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Evaluation of a Real-Time PCR Assay for Simultaneous Detection of *Kingella kingae* and *Staphylococcus aureus* from Synovial Fluid in Suspected Septic Arthritis

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Direct plating of synovial fluid (SF) on agar-based media often fails to identify pathogens in septic arthritis (SA). We developed a PCR assay for the simultaneous detection of *Kingella kingae* and *Staphylococcus aureus* from SF to evaluate molecular detection in SF and to estimate the incidence of *K. kingae* in SA in North America. The assay was based on detection of the *cpn60* gene of *K. kingae* and the spa gene of *S. aureus* in multiplex real-time PCR. *K. kingae* was identified in 50% of patients between 0 and 5 yr of age (n=6) but not in any patients >18 yr old (n=105). Direct plating of SF on agar-based media failed to detect *K. kingae* in all samples. The PCR assay was inferior to the culture-based method for *S. aureus*, detecting only 50% of culture-positive cases. Our findings suggest that *K. kingae* is a common pathogen in pediatric SA in North America, in agreement with previous reports from Europe. PCR-based assays for the detection of *K. kingae* may be considered in children with SA, especially in those with a high degree of clinical suspicion.

Key Words: *Kingella kingae, Staphylococcus aureus*, Septic arthritis, Synovial fluid, Real-time PCR

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Bacterial cultures of synovial fluid (SF) are often negative in suspected cases of septic arthritis (SA) [1-3]. While *Staphylococcus aureus* is the most common causative agent of culture-confirmed SA, *Kingella kingae* is increasingly being recognized as an important cause of SA in young children [3, 4]. *S. aureus* is readily isolated by using conventional culture-based techniques, although the inhibitory nature of SF or treatment with antibiotics prior to specimen collection may lead to false-negative results [5, 8]. *K. kingae* is a fastidious gram-negative bacterium that is rarely recovered by direct plating of SF on agar-based media, and recent PCR-based studies from Europe on SF specimens suggest that the rate of *K. kingae* SA in pediatric patients is grossly underestimated [4, 6-9]. However, the incidence of *K. kingae* SA in North America remains unclear.

We developed a multiplex real-time PCR assay targeting the spa gene of *S. aureus* and the *cpn60* and *rtxA* genes of *K. kingae* [8, 10]. Primers and probes were based on published literature and in-house bioinformatics analyses (Table 1). DNA was extracted from SF by using the MoBio BiOstic bacteremia DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). The reaction volume for PCR was 25 µL, comprising 5 µL of DNA template, 12.5 µL of 2× Qiagen Quantifast PCR master mix (Qiagen, Valencia, CA, USA), 0.2 µmol of primers, and 0.2 µmol of probes. PCR reactions were run for 45 cycles of melting at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 70°C for 15 sec, by using the SmartCyclerII system (Cepheid, Sunnyvale, CA, USA). Fluorescence detection was performed at the annealing step.

Specificity of the assay was established by using DNA from bacteria showing genetic similarity to either *K. kingae* or *S. au-*

Table 1. Primers and probes used in this study

Primer/Probe	Target gene	Primer sequence (5'-3')	Amplified region	Reference
CF (Primer)	*cpn60*	GCAAGAAGTCGGCAAAGAG		8
CR (Primer)	*cpn60*	GTCAAACAACAACACAAATGGG	175 bp	8
RF (Primer)	*rtxA*	GCGCACAAGCAGGTGTACAA		9
RR (Primer)	*rtxA*	ACCTGCTGCTACTGTACCTGTTTTAG	71 bp	9
SF (Primer)	*spa*	TACATGTCGTTAAACCTGGTG		10
SR (Primer)	*spa*	TACAGTTGTACCGATGAATGG	224 bp	10
Cpn-P (Probe)	*cpn60*	TET-CGGTCAAATTGCATACCTTTAACCACTTCTTGACCG-BHQ	N/A	8
RTX-P (Probe)	*rtxA*	FAM-TTGAACAAAGCTGGACACG-BHQ	N/A	9
SPA-P (Probe)	*spa*	FAM-CAAACGGCACTACTGCTGACAAAATTGCTGCA-BHQ	N/A	This study

Table 2. Solid media-based culture or PCR positivity of synovial fluid specimens according to causative organisms and age groups of the patients

Age group	N of specimens	N of specimens (%)					
		K. kingae			*S. aureus*		
		PCR+/Culture+	PCR+/Culture-	PCR-/Culture+	PCR+/Culture+	PCR+/Culture-	PCR-/Culture+
0-5	6	0 (0)	3 (50)	0 (0)	0 (0)	0 (0)	1 (16.7)
6-18	6	0 (0)	1 (16.7)	0 (0)	1 (16.7)	0 (0)	0 (0)
>18	105	0 (0)	0 (0)	0 (0)	3 (2.9)	0 (0)	3 (2.9)
Total	117	0 (0)	4 (3.4)	0 (0)	4 (3.4)	0 (0)	4 (3.4)

reus, and by using known agents of SA, including *Moraxella catarrhalis*, *Haemophilus influenzae, Aggregatibacter actinomycetemcomitans, Micrococcus* spp., *Streptococcus pneumoniae, Staphylococcus epidermidis, Staphylococcus lugdunensis, Neisseria gonorrhoeae, Neisseria flavescens, Eikenella corrodens, S.aureus*, and *K. kingae*. A threshold cycle (Ct) of 34 cycles was selected for reporting a "positive" detection value for *K. kingae* (for both *cpn60* and *rtxA*) and 38 cycles for *S. aureus* detection. By using serial dilutions of DNA, the limit of detection was 56 fg of *K. kingae* DNA for both *cpn60* and *rtxA* and 74.2 fg of *S. aureus* DNA for *spa*. Control culture-negative SF was spiked with serial dilutions of pure *K. kingae* (clinical isolate) or *S. aureus* (ATCC 29213). For *K. kingae*, the limit of detection was 1.5×10^3 colony-forming units (CFU)/mL for *cpn60* and 1.5×10^4 CFU/mL for *rtxA*. For *S. aureus*, the limit of detection was 1.5×10^4 CFU/mL for *spa*. Given the superior detection limit for *cpn60* over *rtxA*, *cpn60,* and *spa* were selected for simultaneous multiplex PCR detection of *K. kingae* and *S. aureus*.

SF samples were collected from 117 patients with suspected SA between 7/16/2009 and 5/29/2011 at 2 major academic medical centers in the United States: St. Louis Children's Hospital/Washington University School of Medicine (11 children and 1 adult) and the Vanderbilt University Hospital (1 child and 104 adults). Samples from the Vanderbilt University hospital were frozen and shipped to St. Louis, where all PCR testing was performed. All samples underwent standard analysis for SF, including direct plating on solid agar-based media at the source institution prior to PCR analysis.

Four of the 117 samples (3.4%) were positive for *K. kingae* by PCR assay (Table 2). Thirteen samples exhibited weak amplification, with a Ct greater than the cut-off value of 34 (Table 3). In the absence of an appropriate gold standard, additional PCR with *rtxA*-specific primers was performed on the 13 samples. As these samples also failed to meet the Ct criteria for *rtxA* PCR, they were deemed negative for *K. kingae* (Table 3). Prolonged Ct (>cut-off value) might represent non-specific amplification from the host genome or might be due to probe degradation at higher cycles. All *K. kingae*-positive samples were from pediatric patients (Table 2). Gram staining of SF or direct plating on solid agar-based media did not detect *K. kingae* in any specimen (Tables 2 and 3). One of the 4 PCR-positive samples was directly inoculated into a blood culture broth (Bactec Peds Plus, aerobic bottle, incubated in the Bactec FX system) (BD, Franklin Lakes, NJ, USA), and under these conditions, *K. kingae* was isolated. The superiority of PCR over direct plating and the 50% PCR positivity in the 0-5 yr age group are consistent with recent studies

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Table 3. Ct values and culture results of culture-positive or Ct value-positive synovial fluid specimens

Specimen No.	Ct of target genes			Solid media-based culture results	Causative organisms
	cpn60	*rtxA*	*spa*		
CH-2	40.9	40.6	Negative	Negative	Negative
Ch-6	36.4	Negative	Negative	Negative	Negative
CH-7	32.5	N/A	Negative	*S. aureus*, rare	*K. kingae* and *S.aureus*
CH-8	29.8	N/A	Negative	Negative	*K. kingae*
CH-9	Negative	N/A	27	*S. aureus*, abundant	*S. aureus*
CH-11	34.6	Negative	33.5	*S. aureus*	*S. aureus*
CH-12	30.5	N/A	39.7	Negative	*K. kingae*
CH-13	31	N/A	Negative	Negative*	*K. kingae*
V-11	Negative	N/A	Negative	*S.aureus*	*S. aureus*
V-12	Negative	N/A	34.4	*S. aureus*	*S. aureus*
V-20	42.7	40.7	Negative	Negative	Negative
V-25	38.1	Negative	Negative	Negative	Negative
V-30	41.6	Negative	Negative	Negative	Negative
V-32	40.3	Negative	Negative	Negative	Negative
V-33	42.5	Negative	Negative	Negative	Negative
V-43	40.9	41	Negative	Negative	Negative
V-50	43.3	Negative	Negative	Negative	Negative
V-70	Negative	N/A	41.5	Negative	Negative
V-71	Negative	N/A	32.4	*S. aureus*	*S. aureus*
V-72	Negative	N/A	41.8	Negative	Negative
V-73	40.9	Negative	Negative	Negative	Negative
V-75	Negative	N/A	42.3	Negative	Negative
V-78	Negative	N/A	42.8	Negative	Negative
V-79	Negative	N/A	43	Negative	Negative
V-80	41.6	Negative	Negative	Negative	Negative
V-82	Negative	Negative	42.2	Negative	Negative
V-87	Negative	N/A	Negative	*S. aureus*, Light growth	*S. aureus*
V-93	43.7	Negative	Negative	Negative	Negative
V-99	Negative	N/A	Negative	*S. aureus*, Light growth	*S. aureus*

*Positive by BACTEC culture.
Abbreviations: N/A, not applicable; Ct, threshold cycle.

from Europe [8]. Inoculation of blood culture broth with SF improves the detection of *K. kingae* when compared to direct plating [6, 7]. We could not compare the PCR assay with the practice of inoculating blood bottles as only a few samples were subjected to the latter during routine clinical care. However, the superiority of PCR-based detection compared to blood broth inoculation has been demonstrated before [8]. Furthermore, the rapid turn-around time for PCR-based assays is an additional advantage over culture-based detection. Therefore, we recommend PCR-based assays for the detection of *K. kingae* in pediatric SA, especially in cases with a high index of clinical suspicion.

Four of the 117 samples (3.4%) were positive for *S. aureus* by PCR (Table 2). Direct plating on agar-based media is the gold standard for the detection of *S. aureus*. The pathogen grew from all 4 PCR-positive samples (Table 2). Growth was reported as "abundant" for 2 samples and "light" for 1 sample. Direct Gram staining of SF demonstrated gram-positive cocci in 3 out of 4 samples. Four additional samples were culture-positive but PCR-negative (Table 2). These samples were negative for direct Gram staining of SF and demonstrated "light growth" of *S. au-*

reus on 3 of the 4 plates (Table 3). Notably, 1 of the 4 PCR-negative culture-positive samples (CH-7) was positive for *K. kingae* by PCR (Table 3). This sample demonstrated "rare" growth of *S. aureus* along with some growth of *Streptococcus anginosus*, suggesting contamination as the probable cause of *S. aureus* culture positivity in this particular sample. Seven additional samples exhibited weak spa amplification, with Ct >38 (Table 3). However, none were culture-positive for *S. aureus*. In summary, conventional plating on agar-based media was more sensitive than our PCR assay for the detection of *S. aureus* in SF, especially with a low pathogen burden. This is not surprising, considering the established limit of detection of this assay. While lowering the cut-off Ct values may enhance sensitivity, we note that none of the PCR-negative (based on Ct ≤38) culture-positive samples demonstrated late Ct positivity (≥38) suggesting an intrinsic limitation of this specific biological matrix (SF). To our knowledge, this is the first report comparing PCR to conventional culture-based methods for detecting *S. aureus* in SF, and thus, the results will be of interest to laboratories considering molecular techniques for the detection of this pathogen.

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