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Molecular Tests That Target the RTX Locus Do Not Distinguish between *Kingella kingae* and the Recently Described *Kingella negevensis* Species

Nawal El Houmami,[a] Janek Bzdrenga,[a,b] Guillaume André Durand,[a] Philippe Minodier,[c] Hervé Seligmann,[a] Elsa Prudent,[a] Sofiane Bakour,[a] Stéphane Bonacorsi,[d] Didier Raoult,[a] Pablo Yagupsky,[e] Pierre-Edouard Fournier[a]

Aix-Marseille Université, Research Unit on Infectious and Emerging Tropical Diseases (URMITE), UM63, CNRS 7278, IRD 198, Inserm 1095, Institut Hospitalo-Universitaire Méditerranée Infection, Assistance Publique-Hôpitaux de Marseille, Marseille, France[a]; Université Grenoble Alpes, CEA, CNRS, IBS, F-38000, Grenoble, France[b]; Department of Pediatric Emergency, North Hospital, Marseille, France[c]; Inserm, IAME, UMR 1137, Université Paris-Diderot, Sorbonne Paris Cité, Laboratoire de Microbiologie, Hôpital Robert-Debré, Assistance Publique-Hôpitaux de Paris, Paris, France[d]; Clinical Microbiology Laboratory, Soroka University Medical Center, Beer-Sheva, Israel[e]

ABSTRACT *Kingella kingae* is an important invasive pathogen in early childhood. The organism elaborates an RTX toxin presumably restricted to this species. Consequently, real-time quantitative PCR (qPCR) assays targeting the RTX locus have been developed in recent years and are gaining increasing use for the molecular diagnosis of *K. kingae* infections. However, the present study shows that *Kingella negevensis*, a *Kingella* species newly identified in young children, harbors an identical *Kingella* RTX locus, raising the question of whether *K. negevensis* can be misidentified as *K. kingae* by clinical microbiology laboratories. *In silico* comparison of *Kingella* sp. RTX and *groEL* genes and *in vitro* studies provided evidence that targeting the *rtxA* and *rtxB* genes could not differentiate between strains of *K. kingae* and *K. negevensis*, whereas targeting the *groEL* gene could. This prompted the design of a highly specific and sensitive qPCR assay targeting *K. negevensis groEL* (*kngroEL*). Ninety-nine culture-negative osteoarticular specimens from 99 children younger than 4 years of age were tested with a conventional 16S rRNA gene-based broad-range PCR assay and *Kingella*-specific *rtxB*, *K. kingae*-specific *groEL* (*kkgroEL*), and *kngroEL* qPCR assays. Forty-two specimens were *rtxB* positive, including 41 that were also *kkgroEL* positive and 1 (the remaining one) that was *kngroEL* positive. Thus, this study discloses an invasive infection caused by *K. negevensis* in humans and demonstrates that targeting the RTX locus cannot be used for the formal diagnosis of *K. kingae* infections. These findings stress the need for further studies on the epidemiology of asymptomatic carriage and invasive infections caused by *K. negevensis* in humans.

KEYWORDS IS*1* family, *Kingella kingae*, *Kingella negevensis*, RTX toxins, osteoarticular infections, pediatrics, qPCR, real-time PCR

For the past 5 decades, since the original characterization of *Kingella kingae* in the 1960s (1, 2), four additional species have been included in the *Kingella* genus, namely, *K. denitrificans* (3), *K. oralis* (4), *K. potus* (5), and (most recently) *K. negevensis* (6). *Kingella* organisms are asymptomatically harbored in the oropharynx in humans and animals, and all members of the genus *Kingella* but *K. negevensis* have been incriminated so far in invasive infections affecting, peculiarly, the musculoskeletal system, and occasionally the cardiac and central nervous systems, in humans (3–7). *Kingella* bacteria belong to the large *Neisseriaceae* family and are notoriously fastidious, and their

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Address correspondence to Nawal El Houmami, nawal.el-houmami@etu.univ-amu.fr.

recovery in routine culture media is suboptimal, which initially made their recognition as human pathogens difficult (7). In the early 1990s, the serendipitous discovery that inoculation of skeletal system exudates into blood culture vials improved the isolation of *K. kingae* revealed that this organism was a common etiology of joint and bone infections in young children (8). Subsequently, the advent of molecular diagnostic tools further improved the detection of *K. kingae* and established the species as the leading cause of skeletal system infections in children aged 6 to 48 months in countries where these modern detection methods are routinely employed (9–15). Initially, PCR assays targeting the small-subunit 16S rRNA gene followed by sequencing of the resulting amplicons enabled improvement of the detection of the organism from osteoarticular samples (9, 10). Thereafter, the development of a *K. kingae*-specific real-time quantitative PCR (qPCR) assay targeting the *groEL* gene (also known as *cpn60*, *hsp60*, or *mopA*), a housekeeping gene encoding a chaperone protein recognized as a universal bacterial marker (16, 17), allowed a further increase in the diagnostic capability for pediatric *K. kingae* arthritis compared to that of traditional PCR or culture methods (11–15). Subsequently, numerous in-house qPCR assays were optimized in an attempt to achieve maximum levels of specificity, sensitivity, and rapid detection of *K. kingae* for a wide array of clinical specimens, including joint fluids, bone (12–15), oropharyngeal specimens (18, 19), and occasionally blood samples (19–21), cerebrospinal fluid (22), or cardiac tissues (20).

In 2007, Kehl-Fie and St. Geme, III, identified within the *K. kingae* genome a locus belonging to the RTX toxin superfamily that comprises 5 genes, namely, *rtxB*, *rtxD*, *rtxC*, *rtxA*, and *tolC*, encoding a cytotoxic and hemolytic toxin that plays a pivotal role in the tissue invasiveness of the bacterium (23, 24). As determined by lactic dehydrogenase (LDH) release assays, Southern blotting (23), and qPCR targeting *rtxA* (15), the less invasive *K. oralis*, *K. denitrificans*, and *K. potus* species were found to lack this RTX locus, paving the way for the development and implementation of qPCR assays targeting *rtxA* and *rtxB* to diagnose *K. kingae* infection (14, 15). Nevertheless, extended analysis unveiled that this *K. kingae* RTX locus is flanked by insertion elements and possesses a reduced GC content, strongly suggesting acquisition by horizontal gene transfer (23). However, the type strain *K. negevensis* Sch538, which shares close phenotypic and genomic relatedness with *K. kingae* (6), harbors an identical *Kingella* RTX locus. Because the *Kingella* RTX locus was previously considered to be restricted to *K. kingae*, this raised the question of whether qPCRs targeting *rtxA*, *rtxB*, and *groEL*, which are in widespread use for the diagnosis of *K. kingae* infection, can differentiate *K. kingae* from *K. negevensis*. The present study provides *in silico* and *in vitro* evidence that qPCR assays targeting the RTX locus cannot discriminate *K. kingae* from *K. negevensis* but that those targeting *K. kingae groEL* (*kkgroEL*) can. Therefore, a specific qPCR targeting *K. negevensis groEL* (*kngroEL*) was designed to discriminate *K. negevensis* from all other members of the *Kingella* genus. This novel and highly sensitive and specific *K. negevensis* qPCR test was then performed on 99 culture-negative osteoarticular samples from children less than 4 years of age, enabling us to disclose the first case of *K. negevensis* arthritis in children (Fig. 1). Finally, the DDER transposase element IS*Kne1*, located in the 3′ region of the *K. negevensis* RTX locus and presumably involved in this interspecies gene transfer, was further identified and characterized.

RESULTS

Validation of *Kingella negevensis*-specific qPCR. The qPCR assay targeting *kngroEL* was positive for all *K. negevensis* strains, while no amplification was observed for *K. kingae* ATCC 23330^T or for *K. denitrificans*, *K. oralis*, and *K. potus* strains (Table 1). Similarly, no amplification was obtained for the other 115 bacterial species tested. To determine the detection limit of the method, 13-fold serial dilutions of a bacterial suspension of strain 538^T at an initial concentration of 10^8 bacteria ml^{-1} in phosphate-buffered saline were evaluated and further quantified by culture on Columbia blood agar (bioMérieux) and colony counting. This method showed a detection threshold of 50 CFU/ml.

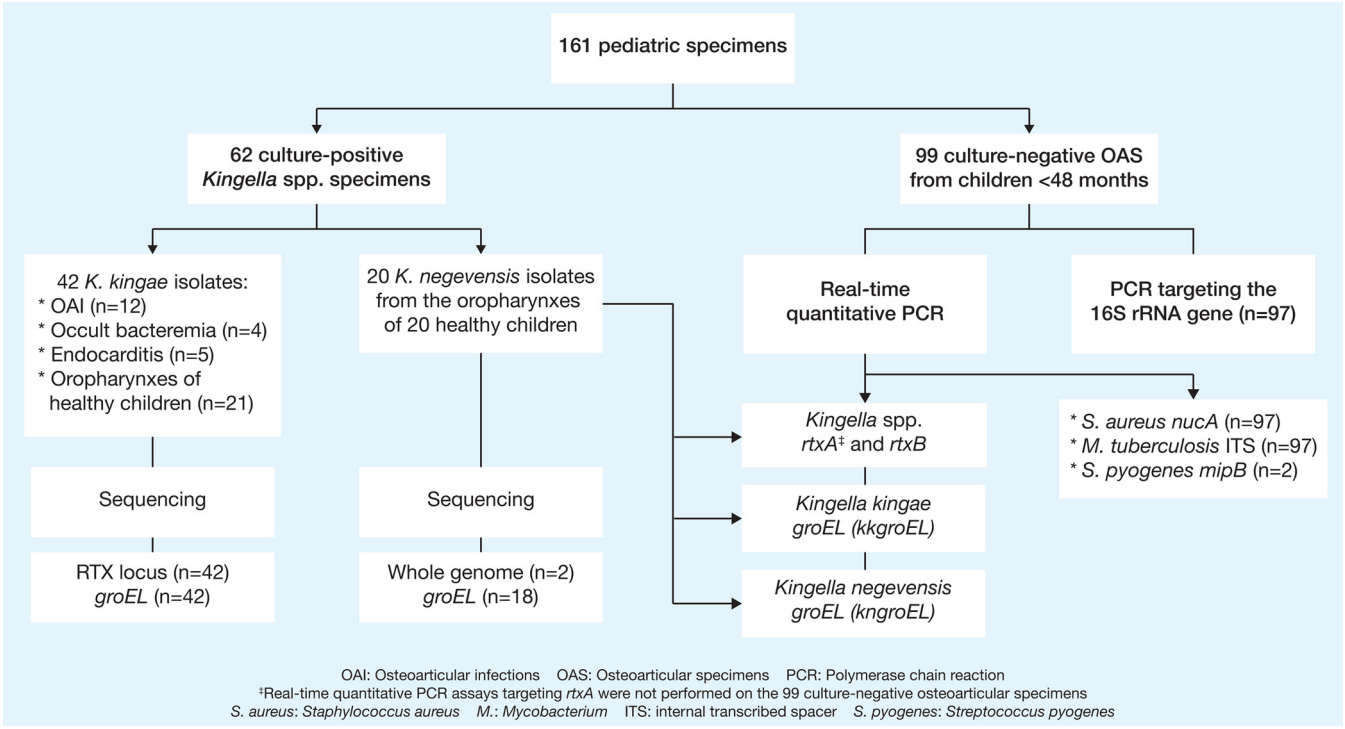

FIG 1 Workflow diagram for this study.

Targeting the RTX locus is not specific to *Kingella kingae*. *In silico* analysis of primers and probes targeting the *rtxA* and *rtxB* genes failed to discriminate between *K. negevensis* and *K. kingae*, whereas those targeting *groEL* showed high discriminative power for both *Kingella* species. As expected, all 20 *K. negevensis* strains were positive for *rtxA* and *rtxB*, whereas no amplification was observed with *kkgroEL* (Table 1), therefore suggesting that *K. negevensis* harbors a constitutional RTX locus.

Patient characteristics and identified pathogens. The median age of the 99 children with culture-negative bone and joint fluids included in this study was 23.1 ± 5.1 months, and the male-to-female ratio was 1.3:1. PCRs were positive for 45 (45%) children (Table 2). The molecular assay targeting the 16S rRNA gene was positive for 8 (8%) patients, in whom *K. kingae* was further identified in 5, *Streptococcus* spp. in 2, and *S. aureus* in 1. *Mycobacterium tuberculosis* was not detected in any specimen. Overall, the *kkgroEL* PCR assay was positive for 41 children, including the 5 cases in which the 16S rRNA gene of *K. kingae* was also detected by conventional PCR and sequencing. One child had a dual infection caused by both *K. kingae* detected by qPCR and *Streptococcus pyogenes* identified by 16S rRNA gene PCR, which was further confirmed by a specific qPCR targeting the *S. pyogenes mipB* gene. Overall, 42 children were

TABLE 1 Primer and probe specificities in qPCR assays targeting the *rtxA*, *rtxB*, *K. negevensis groEL*, and *K. kingae groEL* genes

Gene target	Reference	*K. negevensis* (*n* = 20)		*K. kingae* (*n* = 42)	
		In silico specificity	qPCR result	*In silico* specificity	qPCR result[a]
rtxA	15	No mismatch	+	0 or 1 mismatch	+
	14	1 mismatch	+	0 to 2 mismatches	+
rtxB	14	No mismatch	+	0 to 2 mismatches	+
K. negevensis groEL		No mismatch	+	15 or 16 mismatches	−
K. kingae groEL	12	9 or 10 mismatches	−	0 to 2 mismatches	+
	13	11 or 12 mismatches	−	0 or 1 mismatch	+

[a]Tested on 10 distinct *K. kingae* strains.

TABLE 2 Molecular assays used to identify the 45 pathogens causing culture-negative osteoarticular infections in 99 children between 6 and 48 months of age

Bacterial species	No. of isolates					
	16S rRNA gene[a]	*Kingella rtxB*[b]	*kkgroEL*[a]	*kngroEL*	*S. aureus nucA*[a]	*S. pyogenes mipB*[c]
Kingella kingae	5	41	41	0	0	0
Kingella negevensis	0	1	0	1	0	0
Staphylococcus aureus	1	0	0	0	2	0
Streptococcus pyogenes	2	0	0	0	0	2
Mycobacterium tuberculosis[d]	0	0	0	0	0	0
Total (no. [%])	8 (8)	42 (42)	41 (41)	1 (1)	2 (2)	2 (2)

[a]Data from reference 13.
[b]Data from reference 14.
[c]Determined by use of an in-house real-time PCR assay targeting *mipB* of *Streptococcus pyogenes*, using the forward primer Spyo_mipB_F (CCATACGGTTATAGTAAGGA GCCAAA), the reverse primer Spyo_mipB_R (GGCTATCACATCACAGCAACC), and the probe Spyo_mipB_P (FAM-TCAGCGCCAGCTTCAATGGC).
[d]There were no positive results for detection of the *M. tuberculosis* ITS (36).

positive for *rtxB*, including the 41 previously mentioned *kkgroEL*-positive children and 1 child who was positive for *kngroEL*. Both *rtxB* and *kngroEL* were detected at high threshold cycle (C_T) values in the joint fluid from the right hip of an 8-month-old boy, indicating a low concentration of *K. negevensis*. The DNA content extracted from this one *K. negevensis*-positive specimen was not sufficient for sequencing of the *groEL* gene.

Genomic analysis of the RTX locus in *Kingella* spp. The RTX locus was found in all 42 *K. kingae* strains and, unexpectedly, in *K. negevensis* Sch538^T and SW7208426. The *Kingella negevensis* RTX locus comprises the 5 genes required for the production and secretion of an active toxin, organized in a way similar to that in *K. kingae* (Fig. 2). Nucleotide sequence identities for *rtxA* between strain Sch538^T and *K. kingae* ranged from 97.53% to 99.58% with *K. kingae* AA574 and KK113, respectively, and those for *rtxB* ranged from 99.01% to 99.58% with *K. kingae* KKWG1 and ATCC 23330^T, respectively. At the protein level, *K. negevensis* Sch538^T RtxA exhibited 99.48% identity with *K. kingae* RtxA, whereas RtxB showed 100% identity.

Characterization of a novel IS*Kne1* transposase element. A 705-bp mobile element (IS*Kne1*), located 1,121 bp downstream of the *tolC* gene and flanked by two nearly perfect terminal inverted repeats of 29 bp, was identified within *K. negevensis* strains Sch538^T and SW7204826. Spread in multiple copies within *K. negevensis* genomes and sometimes bordered by an 8-bp direct repeat (TAGCTGTT), IS*Kne1* was present in DNA regions with GC contents ranging from 30.0 to 36.0%, contrasting with the 45.5% GC content of the *K. negevensis* genome (6, 25). The presumed ISKne1 protein contains 234 amino acids, including D106, D165, E191, and R198, which are catalytic amino acid residues highly conserved in IS*1* transposases with a DDER motif (26) (Fig. 2). IS*Kne1* was deposited in the IS Finder database (http://www-is.biotoul.fr).

Phylogeny of *groEL* in *K. negevensis*. All *groEL* genes of *K. negevensis* were 1,638 bp long, and that of strain Sch538^T showed nucleotide sequence identities ranging from 78.33% with *Neisseria meningitidis* Z2491 to 82.97% with *K. kingae* ATCC 23330^T. A neighbor-joining tree created in MEGA7 (27) indicated 5 clusters of *K. negevensis groEL* genes, paralleling those previously described by using pulsed-field gel electrophoresis (PFGE) (Fig. 3) (6).

DISCUSSION

Overall, the measured sensitivity of qPCR assays is 1 order of magnitude higher than that of conventional PCR tests that target the universal 16S rRNA gene (28–30). Because the RTX toxin is elaborated by all *K. kingae* strains examined so far, the encoding RTX locus genes have been used repeatedly as species-specific assay targets for detecting the organism in normally sterile body fluids and tissues in numerous clinical microbiology laboratories worldwide (14, 15, 30–33). *K. kingae* strains exhibiting RTX locus polymorphisms, including nonsynonymous ones, have also been detected (15). Assuming optimal specificity, molecular detection of the *rtxA* and *rtxB* genes in oropharyngeal

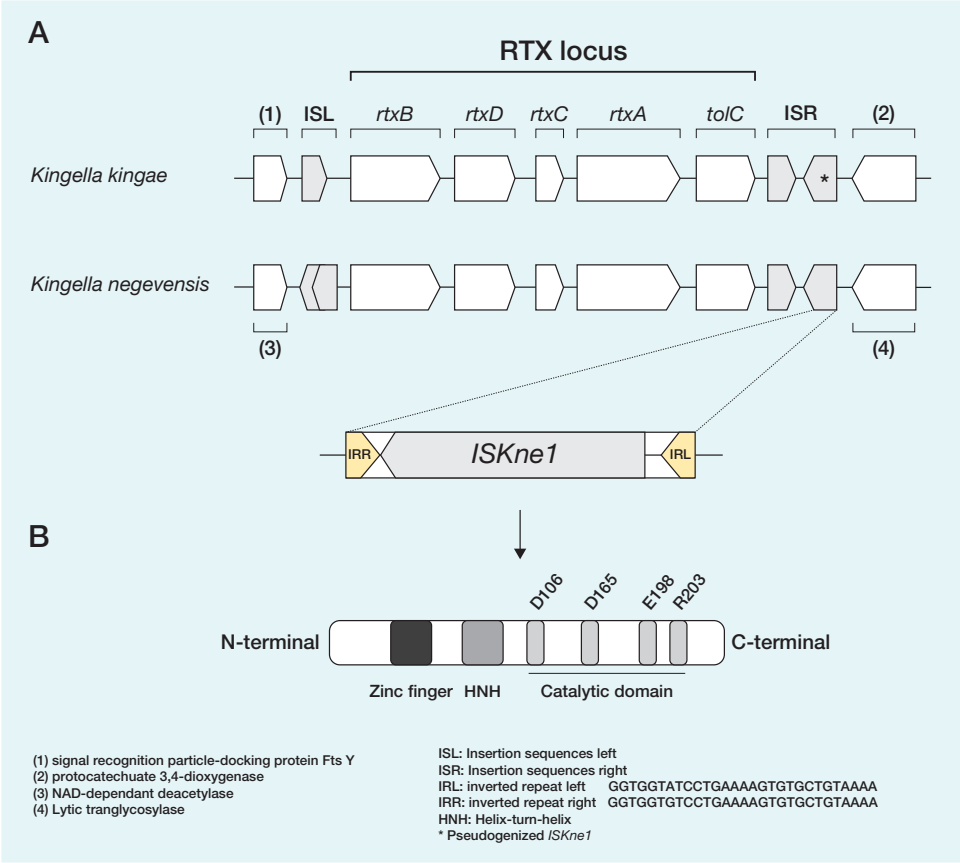

FIG 2 (A) Genomic organization of the RTX locus in *Kingella kingae* strain KKWG1 and *K. negevensis* strain SW7208426 and of the IS*Kne1* transposase element. (B) Schematic representation of the functional domains and catalytic amino acid residues of the DDER transposase ISKne1.

specimens has also been proposed as a strategy to confirm *K. kingae* as the etiology of pediatric skeletal system infections in cases where clinical specimens from joints, bones, or intervertebral disks were not obtained or gave negative culture results (18, 34). The present study provides *in silico* and *in vitro* evidence that genes encoding the RTX toxin are not exclusive to *K. kingae* and shows that qPCR assays designed to detect the RTX locus of *K. kingae* fail to distinguish *K. kingae* from *K. negevensis.* Therefore, the presence of *rtxA* and *rtxB* cannot be considered to formally confirm *K. kingae* infection, raising the pivotal question of whether *K. negevensis* may have been misidentified as *K. kingae* in bone tissue, joint fluid, or oropharyngeal samples.

In contrast, *in silico* analysis of a large panel of orthologous *groEL* genes from closely related *Kingella* and *Neisseria* species, including 43 *K. kingae* and 20 *K. negevensis* strains, showed high discriminative power between all bacterial species for testing of primers and probes routinely used for the molecular identification of *K. kingae*. This was successfully confirmed *in vitro*, as a qPCR targeting *kkgroEL* failed to detect all 20 of the *K. negevensis* strains as well as strains of other *Kingella* spp. Taken together, these results confirm that targeting *groEL* is a valuable strategy for the molecular diagnosis of invasive *K. kingae* infections and carriage (12, 13).

Among the 45 children with positive PCR results in our series, *K. kingae* was identified in 91% (41/45 cases) of cases. Thus, *K. kingae* was the leading cause of culture-negative osteoarticular infections in children aged 6 to 48 months, paralleling the results of numerous prior studies (11–13). Notably, these findings also confirmed the superiority of qPCR targeting *groEL* for the molecular diagnosis of *K. kingae* infection, with an increase in the detection yield of the organism, to 88% (36/41 cases), compared to the results of conventional 16S rRNA gene PCR (28–30). As expected, all

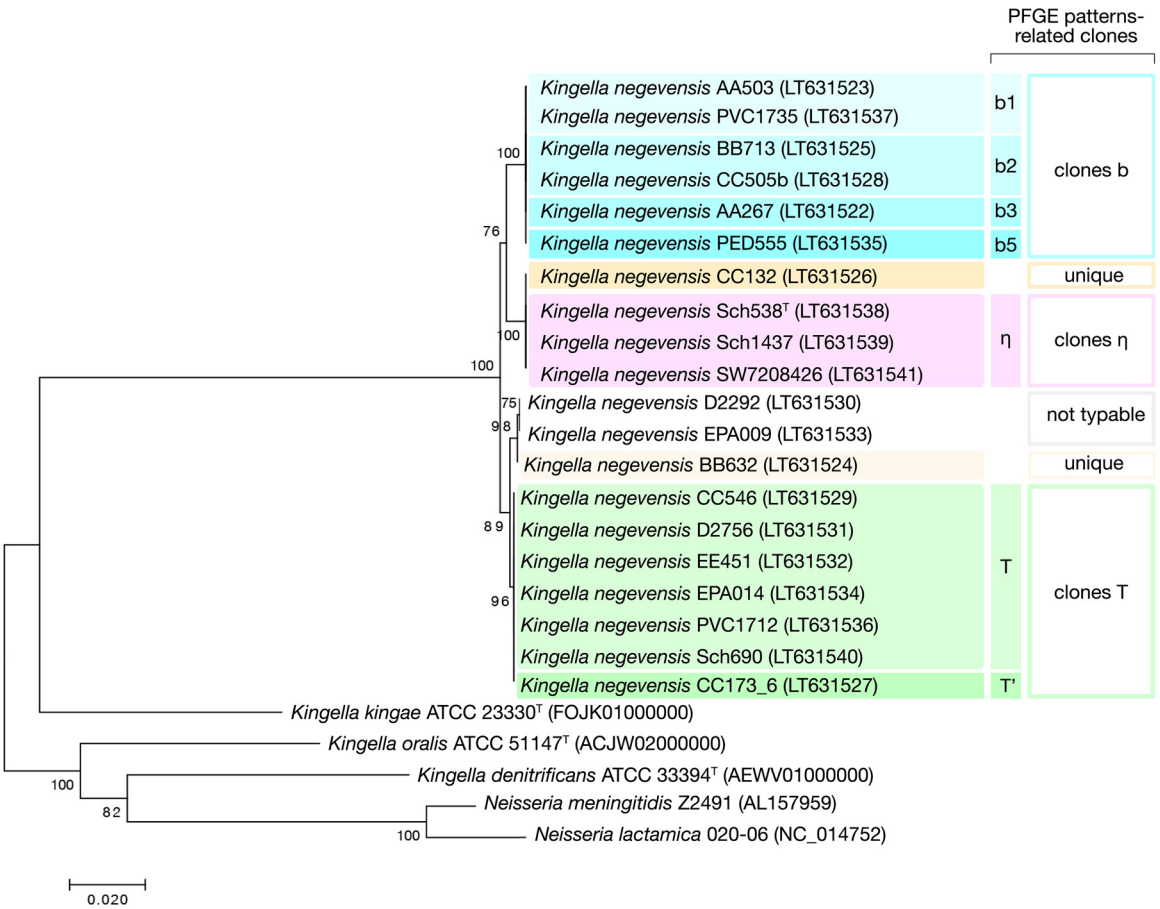

FIG 3 Neighbor-joining tree based on *groEL* nucleotide sequences of 20 strains of *Kingella negevensis* and other *Kingella* and *Neisseria* members. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are presented as numbers of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 8). Bootstrap values (expressed as percentages of 1,000 replications) are displayed at the nodes. All positions containing gaps and missing data were eliminated. There was a total of 1,635 positions in the final data set. Clones related by PFGE pattern are indicated on the right and include clones η ($n = 3$), T ($n = 6$), T′ ($n = 1$), unique ($n = 2$), and b (composed of b1 [$n = 2$], b2 [$n = 2$], b3 [$n = 1$], and b5 [$n = 1$]).

the *kkgroEL*-positive samples were also positive for *rtxB*, which is consistent with studies reporting joint fluids positive for both *K. kingae kkgroEL* and *rtxA* (12, 15).

To further fill the gap in knowledge for cases where culture-negative osteoarticular specimens from children younger than 4 years old are also negative for *kkgroEL* and other pediatric pathogens, a highly sensitive and specific qPCR targeting *kngroEL* that enabled discrimination of *K. negevensis* from *K. kingae* was developed. Note that one joint fluid aspirate from the hip of an 8-month-old boy was positive for both *kngroEL* and *rtxB*, thus providing the first clinical evidence of *K. negevensis* arthritis in humans, which highlights the fact that the molecular detection of the RTX locus alone cannot be considered irrefutable proof of *K. kingae* infection.

Finally, we found a conserved synteny of the chromosomal region carrying the RTX locus in *Kingella* spp., in which we identified a novel DDER transposase element (IS*Kne1*) present in multiple copies within the whole genome of *K. negevensis*, suggesting a transposase activity (26, 35). IS*Kne1* has presumably acted with neighboring insertion sequences to enable RTX locus accretion, thus facilitating its inter-*Kingella*-species transfer, though the precise mechanism of transposition remains to be elucidated. Functional genomics studies previously showed that *rtxA* is required for the cytotoxic activity of *K. kingae* on human epithelial, synovial, and macrophage-like cells (23). Given the percentage of amino acid sequence identity of the RTX toxin of *K. negevensis* and that of *K. kingae*, their close genomic characteristics, and their shared habitat in the

oropharynx of healthy children (6), it is possible that *K. negevensis* invades human tissues in a manner similar to that of *K. kingae*, with oropharyngeal colonization being a required step prior to invasive disease.

In conclusion, *K. negevensis* harbors a constitutional RTX locus similar to that of *K. kingae*, and as a result, positive assays targeting *rtxA* and *rtxB* only cannot be considered definitive evidence of *K. kingae* infection. Similarly, there is a high risk that previous epidemiological data on *K. kingae* oropharyngeal carriage in children aged 6 to 48 months originating from studies using assays targeting *rtxA* and *rtxB* may be slightly overestimated because of the detection of both *Kingella* species. However, this study also emphasizes that the predictive value of a positive *rtxB* assay remains high (98%) for the diagnosis of osteoarticular infections in children younger than 4 years old. Additionally, the first invasive infection caused by *K. negevensis* in a young patient suggests that this organism may be an occasional player in bone and joint infections in early childhood. Taken together, these findings highlight the need for further studies to better understand the epidemiology of *K. negevensis* carriage and disease, as well as the clinical presentation and pathogenesis of infections in humans, for which the highly sensitive and specific qPCR assay described herein may be highly relevant.

MATERIALS AND METHODS

A workflow diagram for this study is provided in Fig. 1.

Bacterial strains. In the 2000s, epidemiological studies were conducted in the Negev Desert region of southern Israel on 7,217 healthy children younger than 8 years old, from whom *K. kingae* and *K. negevensis* strains were isolated at the clinical microbiology laboratory in Beer-Sheva, Israel (6). From these isolates, 20 strains of *K. negevensis* and 42 strains of *K. kingae* were used in the present study. All 20 *K. negevensis* strains were isolated from the oropharynxes of healthy children aged between 6 months and 8 years, after pharyngeal specimens were inoculated onto BAV medium, a selective vancomycin-containing medium, to inhibit the competing Gram-positive flora and facilitate the recognition of *K. kingae* colonies (6). *K. negevensis* isolates showed an atypical phenotype consisting of unusually long chains of coccobacilli, early autolysis, poor growth as pinpoint β-hemolytic colonies on blood-agar plates, and excellent growth on GC-base medium. The 42 *K. kingae* strains were derived from children aged 6 to 48 months with osteoarticular infections ($n = 12$), occult bacteremia ($n = 4$), endocarditis ($n = 5$), or asymptomatic oropharyngeal colonization ($n = 21$). Additionally, 115 other bacterial strains belonging to 43 genera, including other *Kingella* species and members of the *Neisseria*, *Haemophilus*, *Staphylococcus*, *Streptococcus*, and *Mycobacterium* genera, were used to determine the specificity of the qPCR primers and probes targeting *K. negevensis groEL* (see Table S1 in the supplemental material). *Kingella* species strains were cultured on 5% sheep blood-enriched Columbia agar for 24 to 36 h at 37°C in a 5% enriched CO_2 atmosphere.

Culture-negative osteoarticular samples. From 2014 to 2016, 99 culture-negative bone and joint samples from children aged 6 to 48 months with suspected osteoarticular infection were retrieved at the URMITE Laboratory, Marseille, France, where they were stored at −20°C. By applying a diagnostic approach consisting of systematic PCR for the diagnosis of osteoarticular infections in culture-negative patients (13), samples were tested by conventional broad-range PCR targeting the 16S rRNA gene (13) and specific qPCRs targeting *K. kingae groEL* (*kkgroEL*), *Staphylococcus aureus nucA* (13), and the *M. tuberculosis* internal transcribed spacer (ITS) (36) (Fig. 1). When the conventional broad-range PCR targeting the 16S rRNA gene was positive for *Streptococcus* spp., a second control was obtained by an in-house specific qPCR assay targeting the *mipB* gene of *Streptococcus pyogenes* (Table 2). In cases where negative controls gave a positive result, the PCR was considered invalid and was repeated. The efficiency of DNA extraction and the possible presence of inhibitors in the samples were evaluated using the RS42-Km primer pair, targeting a fragment of the human β-globin gene, as previously described (13).

Genomic DNA extraction. Genomic DNAs of all bacterial strains and clinical samples were extracted with a BioRobot EZ1 workstation and an EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations. DNA was stored at −80°C until required for analysis. To negate the effects of PCR inhibitors, extracted DNAs of *Kingella* species strains were tested both undiluted and diluted 1:10, 1:100, and 1:1,000, and those of clinical samples were tested both undiluted and diluted 1:10.

Sequencing of the *groEL* genes and RTX loci of *Kingella kingae* strains. Paired-end sequencing of the complete *groEL* genes and RTX loci of the 42 *K. kingae* strains by use of an Illumina MiSeq instrument was performed as previously described (6). In addition, *groEL* and RTX locus nucleotide sequences from *K. kingae* KKWG1 (LN869922), for which the genome is available in one scaffold (37), were included in this study and used as references for the comparative analysis of *Kingella* spp.

Sequencing of the *groEL* genes of *Kingella negevensis* strains. The set of *K. negevensis* primers for conventional PCR, namely, groEL_Knegev_F1 (5′-CTGGTGATGCGTGAAGAAG-3′) and groEL_Knegev_R3 (5′-TCCTATATGAAACAATTGCC-3′), located 106 bp upstream and 155 bp downstream of the *groEL* gene of *K. negevensis* Sch538^T (accession no. CCNJ01000030) and SW7208426 (accession no. FXBH01000000), were manually designed. For the 18 remaining *K. negevensis* strains, an 1,899-bp PCR product was

amplified by using HotStarTaq DNA polymerase (Qiagen). Amplification parameters consisted of 1 cycle of 95°C for 5 min followed by 39 cycles of 94°C for 1 min, 55°C for 30 s, and 72°C for 2 min 30 s, with 1 final extension cycle of 72°C for 10 min. The amplicons were purified using a NucleoFast 96 PCR kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Sequencing reactions were carried out by using a BigDye Terminator v1.1 cycle sequencing kit (Perkin-Elmer, Shelton, CT) according to the manufacturer's instructions. Sequencing of the products was achieved on both strands by using the initial set of PCR primers and the internal primers groEL_Knegev_F2 (5′-CGCAAG TAGGTTCTATCTCTGC-3′), groEL_Knegev_F3 (5′-GGCTGTGATTAAAGTGGG-3′), groEL_Knegev_R1 (5′-CCA ATGATTTGCCGTCTTCC-3′), and groEL_Knegev_R2 (5′-CACCACCTGCAACGATACC-3′), using an ABI Prism 3130 genetic analyzer. Sequence assembly was performed in Geneious R9.1.8 (Biomatters).

qPCR targeting the *Kingella negevensis groEL* gene (*kngroEL*). (i) Design of primers and probe. To design specific primers and a probe for *K. negevensis* qPCR, an alignment of the *groEL* nucleotide sequences obtained from 20 *K. negevensis* strains, 42 *K. kingae* strains, *K. kingae* KKWG1 (LN869922), *K. denitrificans* ATCC 33394^T (accession no. AEWV01000000), *K. oralis* ATCC 51147^T (accession no. ACJW02000000), *N. meningitidis* Z2491 (accession no. AL157959), *Neisseria elongata* subsp. *glycolytica* ATCC 29315^T (accession no. NZ_CP007726), and *Neisseria lactamica* 020-06 (accession no. NC_014752) was performed by using MAFFT, version 7.22.2, in Geneious R9.1.8 (38). Thereafter, the primers groEL_F_Knegev (5′-CACGTTCTGCATTGAAATCTG-3′) and groEL_R_Knegev (5′-GTTCACTACTACAGACGC TTC-5′), located at nucleotides 1265 and 1401, respectively, within the *groEL* gene of *K. negevensis*, and the probe groEL_P_Knegev (5′-6-carboxyfluorescein [FAM]-CGCTGACCAAGAAGCTGGCGTG), located at nucleotide 1299, were manually designed. Particular care was taken in order to (i) minimize mismatches in the primers and probe between *K. negevensis* strains, especially at the 3′ end, and (ii) maximize mismatches with other *Kingella* and *Neisseria* species. The primer and probe specificity was confirmed *in silico* by using the BLAST tool (http://blast.ncbi.nlm.nih.gov).

(ii) *Kingella negevensis*-specific qPCR. TaqMan real-time PCR amplification and hybridization reactions were carried out in a final volume of 20 μl of reaction mixture containing 10 μl of Takyon No Rox Probe MasterMix dTTP (Eurogentec), 0.25 μM (each) primers, 0.25 μM labeled probe, and 5 μl of purified DNA. Amplification of a 137-bp product was performed on a Bio-Rad CFX96 platform in a C1000 Touch thermal cycler, using the following cycling parameters: heating at 50°C for 2 min and 95°C for 5 min, followed by 40 cycles of a two-stage temperature profile of 95°C for 10 s and 55°C for 45 s.

***Kingella* sp. qPCR assays.** *In silico* analysis of the specificities of primers and probes targeting *rtxA* (14, 15), *rtxB* (14), and *K. kingae groEL* (*kkgroEL*) (12, 13), all used routinely for the molecular diagnosis of *K. kingae* infection, was first performed. All 20 *K. negevensis* strains were then tested using qPCR assays targeting the *kngroEL*, *kkgroEL*, *rtxA*, and *rtxB* genes, as previously described (12–15). In addition to the above-mentioned qPCR tests, all 99 clinical samples were tested for *kngroEL* and *rtxB*.

Genomic analysis of the RTX loci of *Kingella* spp. MAFFT alignment of the RTX loci of *K. negevensis* Sch538^T (6), *K. negevensis* SW7208426 (25), and *K. kingae* KKWG1 (37) was performed in Geneious R9.1.8.

Ethics statement. This study was approved by the Ethics Committee of the IHU Méditerranée Infection under reference number 2016-024. Epidemiological studies performed in the 2000s were approved by the Ethics Committee of the Soroka University Medical Center, as well as by the Israel Ministry of Health.

Accession number(s). The GenBank accession numbers for the *groEL* genes from the 43 *K. kingae* strains analyzed in this study are LT838407 to LT838420, and those for the *groEL* genes from the 20 *K. negevensis* strains are LT631522 to LT631541. The GenBank accession numbers for the *rtxA* genes from the 43 *K. kingae* strains are LT841363 to LT841378, CCNJ01000000, FXBH01000000, FOJK01000000, CCJT01000000, and NZ_LN869922, and those for the *rtxB* genes are LT841334 to LT841346, CCNJ01000000, FXBH01000000, FOJK01000000, CCJT01000000, and NZ_LN869922.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00736-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

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