Molecular diagnosis of *Kingella kingae* osteoarticular infections by specific real-time PCR assay

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Kingella kingae is an emerging pathogen that is recognized as a causative agent of septic arthritis and osteomyelitis, primarily in infants and children. The bacterium is best detected by rapid inoculation in blood culture systems or by real-time PCR assays. Pathogenesis of the agent was linked recently to the production of a potent cytotoxin, known as RTX, which is toxic to a variety of human cell types. The locus encoding the RTX toxin is thought to be a putative virulence factor, and is, apparently, essential for inducing cytotoxic effects on respiratory epithelial, synovial and macrophage-like cells. Herein, we describe a novel real-time PCR assay that targets the RTX toxin gene and illustrate its use in two clinical cases. The assay exhibited a sensitivity of 30 c.f.u., which is 10-fold more sensitive than a previously published semi-nested broad-range 16S rRNA gene PCR, and showed no cross-reactivity with several related species and common osteoarticular pathogens.

INTRODUCTION

Since its characterization in 1960, *Kingella kingae* has been implicated rarely as a causative agent of endocarditis in adults and it is included in the HACEK group (Kiang *et al.*, 2005; Matta *et al.*, 2007). More recently, it has been reported as a causative agent of osteoarticular paediatric infections (Lebel *et al.*, 2006). There are currently five known *Kingella* species: *Kingella kingae*, *Kingella indologenes*, *Kingella denitrificans*, *Kingella potus* and *Kingella oralis*. Among these species, *K. kingae* has been most commonly reported to cause endocarditis (Wells *et al.*, 2001), sepsis, and bone and joint infections (Kiang *et al.*, 2005; Berkun *et al.*, 2004) in humans. The bacterium is occasionally found to be a normal inhabitant of the upper respiratory tract (Yagupsky *et al.*, 2002). Interestingly, *K. kingae* has been implicated in concomitant upper respiratory tract infections or stomatitis in children with invasive viral infections (Costers *et al.*, 2003; Dodman *et al.*, 2000). It is believed that damage to the respiratory mucosa (e.g. due to the viral infection) allows haematogenous spread of *K. kingae* to distant organs, especially joints and bones. For example, *K. kingae* was isolated from the blood of 4 out of 29 young children with culture-proven herpetic gingivostomatitis (Yagupsky *et al.*, 2002; Amir & Yagupsky, 1998).

The pathogenesis of *K. kingae* was recently attributed to the production of a potent cytotoxin (RTX) that may play a role in colonization of the respiratory tract, invasion of the bloodstream, and damage to the joints (Kehl-Fie & St Geme, 2007).

We describe here a novel real-time PCR assay that targets the RTX toxin gene and report its use in two clinical cases of invasive *K. kingae* infections.

METHODS

Strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All strains were stored at –80 °C in skim milk with 15 % glycerol. *K. kingae* was grown on Columbia blood agar and chocolate agar at 37 °C with 5 % CO₂.

DNA extraction and purification. *K. kingae* strains, osteoarticular fluids and synovial biopsies were incubated for 1 h at 55 °C with proteinase K and lysis buffer. DNA was then extracted with a MagNAPure LC instrument using the MagNAPure LC DNA isolation kit II (Roche Molecular Biochemicals) according to the manufacturer's instructions. The final elution volume was 100 µl. For each PCR analysis, we used 5 µl DNA extract.

Broad-range PCR amplification of 16S rRNA. Broad-range PCR amplification of the 16S rRNA gene was performed as previously

Table 1. Strains used in this study

Patient	Gender* and age (years)	Diagnosis	Sample type	Culture	<i>K. kingae</i> strain confirmation by 16S sequencing†	qPCR assay	
						<i>rtxA</i>	<i>rtxB</i>
1	M (3)	Septicaemia	Blood	<i>K. kingae</i>	Yes	+	+
2	M (3)	Septicaemia	Blood	<i>K. kingae</i>	Yes	+	+
3	UNK (UNK)	Septic arthritis	Synovial fluid	<i>K. kingae</i>	Yes	+	+
4	UNK (UNK)	Septic arthritis	Synovial fluid	<i>K. kingae</i>	Yes	+	+
5	UNK (UNK)	Septic arthritis	Joint biopsy	<i>K. kingae</i>	Yes	+	+
6	F (4)	Septic arthritis	Joint biopsy	<i>K. kingae</i>	Yes	+	+
7	F (3)	Septic arthritis	Joint biopsy	<i>K. kingae</i>	Yes	+	+
8	M (17)	Septicaemia	Blood	<i>N. meningitidis</i>	ND	—	—
9	UNK (UNK)		Strain	<i>N. perflava</i>	ND	—	—
10	M (34)	Septic arthritis	Synovial fluid	<i>N. gonorrhoeae</i>	ND	—	—
11	UNK (UNK)		Strain	<i>M. bovis</i>	ND	—	—
12	F (70)	Septic arthritis	Synovial fluid	<i>S. aureus</i>	ND	—	—
13	M (57)	Septicaemia	Blood	<i>S. pneumoniae</i>	ND	—	—
14	M (40)	Septicaemia	Blood	<i>S. pyogenes</i>	ND	—	—

*F, Female; M, male; UNK, unknown.

†ND, Not done.

described (Bosshard *et al.*, 2006; Goldenberger *et al.*, 1997), using primers BAK11w, BAK2 and BAK533r (Eurogentec). Briefly, the first round of amplification was conducted with 30 pmol primers BAK11w and BAK2 with an initial denaturation at 95 °C (10 min) and cycled as follows: 95 °C for 20 s, 48 °C for 45 s and 72 °C for 1 min for 40 cycles. Five microlitres of the first round of amplification was amplified in a semi-nested reaction with primers BAK11w and BAK533r. The second round of amplification was carried out in a similar manner, except that cycling was as follows: 95 °C for 20 s, 50 °C for 45 s and 72 °C for 1 min for 40 cycles.

A PCR-positive sample collected from a culture-proven *K. kingae* sample was used as a positive control, and a mixture of all reagents used for DNA extraction was processed as a negative control. One negative control was included for every five samples. Reagent controls from the first round were always subjected to a second round of amplification to control for any contamination. Amplicons were resolved on a 2% agarose EDTA gel, visualized by using ethidium bromide staining under UV illumination, using Φ 174/*Hae*III size markers (Promega). Each PCR analysis was performed in duplicate.

16S rRNA gene sequencing. Amplicons were purified and sequenced using primer BAK11w. Fragments were analysed using an automatic DNA sequencer (ABI PRISM 3130 XL Genetic Analyzer; Applied Biosystems).

***rtxA* and *rtxB* qPCR assays.** The qPCR primers used in the present study are shown in Table 2. The assays were designed to detect two independent gene targets from the *K. kingae* RTX toxin locus, genes *rtxA* and *rtxB*. Primers and probes were designed using Primer Express (version 2.0; Applied Biosystems) on the basis of the *K. kingae* strain 269-492 RTX gene locus (accession number EF067866). BLAST searches across public databases failed to reveal any significant cross-homology. TaqMan Universal PCR Master Mix with AmpErase UNG (Applied Biosystems) was used with 0.5 μ M of each primer, 0.25 μ M of the probe, 5 μ l input DNA and nuclease-free water (Promega). Each PCR analysis was performed in duplicate.

To determine the specificity of the qPCR assays, we tested DNA extracted from related species and organisms that are frequently encountered in osteoarticular infections (Table 1). To determine the analytical sensitivity of the *rtxA* and *rtxB* qPCR assays and that of a semi-nested broad-range 16S rRNA gene PCR, we used a range of serially diluted *K. kingae* genomic DNA (3×10^6 – 3×10^{-2} c.f.u.).

qPCR assays have a typical turnaround time of 4 h: 1 h for DNA extraction/purification and 3 h for qPCR amplification and analysis. Broad-range PCR amplification of the 16S rRNA requires at least 2 days. Reagent costs are approximately US\$5 for the qPCR assay and US\$50 for the broad-range PCR.

Table 2. Primers and probes used for amplifying *rtxA* and *rtxB* in *K. kingae*

Gene	Primer/probe	Primer sequence (5'→3')	Accession no.
<i>rtxA</i>	<i>rtxA</i> -F	TGCCAAAGTAAAACCAGCTGAA	EF067866
	<i>rtxA</i> -R	AACTTACCTAATTTTGGCAAAGCAA	EF067866
	<i>rtxA</i> P*	TGACAACAAACCGCTAATCATTTCTAAGGCC	EF067866
<i>rtxB</i>	<i>rtxB</i> -F	CAACATAAGCCGCCAGTTGA	EF067866
	<i>rtxB</i> -R	ACAATTAAGCAATGGCAGTTGAG	EF067866
	<i>rtxB</i> -P*	ATCCCAACGGCGCGTCATTTGT	EF067866

*Fluorophore and quencher: 5', 6-carboxyfluorescein; 3', 6-carboxytetramethylrhodamine.

Case 1. A 2-year-old-girl was admitted to our hospital with pain in the left foot. Symptoms began 4 weeks earlier with fever (40 °C rectal) and left foot pain. There was no history of trauma or viral infection. The left mid-foot was tender on palpation but there was no local swelling. Anteroposterior and oblique X-rays of the left foot did not reveal any abnormality. The C-reactive protein was less than 10 mg l⁻¹, the sedimentation rate was 16 mm h⁻¹, and the complete blood count was normal. The child received anti-inflammatory therapy for 1 week with complete resolution of symptoms. However, soon after completion of the treatment, we noted a recurrence of her left foot pain and she had developed a limp. Despite foot immobilization, there was no improvement and an MRI was performed. On T1-weighted images, the first cuneiform cartilage and adjacent soft tissues appeared hypointense but strongly enhanced after gadolinium infusion. Signal alterations were centred by an abscess located in the body of the first cuneiform cartilage. The child underwent surgical drainage of the abscess. During the operation, synovial fluid was obtained for cultures and PCR. Aerobic and anaerobic cultures were performed on selective and non-selective media (Columbia blood agar, MacConkey agar, Columbia CNA agar and Brain Heart infusion broth) but all remained negative. There was no direct inoculation of blood culture vials. Broad-range PCR detected the presence of *K. kingae* and real-time PCR confirmed that this strain contained the RTX toxin locus, as assessed by the presence of both *rtxA* and *rtxB* with C_t values of 20. Intravenous cefuroxime was started immediately after microbiology samples were obtained and replaced after 6 days by oral cefuroxime-axetil for another 2 weeks. Two months later, the clinical examination was normal and the child walked without limping.

Case 2. A 23-month-old infant presented with a 4-day history of fever (up to 39 °C) in the context of a 'common cold', accompanied by pain involving the right elbow. There was no history of trauma. The right elbow was tender on palpation and movements were severely restricted by an articular effusion. Plain radiographs of the elbow confirmed the intra-articular effusion, but showed no bone lesion. The C-reactive protein was 15 mg l⁻¹, the sedimentation rate was 32 mm h⁻¹, and the full blood count was normal. The elbow was immobilized and the infant received anti-inflammatory therapy. Forty-eight hours later, he was still feverish (37.8 °C) and the clinical examination was unchanged. An MRI revealed intra-articular effusion with spontaneously visible hyper-signal of the elbow on T1-weighted sequences. There was no signal abnormality of the cartilage or bone structures. The infant underwent an elbow joint aspiration that collected 3 ml purulent fluid. A small arthrotomy was subsequently conducted and the joint was carefully washed. Synovial liquid was subjected to bacterial identification by culture (same as in Case 1) and PCR. Aerobic and anaerobic cultures remained negative. Broad-range PCR remained negative but both *rtx*-specific qPCR assays detected the *K. kingae* RTX toxin gene with C_t values of 34. Intravenous cefuroxime was started immediately after microbiology samples were obtained. After 6 days of parenteral antibiotics (cefuroxime followed by amoxicillin), we noted complete resolution of the complaints and the child left the hospital. Oral amoxicillin was continued for another 2 weeks. Two months later, the child had recovered and exhibited normal elbow function.

RESULTS AND DISCUSSION

The apparent increase in reported cases of *K. kingae* infections can be partly explained by improved isolation methods and better recognition of this emerging pathogen. Unfortunately, this fastidious bacterium is difficult to isolate on solid medium. Rapid inoculation of clinical specimens into enriched blood culture systems was

reported to enhance the recovery rate (Host *et al.*, 2000; Yagupsky, 1999). Some authors then reported detection of *K. kingae* from culture-negative specimens by using broad-range PCR amplification (Matta *et al.*, 2007; Chometon *et al.*, 2007; Moumille *et al.*, 2003; Rosey *et al.*, 2007; Verdier *et al.*, 2005). Recently, the pathogenesis of *K. kingae*, including colonization of the respiratory tract, invasion of the bloodstream, and damage to the joints, was related to the production of a potent cytotoxin (RTX). The *K. kingae* RTX locus contains five genes designated *rtxA*, *rtxC*, *rtxD*, *rtxB* and *tolC* (Kehl-Fie & St Geme, 2007). To confirm the presence of the RTX toxin in clinical cases, two qPCR assays targeting *rtxA* and *rtxB* were developed. First, we validated these assays on seven invasive *K. kingae* isolates from our strain collection of invasive cases (Table 1). We then documented their specificity by showing the absence of cross-reaction when testing related species and other osteoarticular pathogens (not shown). The analytical sensitivities of the qPCR assays and of the semi-nested broad-range 16S rRNA gene PCR, as determined with serially diluted *K. kingae* genomic DNA, were 30 and 300 c.f.u., respectively. For comparison, the analytical sensitivity of the *K. kingae*-specific real-time PCR assay developed by Chometon *et al.* (2007) was 300 c.f.u.

The use of a broad-range 16S rRNA gene assay offers the tremendous advantage of not requiring any a priori knowledge of the causative bacteria. Despite the use of a semi-nested amplification strategy, this method is hampered by lower detection sensitivity than that of a target-specific assay. We therefore reasoned that a broad-range assay might not have sufficient sensitivity to detect the agent in clinical samples. This may also explain why some cases of paediatric *K. kingae* bone and joint infections go undetected. In this study, we simultaneously looked for the presence of *K. kingae* by broad-range PCR and the toxin-specific *rtxA* and *rtxB* assays. In the two reported paediatric cases, many investigations and treatments were performed without success, until an MRI, intervention and novel PCR permitted a definite diagnosis and efficient treatment. This illustrates that modern medicine challenges us to seek new techniques to improve the care of our patients. The second case illustrates that low inoculum might cause genetic targets to remain undetected by a semi-nested broad range PCR and would have precluded aetiological diagnosis.

In conclusion, a real-time PCR assay that is specific to the *K. kingae* RTX toxin was more sensitive than a semi-nested broad-range 16S rRNA gene PCR, thus providing better diagnostic performance when implemented in the routine clinical microbiology laboratory. We expect that the implementation of this method will increase knowledge of *K. kingae* infections, by significantly increasing the number of detected cases.

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