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New Real-Time PCR-Based Method for *Kingella kingae* DNA Detection: Application to Samples Collected from 89 Children with Acute Arthritis▽†

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Inoculation of blood culture vials with joint fluid samples has revealed the important pathogenic role of *Kingella kingae* in pediatric arthritis. However, recent studies based on broad-range 16S ribosomal DNA PCR and real-time PCR without a probe suggest that conventional methods remain suboptimal. We developed a new real-time PCR method with a probe that is highly specific for *K. kingae* and applied it to joint fluid samples collected from 89 children with suspected arthritis admitted to our institution during a 2-year period. Real-time PCR was also applied to blood samples obtained before surgery and to joint drainage fluid samples obtained during several days after surgery. Thirty-six (40%) of the 89 cases of suspected septic arthritis had positive culture. *Staphylococcus aureus* was the main isolate ($n = 19/36$, 53%), followed by *K. kingae* ($n = 7/36$, 19%). Specific real-time PCR identified *K. kingae* in 24 of the 53 culture-negative cases. Thus, *K. kingae* was present in 31 (52%) of the 60 documented cases, making it the leading pathogen. Real-time PCR on all 15 blood DNA extracts from patients with *K. kingae* infection was negative, demonstrating that joint fluid positivity did not result from DNA circulating in blood. Real-time PCR amplification of drainage fluid samples showed that the pathogen could be detected for up to 6 days after antibiotic initiation. *K. kingae* real-time PCR applied to DNA extracted from joint fluid samples, but not from blood samples, markedly improved the etiological diagnosis of septic arthritis in children. Retrospective diagnosis is feasible for up to 6 days after treatment initiation.

Acute septic arthritis in children must be diagnosed and treated urgently because of the risk of long-term sequelae. Identification of the causative organism is required to optimize the choice of antibiotics, but cultures are negative in one-third to two-thirds of patients (13, 19, 29). *Kingella kingae*, a gram-negative coccobacillus, is part of the normal oropharyngeal flora of young children from 6 months to 4 years (10, 30). It was initially considered a rare cause of invasive diseases, including skeletal infections in children and endocarditis in adults. However, the reported number of cases of *K. kingae* arthritis has increased markedly since the 1990s, mainly owing to improvements in culture techniques, such as inoculation of blood culture vials with joint specimens (16, 29). In the literature, *K. kingae* currently accounts for 5% to 29% (5, 14, 19, 24) of culture-positive osteoarticular infections (OAI) and for up to 48% of cases of septic arthritis in children under 2 years of age (27).

K. kingae is a fastidious microorganism, and its frequency in OAI may still be underestimated. Indeed, Stahelin et al. for the first time described the potential benefits of a molecular method, the universal 16S ribosomal DNA PCR method, in a case report of culture-negative arthritis due to *K. kingae* (21). Since then, several molecular methods, applied to a large series of cases, have recently shown a higher prevalence of *K. kingae* than previously reported in this setting. Rosey et al. and Verdier et al., using a universal 16S ribosomal DNA PCR method, found *K. kingae* sequences in, respectively, 18% and 14% of culture-negative specimens from infants with OAI (19, 24). Recently, Chometon et al., using a real-time PCR method without a probe, found that *K. kingae* was the leading cause of OAI in children in Lyon, France (5).

Detection of bacterial DNA does not provide irrefutable proof that the relevant bacterium has a pathogenic role (26). Indeed, Dagan et al. have shown that the DNA of organisms colonizing the respiratory tract, such as pneumococci, can be detected by PCR in serum of uninfected patients (6). Thus, given the ability of *K. kingae* to colonize the respiratory tracts of young children (30), a suitable control, allowing for exclusion of contamination of joint fluid samples by circulating DNA of *K. kingae* in blood, is needed to confirm the relevance of PCR-based diagnoses.

Unlike culture, molecular methods can detect a pathogen after the outset of effective antibiotic therapy for various infectious diseases (3, 15, 18, 23). However, this property has been assessed in very few cases of septic arthritis in adults (23). In this respect, it would be of interest to determine the contribution of PCR to the diagnosis of *K. kingae* infection in

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children who have already received antibiotics before joint fluid aspiration.

The objectives of this prospective study were to describe and evaluate a new *K. kingae*-specific real-time PCR method with a fluorogenic probe and to apply it to blood and joint fluid samples from children with suspected acute septic arthritis. We also describe, in the largest series of cases published to date, the characteristics of *K. kingae* arthritis in this age group.

MATERIALS AND METHODS

Patients and diagnosis. This study involved all children admitted consecutively to our institution between January 2006 and January 2008 for suspected acute septic arthritis. This diagnosis was defined by joint pain and limited limb movement with or without fever (≥38°C) and joint effusion visualized by radiography or sonography. All the children with suspected acute septic arthritis had fluoroscopically guided joint fluid aspiration to document the infection. Biological evaluation included the peripheral blood white cell count (WBC) and the C-reactive protein (CRP) and fibrinogen levels.

Microbiological methods. Before surgery, a blood sample was inoculated into an aerobic blood culture vial. During surgery, joint fluid was immediately inoculated into aerobic blood culture bottles. The blood culture vials were incubated in a continually monitored instrument (BacT/Alert 3D; BioMérieux) and were not blindly subcultured. The remainder of the joint fluid sample was sent to the laboratory for Gram staining, cell count, and immediate inoculation onto Columbia blood agar (incubated in anaerobic conditions), chocolate agar (incubated in CO_2-enriched air), and brain-heart broth. Aliquots (100 to 200 μl) were stored at −80°C for DNA extraction. Blood culture bottles and the other media were incubated for, respectively, 5 and 10 days. When sufficient sample was available, 100 to 200 μl of plasma was separated from blood samples obtained for WBC or clotting tests and was stored at −80°C for DNA extraction. Joint fluid drainage samples were collected 2, 4, and 6 days after surgery and stored at −80°C for DNA extraction. *K. kingae* identification was based on the microbiological characteristics (27).

Preliminary molecular investigations. In order to develop a highly specific PCR-based diagnostic method for *K. kingae*, two primer pairs, Ksm1 (5′ GCA AGAAGTCGGCAAAGAG 3′) and Ksm2 (5′ GTCAAACAACAACACAAA TGGG 3′), amplifying a 175-bp fragment, and KingF (TGTTGGCGCAAGCG ATTGTTGCTG) and KingR (CGCCCACTTGAGCGATTTGCTCG), amplifying a 169-bp fragment, were first designed using the sequence of the nonribosomal gene *cpn60*, which was the only sequence available in the Public Database at the outset of the study (accession number AY123650). The specificity of the primers was assessed by conventional PCR on four reference strains of *K. kingae*, 12 genetically related species and genera, and four non-*K. kingae* isolates from patients with septic arthritis (Table 1). Amplification products obtained with the most specific primers were sequenced, yielding a consensus sequence and allowing us to design a specific "Kingprobe": 6-carboxyfluorescein-CGCGATCGCGACAAGTAGCCACGGTCA AGATCGCG-black hole quencher 1.

Molecular diagnosis. Once a week, DNA was extracted from specimens with the BioRobot EZ1 workstation using the EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. Part of the DNA extract was immediately amplified, and the remainder was stored at −80°C. A negative extraction control was included in each run, consisting of all the reagents used for DNA extraction minus the sample. The real-time PCR mixture contained 5 μl of DNA, 1 μl of each primer (Ksm1 and Ksm2) at 10 μM, 1 μl of probe at 10 μM, and 25 μl of IQ qPCR Mastermix (Eurogentec, Seraing, Belgium) in a final volume of 50 μl. Amplification was performed in an iCycler (Bio-Rad, Marnes la Coquette, France), with an initial step of 15 min at 95°C, followed by 45 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C and a final extension step of 10 min at 72°C. A positive control consisting of DNA extracted from *K. kingae* CIP 80.16 was included in each PCR test run. For each sample, amplification of the human beta globulin gene (262 bp) with primers B2M-TR-1 (5′ GCAAGGACTGGTCTTTCTATC 3′) and B2M-TR-2 (5′ TACACAACTT TCAGCAGCTTACA 3′) and the probe B2M-TR-sde (6-carboxyfluorescein-C GTGCCCTGCCGTGTGAACCATGTGACTTTGGCACG-black hole quencher 1) served as an internal positive extraction control and was used to detect the presence of PCR inhibitors as previously described (1).

Treatment. If purulent fluid was recovered from joint fluid aspiration, open arthrotomy or arthroscopy was performed for joint lavage. After surgery, all the patients received a standardized treatment protocol. Empirical antibiotic therapy was started with intravenous cefotaxime (200 mg/kg of body weight/24 h) and fosfomycin (200 mg/kg/24 h) for 7 days, followed in case of no documented arthritis by oral amoxicillin (amoxicilline)-clavulanic acid (80 mg/kg/24 h) and rifampin (rifampicin) (20 mg/kg/24 h) for 5 weeks. In case of documented *K. kingae* arthritis, the antibiotic regimen consisted of amoxicillin alone (150 mg/kg/24 h in three doses) for the same length of time. Cast immobilization was not used. All the patients had a minimum of 6 months of follow-up.

TABLE 1. Primer specificity for *K. kingae* DNA amplification

Species (serogroup)	Origin	Amplification with primer pair[a]:	
		KingF-KingR	Ksm1-Ksm2
Kingella kingae	CIP 80.16	+	+
Kingella kingae	CIP 68.12	+	+
Kingella kingae	CIP 73.01	+	+
Kingella kingae	CIP 102470	+	+
Kingella denitrificans	CIP 103473	+	−
Kingella oralis	CIP 103803	+	−
Capnocytophaga canimorsus	CIP 103936	−	−
Capnocytophaga ochracea	CIP 103448	−	−
Aggregatibacter actinomycetemcomitans	CIP 52.106	NS band	−
Aggregatibacter aphrophilus	CIP 70.73	−	−
Haemophilus influenzae	CIP 52.152	−	−
Cardiobacterium hominis	CIP 70.70	−	−
Eikenella corrodens	CIP 70.75	NS band	−
Neisseria polysaccharea	CIP 100113	−	−
Alysiella crassa	CIP 103341	+	−
Conchiformibius steedae	CIP 103435	−	−
Neisseria meningitidis (W135)	Clinical isolate	−	−
Streptococcus pneumoniae	Clinical isolate	−	−
Streptococcus pyogenes	Clinical isolate	−	−
Staphylococcus aureus	Clinical isolate	−	−

[a] +, positive amplification with a band of the expected length; −, no band observed; NS band, nonspecific band (of unexpected length).

Statistical analysis. Means and frequencies were compared using the Mann-Whitney U test and the chi-square test. *P* values below 0.05 were considered to denote significant differences.

Nucleotide sequence accession numbers. *cpn60* gene amplification region sequences of *K. kingae* strains and *K. kingae*-positive samples have been assigned the following GenBank accession numbers: for *K. kingae* strain ATCC 23331, EU864312; for *K. kingae* strain CIP 73.1, EU864313; for *K. kingae* strain CIP 102473; EU864314; for sample 80700158 (collected from a 16-month-old girl with knee arthritis), EU864315; and for sample 80900052 (collected from a 13-month-old boy with ankle arthritis), EU864315.

RESULTS

Development of real-time *K. kingae*-specific PCR. Of the two primer pairs, only Ksm1-Ksm2 was highly specific for *K. kingae* (Table 1). This pair was used to obtain partial *cpn60* gene sequences from four *K. kingae* reference strains and from two of our clinical isolates. The resulting sequences, excluding regions that we found contained several point mutations ($n = 8$) (GenBank accession numbers EU864312 to EU864316 and AY123650), were then used to design a probe (see the figure in the supplemental material). The real-time PCR was assessed against the panel of test strains and was positive only with *K. kingae* strains. The detection limit of the real-time PCR method, determined with serial dilutions of *K. kingae* genomic DNA containing 10^6 copies to 1 copy, was 200 copies by PCR.

Demographic and microbial characteristics. During the study period, 89 patients, with ages from 1 month to 14 years (mean: 45 months) underwent surgery in our institution for suspected septic arthritis. For each patient, culture and real-

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TABLE 2. Microbiological diagnosis of 31 pediatric cases of acute *Kingella kingae* arthritis

Diagnostic method	Proportion (%) of positive results
Cultures of joint fluid on solid media	3/31 (10)
Cultures of joint fluid in blood culture vials	7/31 (22)
Peripheral blood culture	0/31
Real-time PCR on joint fluid at admission	31/31 (100)
Real-time PCR on blood samples at admission	0/15
Real-time PCR on joint drainage fluid	
2 days after treatment initiation	9/9 (100)
4 days after treatment initiation	4/5 (80)
6 days after treatment initiation	3/4 (75)

time PCR were both performed on joint fluid aspirates. None had received antibiotics in the week before their admission. A microorganism was cultured in 36 (40%) cases. *Staphylococcus aureus* was the most prevalent (19 cases, 53%), followed by *K. kingae* (7 cases, 19%). The performance of the different culture methods for *K. kingae* isolation is indicated in Table 2. *K. kingae* was never isolated by peripheral blood culture. None of the seven *K. kingae* isolates produced β-lactamases. The other pathogens were *Streptococcus pneumoniae* (four cases, 11%); *Salmonella* spp. (three cases, 8.3%); and nonencapsulated *Haemophilus influenzae*, *Neisseria meningitidis* serogroup W135, and *Escherichia coli* K1 (one case each).

Our specific real-time PCR method was positive for all the patients with *K. kingae*-positive culture and negative for all other patients with microbiologically documented arthritis. Among the 53 culture-negative patients, the real-time PCR assay identified *K. kingae* in 24 cases (45%). Thus, when culture and real-time PCR were combined, *K. kingae* was found to be the primary pathogen among the documented cases (31/[36 + 24]; 52%), ahead of *S. aureus* (19/60; 32%). Real-time PCR was also applied to DNA extracts from blood samples of 15 patients with a molecular diagnosis of *K. kingae* infection (Table 2). None was positive. Real-time PCR was also applied to joint drainage fluid samples from nine patients. All nine samples obtained 48 h after treatment initiation were positive. Four (80%) of the five samples obtained after 4 days were positive, and so were three (75%) of the four samples obtained after 6 days (Table 2).

Baseline clinical and biological characteristics of patients with *K. kingae* arthritis. Twenty-seven (87%) of the 31 children with *K. kingae* arthritis were less than 24 months old, compared to only 3 (16%) of the 19 children with *S. aureus* arthritis ($P < 0.001$). Symptoms started an average of 3 days before admission (range, 1 to 10 days). The most frequently affected joint was the knee (52%), followed by the hip (26%) (Table 3). One-third of the patients were not febrile at admission. The CRP level was slightly elevated in all but one case. The fibrinogen level was elevated in all the patients tested. In contrast, the WBC was abnormal in only three cases. Gram staining of joint fluid showed *K. kingae* in only one case. Joint fluids were hematic in six cases and purulent in others, with a median cell count of 106,000 cells/mm^3 (range, 5,900 to 3,200,000 cells/mm^3). The seasonal distribution of *K. kingae* arthritis was as follows: 9 cases in fall, 7 cases in winter, 13 cases in spring, and 2 cases in summer.

Outcome. After 3 days of intravenous antibiotic therapy the CRP was normal in only eight cases but the temperature was below 37.5°C in every case (Table 3). After 7 days the CRP level was normal in all 30 patients tested. The hospital stay lasted 7 days in every case, owing to the use of a standard treatment protocol. No complications, including epiphyseal arrest, were reported during follow-up. None of the patients had a revision procedure.

TABLE 3. Clinical and biological characteristics of 31 pediatric cases of acute *Kingella kingae* arthritis

Characteristic	Proportion (%) of patients	Value		
		Median/mean	10th–90th percentile	Range
Age (mo)		16/19.3	10–38	8–68
Boys	18/31 (58)			
Location of arthritis				
Knee	16/31 (52)			
Hip	8/31 (26)			
Ankle	3/31 (10)			
Shoulder	1/31 (3)			
Elbow	1/31 (3)			
Wrist	1/31 (3)			
Proximal interphalangeal joint	1/31 (3)			
Temp (°C)	19/31 (61)[a]	38/38	37–38.8	36.3–39.9
At admission				
On day 3	0/31 (0)[a]			
CRP (mg/liter)	30/31 (97)[a]	32/39	18–69	10–133
At admission				
On day 3	23/31 (74)[a]	22/28	10–63	10–122
On day 7	0/30 (0)[a]			
WBC (1,000/mm^3) at admission	3/31 (10)[a]	12.3/12.4	9.3–16.3	6–19.6
Fibrinogen (g/liter) at admission	27/27 (100)[a]	5.4/5.8	5–7.1	4.2–9.1

[a] Proportion of patients with abnormal values (temperature, >38°C; CRP, >10 mg/liter; WBC, >17,000/mm^3 between 6 months and 2 years and >15,000/mm^3 between 4 and 6 years; fibrinogen, >4 g).

DISCUSSION

In this study, we evaluated a new *K. kingae*-specific real-time PCR method in a prospective series of consecutive hospitalized children with acute septic arthritis. We also tested, for the first time, blood samples obtained at admission and joint fluid obtained during treatment.

The specificity of our *K. kingae* real-time PCR method with a fluorogenic probe was assessed by testing not only pathogens frequently involved in septic arthritis but also other *Kingella* species and a phylogenetically related genus. We found that a homologous *cpn60* gene is probably shared by *K. kingae*, *Kingella oralis*, and *Kingella denitrificans* and by *Alyssiela* spp. However, one primer pair (Ksm1-Ksm2) was highly specific for *K. kingae*. The probe design was based on the sequences of the amplicons obtained with this primer pair and six strains of *K. kingae* and took into account the presence of several regions bearing point mutations. These sequence variations clearly underlined the necessity of sequencing the target gene to optimize the probe design, and, to our knowledge, this has not be previously performed on *K. kingae*. To further evaluate the specificity of our method, we also tested another primer pair based on the recently sequenced hemolysin gene *rtxA* (9). All our real-time PCR-positive samples were also positive for *rtxA* (not shown), providing evidence of the high specificity of our PCR method.

Thirty-six (40%) of the 89 cases of suspected acute septic arthritis were documented by culture, a proportion consistent with other studies (13, 19, 29). *S. aureus* was the main pathogen detected by culture (19 cases, 53%). *K. kingae* accounted for

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19% (7/36) of culture-positive cases, a proportion similar to that reported by Yagupsky et al. (22%) (29) and higher than that reported by Luhmann and Luhmann (8%) (13). However, when we added the results of our real-time PCR method, the rate of documented arthritis rose from 40% to 67% and *K. kingae* became the most prevalent pathogen (31 cases, 52%). In children younger than 24 months, *K. kingae* accounted for 75% (27/36) of the documented cases.

To our knowledge, this is the second study to evaluate the potential benefit of a specific PCR method for the diagnosis of *K. kingae* infections on a large series of cases (5). Very recently, Cherkaoui et al. developed a real-time PCR with a probe, described in two case reports (4). By comparison with conventional culture, our PCR method increased the *K. kingae* detection rate by a factor of more than four, from 7 to 31 cases. Chometon et al. recently reported that the use of a different specific PCR method increased the number of identified cases of *K. kingae* osteomyelitis and arthritis by a factor of approximately two (from 17 to 39), making this the most prevalent pathogen in children with these diseases (5). Rosey et al., using a broad-range PCR method, found that 30% of cases of septic arthritis in a pediatric population with a median age of 32 months were due to *K. kingae* (19). The higher rate observed here (52%) may be due in part to the better sensitivity of specific PCR methods (5).

DNA from *S. pneumoniae* colonizing the throats of young infants can enter the bloodstream and give false-positive diagnosis of infection by this organism from PCR on nucleic acids extracted from blood (6). As *K. kingae* colonizes the throat in up to 20% of young infants, Yagupsky et al. pointed out that a positive PCR result for joint fluid might not necessarily prove the responsibility of *K. kingae* (26). Interestingly, however, we tested presurgical blood samples of 15 patients with a diagnosis of *K. kingae* arthritis and all were PCR negative without detection of PCR inhibitors. These results indicate that real-time PCR on blood samples does not contribute to the etiological diagnosis and that PCR positivity of joint fluid is not due to bacterial DNA derived from blood, at least at the time when the specimens were collected. Therefore, we think that we provided for the first time a control set which gives evidence of the true benefit of real-time PCR for diagnosis of *K. kingae* arthritis. In addition, real-time PCR was always negative for patients with arthritis due to other pathogens, serving also as a control set (26), although they tended to be older than the children with *K. kingae* infection.

The main demographic and clinical characteristics of the 31 cases of acute *K. kingae* arthritis are in keeping with previous reports (5, 7, 8, 12, 14, 16, 24, 28). In particular, patients with *K. kingae* infection tended to be young (mean age, 19.3 months versus 98 months for patients with *S. aureus* arthritis; $P < 0.001$), confirming the higher prevalence of this microorganism in children less than 3 years of age (5, 7, 8, 12, 14, 16, 24, 28). Clinical and biological signs at admission were often mild, consisting of slightly elevated temperature, CRP, and fibrinogen values. Fibrinogen was the most sensitive marker of inflammation in our patients, all of whom had abnormal values. The frequency of *K. kingae* infection was lowest in summer (2 cases), as also noted in previous studies (5, 8, 24) except one (7). Again in keeping with the literature, we found that *Kingella* arthritis was always monoarticular and mainly affected the lower extremities (27/31 cases in our series) (27). *K. kingae* mainly infected the knee, as expected (27), whereas *S. aureus* and *S. pneumoniae* mainly infected the hip in other series (20, 25). Indeed in our series *S. aureus* infected the hip in 9 of the 19 cases (not shown).

One limit of PCR-based diagnosis is that it provides no information on antimicrobial susceptibility. However, *K. kingae* is highly susceptible to β-lactam antibiotics: reported MIC_{50} values of penicillin G and amoxicillin are 0.023 and 0.16 mg/liter, respectively (11, 31). Very few isolates, including three in Iceland, have been reported to produce β-lactamases (2). Therefore, we believe that, in most countries, *K. kingae* arthritis diagnosed by PCR may be treated confidently with a β-lactam drug such as amoxicillin, providing an easy switch from intravenous to oral antibiotic therapy.

One other advantage of PCR methods is that they can yield an etiological diagnosis after treatment initiation. Depending on the sites of infection and the pathogens, DNA may persist for several weeks (3, 15, 18) or less than 48 h (17, 22). To our knowledge only one study has investigated this property in cases of septic arthritis (23). In this study, broad-range PCR was applied to serial joint fluid samples from six adults with septic arthritis due to various bacteria. *Streptococcus pyogenes*, *Neisseria meningitidis*, and *Peptostreptococcus anaerobius* DNA was detected 1 week after treatment initiation. Likewise, we show here for the first time that *K. kingae* DNA can be detected in joint fluid for up to 6 days after treatment initiation.

In conclusion, our new highly specific real-time PCR confirms that *K. kingae* is the major bacterial cause of arthritis in children. We provide strong evidence that *K. kingae* DNA in joint fluids, at least at the time when the specimens were collected, does not arise from DNA circulating in blood and that the bacterial DNA persists for several days after treatment initiation, allowing retrospective diagnosis. In a more general consideration, our real-time PCR may also contribute in the future to the diagnosis of endocarditis, to knowledge on the colonization characteristics of this pathogen, and to outbreak investigation (10).

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AUTHOR'S CORRECTION

New Real-Time PCR-Based Method for *Kingella kingae* DNA Detection: Application to Samples Collected from 89 Children with Acute Arthritis

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Vol. 47, no. 6, p. 1837–1841, 2009. Page 1838, column 1, lines 36 and 37 of Materials and Methods: "6-carboxyfluorescein-CGCGATCGCGACAAGTAGCCACGGTCAAGATCGCG-black hole quencher 1" should read "6-carboxyfluorescein-CGGTCAAATTGCATACCTTTAACCACTTCTTGACCG-black hole quencher 1."