throughput method. It can be performed directly on isolated bacterial gDNA with a processing time of less than 2 hours after gDNA extraction[22]. Another advantage of this qPCR assay is that amplification reactions are analysed in a closed system, eliminating the need for post-amplification manipulation and therefore reducing opportunities for contamination[27].

The *nuc* gene primers used in this study were selected based on their specificity for the *S. aureus* genome[34,35]. The 270-bp fragment of the *nuc* gene was successfully amplified using conventional PCR and optimised for the qPCR assay. Brakstad and colleagues (1992) originally proved the suitability of the *nuc* gene for the detection of *S. aureus* and the results of this study agree with their claims[24].

The sensitivity threshold of this qPCR assay, with the reference strain *S. aureus* AU-19, was equivalent to 10^2 CFU/mL^{-1} (Cq 33.63 ± 0.58) after overnight enrichment culture. Non-specific amplification was observed after 35 cycles. The occurrence of non-specific amplification, such as primer dimer, can be overcome with further optimisation of the qPCR assay or the incorporation of a fluorescently labelled oligonucleotide probe[36]. Hoegh and colleagues (2014) reported that using qPCR *nuc* gene-specific primers alone for detection and identification of *S. aureus* may result in misidentification of certain strains of *S. aureus* and that additional species-specific targets should be considered to increase assay efficiency[37]. Numerous qPCR-based assays targeting the *nuc* gene alone or in combination with the *mec*A gene have been described for the rapid screening or identification of *S. aureus,* including both methicillin-sensitive and methicillin-resistant strains of *S. aureus*[25,27,38].

In total, 41 clinical PICC samples were used in this study to validate the sensitivity of the qPCR assay by determining the detection level of *S. aureus*. One of the 41 clinical PICC samples was found to be colonised (10^3 CFU/mL^{-1}) by both the qPCR assay and the semi-quantitative roll-plate culture method. On further analysis, the patient was diagnosed with CLABSI and isolates from both the PICC and patient's bloodstream were identified as *S. aureus*. Therefore, this qPCR assay proved to be an accurate and rapid detection method for *S. aureus* PICC colonisation.

Although this study had limitations, including a small sample size, and only one patient with confirmed *S. aureus* PICC colonisation and subsequent CLABSI, it does present a good model and demonstrates promise as a suitable method for the detection of *S. aureus* PICC colonisation.

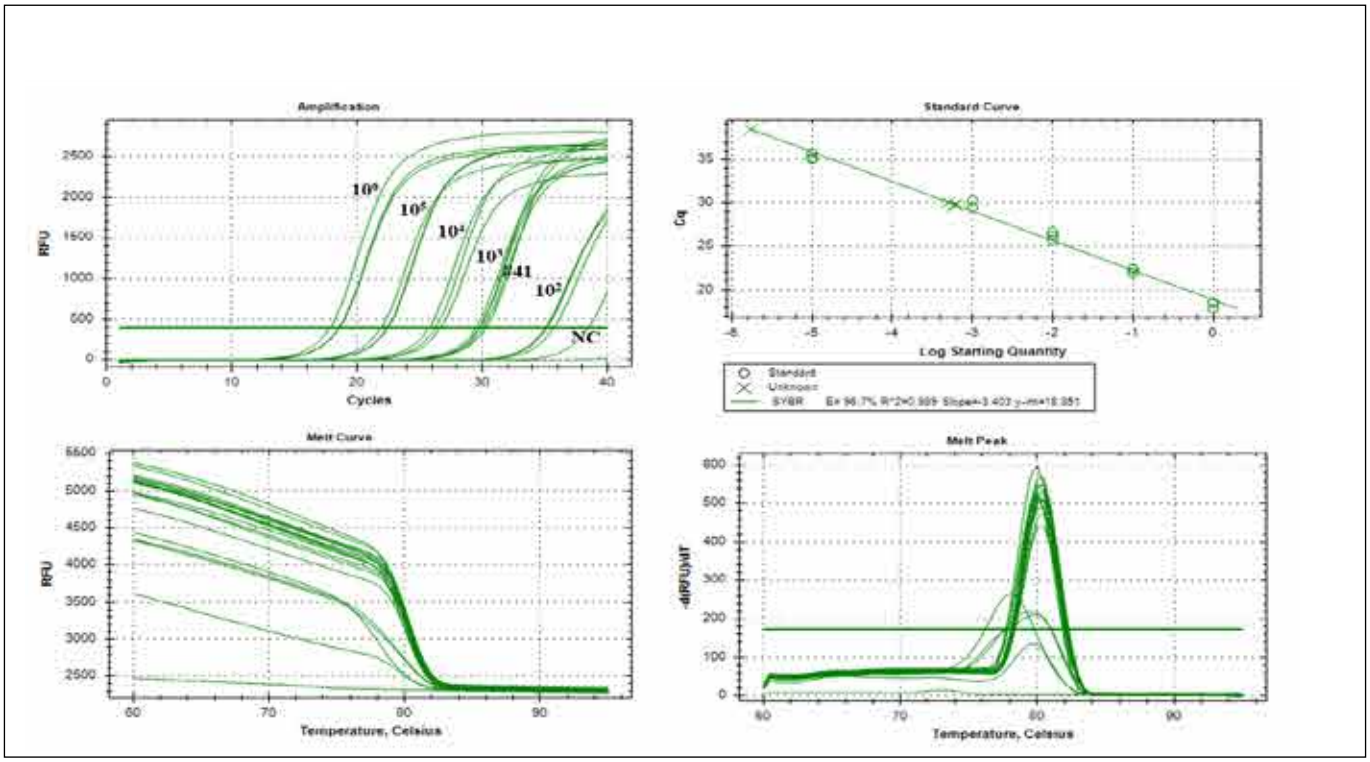

Figure 3. Real-time qPCR assay. Amplification of the nuc *gene (106–102* nuc *gene copy) using gDNA isolated from an overnight culture of* S. aureus *AU-19. One clinical PICC sample was amplified at Cq 29.82 ± 0.18. The standard curve plotted using the log starting quantity against the mean Cq. The straight lines calculated by linear regression and the data yielded an efficiency of 96.7%, correlation coefficient (R2)=0.989 and slope of the graph (y)=-3.403. Error bars indicate the standard deviations based on three replications.*

The qPCR assay, using primers specific to the *nuc* gene used in this study, accurately and rapidly detected *S. aureus* PICC colonisation. With further optimisation and validation with a larger sample size, this qPCR assay has the potential to be used as a diagnostic method in a clinical setting.

ACKNOWLEDGEMENT

The authors thank all clinical staff within the Vascular Assessment and Management Service in Queensland Children's Hospital and AVATAR group for their efforts in recruiting patients, data collecting, and ensuring protocol adherence.

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