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A New Highly Sensitive and Specific Real-Time PCR Assay Targeting the Malate Dehydrogenase Gene of *Kingella kingae* and Application to 201 Pediatric Clinical Specimens

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ABSTRACT *Kingella kingae* is a significant pediatric pathogen responsible for bone and joint infections, occult bacteremia, and endocarditis in early childhood. Past efforts to detect this bacterium using culture and broad-range 16S rRNA gene PCR assays from clinical specimens have proven unsatisfactory; therefore, by the late 2000s, these were gradually phased out to explore the benefits of specific real-time PCR tests targeting the *groEL* gene and the RTX locus of *K. kingae*. However, recent studies showed that real-time PCR (RT-PCR) assays targeting the *Kingella* sp. RTX locus that are currently available for the diagnosis of *K. kingae* infection lack specificity because they could not distinguish between *K. kingae* and the recently described *Kingella negevensis* species. Furthermore, *in silico* analysis of the *groEL* gene from a large collection of 45 *K. kingae* strains showed that primers and probes from *K. kingae groEL*-based RT-PCR assays display a few mismatches with *K. kingae groEL* variations that may result in decreased detection sensitivity, especially in paucibacillary clinical specimens. In order to provide an alternative to *groEL*- and RTX-targeting RT-PCR assays that may suffer from suboptimal specificity and sensitivity, a *K. kingae*-specific RT-PCR assay targeting the malate dehydrogenase (*mdh*) gene was developed for predicting no mismatch between primers and probe and 18 variants of the *K. kingae mdh* gene from 20 distinct sequence types of *K. kingae*. This novel *K. kingae*-specific RT-PCR assay demonstrated high specificity and sensitivity and was successfully used to diagnose *K. kingae* infections and carriage in 104 clinical specimens from children between 7 months and 7 years old.

KEYWORDS RTX locus, *groEL* gene, *Kingella kingae*, *Kingella negevensis*, malate dehydrogenase, *mdh* gene, pediatrics, real-time PCR

Kingella kingae is a significant pediatric pathogen responsible for bone and joint infections, occult bacteremia, and, more rarely, endocarditis that may occur either sporadically or in the context of outbreaks in daycare centers (1, 2). Past efforts to detect this organism by culture have proven unsatisfactory, and molecular diagnostics were gradually used increasingly throughout the 2000s to successfully diagnose *K. kingae* disease (3–5). Consequently, the increasing number of molecularly confirmed *K. kingae* infections in infants led to this organism being recognized as the primary agent of septic arthritis, osteomyelitis, and tenosynovitis in children between age 6 and 36 months in countries where *K. kingae*-specific real-time PCR assays are routinely employed (4–7). This contributed to a significant improvement in our knowledge of the etiology of infantile bone and joint infections (5, 8, 9). In addition to increasing the detection yield of microorganisms from osteoarticular samples, molecular assays have

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contributed to a better understanding of the epidemiology of *K. kingae* carriage among healthy carriers and ill children (10–12). Although PCR assays targeting the 16S rRNA gene made it possible to moderately enhance the detection of the organism from osteoarticular samples (13), the development of *K. kingae*-specific RT-PCR assays allowed a substantial increase in the diagnosis of *K. kingae* infections and oropharyngeal carriage (5, 14, 15).

To date, only the *groEL* (also known as *cpn60*) gene and those located in the RTX locus, namely, *rtxA* and *rtxB*, have been targeted in the development of *K. kingae*-specific RT-PCR assays (4, 5, 16, 17). A comprehensively studied gene, *groEL* encodes a chaperone protein that is considered a universal bacterial marker (18), and PCR assays targeting this gene are widely used for the molecular diagnosis of infectious diseases (19–21). However, although recent studies have confirmed that targeting the *groEL* gene from *K. kingae* (*KkigroEL*) is a reliable strategy for the molecular detection of this bacterium in clinical specimens (15, 22), primers and probes from *groEL*-based RT-PCR assays that were reported by Ilharreborde et al. (4) and Levy et al. (5) display a few mismatches with *KkigroEL* variations that may result in a decreased detection sensitivity (15, 23).

In contrast, RTX-targeting RT-PCR assays have gained great popularity worldwide because they were initially believed to be highly specific for *K. kingae* (16, 17). However, the RTX locus of *K. kingae* is flanked by mobile genetic elements that are present in genomic regions of decreased GC content (30%, versus an average of 46.6% for the whole genome of *K. kingae*). Because such a GC content difference is a meaningful genetic marker of the mobilome, Kehl-Fie and St. Geme suggested that this RTX locus was horizontally acquired (24). This assumption was recently confirmed by the presence of an identical RTX locus in the genome of *K. negevensis* (15, 25), a newly described *Kingella* species isolated from the oropharynx of Israeli and Swiss children (26, 27) and from the vaginal discharge of a young woman (25). Furthermore, *in vitro* studies indicated that RT-PCR assays targeting the RTX locus of *K. kingae* were also positive for *K. negevensis* and hence could not formally discriminate between the two species when used alone (15). *Kingella negevensis* has also been identified in the hip of an 8-month-old boy with a specific quantitative PCR (qPCR) targeting the *K. negevensis groEL* gene, indicating that this novel described *Kingella* species may occasionally be a pediatric pathogen (15).

In order to provide an alternative to *groEL*- and RTX-targeting RT-PCR assays that may suffer from suboptimal specificity and sensitivity, a *K. kingae*-specific RT-PCR assay targeting the malate dehydrogenase (*mdh*) gene, a housekeeping gene, was developed. This novel RT-PCR assay targeting the *mdh* gene from *K. kingae* (*Kkimdh*) demonstrated high specificity and sensitivity and was successfully used to diagnose *K. kingae* infections and carriage in 104 clinical specimens from young children.

MATERIALS AND METHODS

Bacterial isolates. In the 2000s, epidemiological studies were conducted in southern Israel on 7,217 healthy children from whom *K. kingae* strains were isolated at the Soroka University Medical Center, Beer-Sheva, Israel (26). Forty of these *K. kingae* strains, cultivated from children between age 6 and 48 months suffering from osteoarticular infections ($n = 12$), occult bacteremia ($n = 4$), endocarditis ($n = 3$), or asymptomatic oropharyngeal colonization ($n = 21$), were used in this study (see Table S1 in the supplemental material). Oropharyngeal swabs from healthy children were first inoculated onto a selective vancomycin-containing agar (also named BAV medium) to inhibit the competing Gram-positive flora and facilitate the recognition of hemolytic *K. kingae* colonies (28). All *K. kingae* isolates were then subcultured on 5% sheep blood-enriched Columbia agar for 24 to 36 h at 37°C in a 5% CO_2-enriched atmosphere. Additionally, 86 other bacterial strains, including all other *Kingella* species, namely, *K. negevensis* strain Sch538^T, *K. oralis* CIP103803^T, *K. denitrificans* CIP103473^T, and *K. potus* CIP108935^T, as well as members of the *Neisseria*, *Haemophilus*, *Staphylococcus*, *Streptococcus*, and *Mycobacterium* genera, were used to determine the specificities of the RT-PCR primers and probes targeting the *Kkimdh* gene (Table S2).

Clinical specimens of *K. kingae* infection and carriage. Between December 2013 and December 2017, 106 children with *K. kingae* infections and/or carriage originally from Europe, South America, Africa, and the South Pacific were diagnosed by using *KkigroEL*-specific RT-PCR (5) at the IHU Mediterranée Infection, Marseille, France. Of those, 96 clinical specimens or extracted DNA (from 95 children) that were stored at −80°C were retrieved, which were derived from joint fluid ($n = 64$), bone tissue ($n = 6$),

tenosynovial fluid ($n = 2$), soft tissue ($n = 2$), endocardial cushion ($n = 1$), and pharyngeal swabs ($n = 25$) (Table S3). The efficiency of DNA extraction and the possible presence of inhibitors were evaluated in all clinical specimens using the RS42-Km primer pair, which targets a fragment of the human β-globin gene (5).

Genomic DNA extraction. Genomic DNA from all bacterial strains and clinical samples was extracted with a BioRobot EZ1 workstation and an EZ1 DNA tissue kit (Qiagen, Courtaboeuf, France), according to the manufacturer's recommendations (15). DNA was stored at −80°C prior to the molecular assays. To limit the effects of PCR inhibitors, all extracted DNAs were tested both undiluted and diluted 1:10.

Selection of the *mdh* gene from *K. kingae* genomes. In order to select a relevant target gene for the development of a *K. kingae*-specific RT-PCR assay, a comparison of five *K. kingae* genomes available in GenBank, namely, those of *K. kingae* ATCC 23330^T (accession no. FOJK01000000) (26), *K. kingae* KKWG1 (accession no. LN869922) (29), *K. kingae* PYKK081 (accession no. NZ_JH621344), *K. kingae* 11220434 (accession no. JH768595) (30), and *K. kingae* KK247 (accession no. CCJT01000000) (31), was performed using the Geneious R11.0.5 software (32). Genes and their flanking regions belonging to the core genome of *K. kingae* and exhibiting a GC content close to 50% were screened to facilitate the primer and probe design according to the Takyon polymerase protocol (Eurogentec, Seraing, Belgium). The *mdh* gene of *K. kingae* (*Kkimdh*) encoding the malate dehydrogenase met the above-mentioned criteria and was thus selected. Thereafter, paired-end sequencing of the *Kkimdh* gene and its flanking regions from 40 *K. kingae* strains using a MiSeq sequencer (Illumina, Inc., San Diego, CA, USA) and genome assembly were performed as previously described (26).

Characterization of the *Kingella kingae mdh* gene. A MAFFT alignment of the *Kkimdh* nucleotide sequences and its flanking regions from the 45 studied *K. kingae* strains was performed using Geneious R11.0.5 (32, 33). The related distance matrix of the 45 distinct *Kkimdh* genes was obtained using Geneious R11.0.5 (Table S4). To detect possible lateral gene transfer from or within the genome of other bacterial species, a MegaBlastN search with default parameters (http://blast.ncbi.nlm.nih.gov) was then conducted by comparing the obtained *mdh* orthologous sequences and their genomic environment to public databases. A neighbor-joining tree of *Kkimdh* gene sequences was then created using MEGA7, with default parameters (34).

RT-PCR assay targeting the *Kkimdh* gene. (i) Design of primers and probe. To design specific primers and probes, a MAFFT alignment of *mdh* nucleotide sequences from the 45 studied *K. kingae* strains was first performed. Thereafter, the primers Fwd_Kkimdh (5′-TGTTCCGCATTGCTTCTG-3′) and Rev_Kkimdh (5′-TCATGCCGTCCAACAATG-3′), amplifying a 144-bp fragment, and the probe P_Kkimdh (5′-FAM-CATCATCACGCCCTGAACGGCTT-3′; FAM, 6-carboxyfluorescein) were manually designed. Particular care was taken in order to avoid nucleotide mismatches between all *K. kingae* strains and to maximize mismatches with *mdh* orthologous detected from other bacterial species. Primer and probe specificity was confirmed *in silico* using the BLAST tool (http://blast.ncbi.nlm.nih.gov).

(ii) *Kkimdh* RT-PCR protocol. Real-time PCR amplification 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), 0.45 μM (each) primers, 0.45 μM labeled probe, and 5 μl of purified DNA. Amplification was performed using a Bio-Rad CFX96 platform and the following cycling parameters: heating at 50°C for 2 min and 95°C for 3 min, followed by 45 cycles of a two-stage temperature profile of 95°C for 3 s and 60°C for 30 s.

Evaluation of the sensitivity and specificity of the *Kkimdh* RT-PCR assay. Twenty *K. kingae* strains belonging to 20 distinct sequence types (STs) (Table S2) which had previously tested positive for *KkigroEL*, *rtxA*, and *rtxB* (15) and 96 specimens which had previously tested positive for *KkigroEL* were tested using the *Kkimdh* RT-PCR assay. In addition, 105 various *KkigroEL*-negative clinical specimens derived from children with suspected osteoarticular infections were added to the analysis. Furthermore, 87 other bacterial strains, including strains from *K. negevensis*, *K. oralis*, *K. denitrificans*, and *K. potus*, as well as members of the *Neisseria*, *Haemophilus*, *Staphylococcus*, *Streptococcus*, and *Mycobacterium* genera, were tested to assess the specificity of the assay (Table S2). To determine the detection limit of the method, 12-fold serial dilutions of a bacterial suspension of *K. kingae* strain ATCC 23330^T at an initial concentration of 10^8 bacteria · ml^{-1} in phosphate-buffered saline were evaluated and further quantified by culture on 5% sheep blood-enriched Columbia agar (bioMérieux) and colony counting.

Ethics statement. This study was approved by the ethics committee of the IHU Méditerranée Infection under reference number 2017-006. 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 *mdh* genes from the 45 studied *K. kingae* strains analyzed in this study are LT985480 to LT985523 and LS453284 (Table S1).

RESULTS

Genomic analysis of the *mdh* gene of *K. kingae* and its environment. A 978-bp *Kkimdh* gene was identified in all 45 *K. kingae* genomes. The chromosomal region carrying the *Kkimdh* gene is surrounded by the ribosomal small subunit-dependent GTPase gene located 218 bp upstream, and downstream by a locus containing genes coding for the GTP cyclohydrolase FolE2 and sensor histidine kinase (Fig. 1). The synteny of this genomic architecture was conserved in all 45 *K. kingae* strains. The distance matrix calculated from the 45 *Kkimdh* DNA sequences displayed 18 distinct

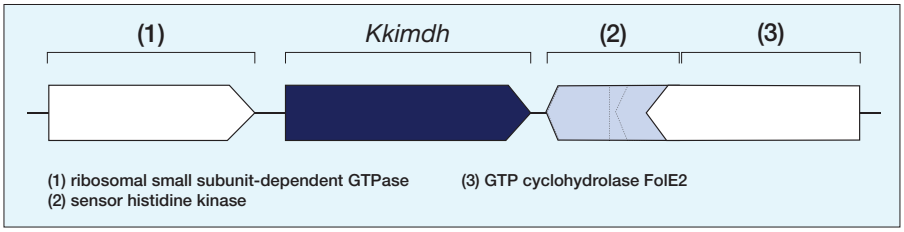

FIG 1 Genomic architecture of the region carrying the *mdh* gene of *Kingella kingae* (*Kkimdh*). The genomic elements are not drawn to scale. The dashed lines indicate that the sensor histidine kinase may be encoded by either a single gene or two-component genes.

variants (Fig. 2), with a maximum distance of 98.4% between *K. kingae* strains ATCC 23330^T and D2363 (Table S4).

***In silico* analysis of the *Kkimdh* gene and design of the *Kkimdh*-specific RT-PCR assay.** The MegaBlastN search indicated the presence of an *mdh* gene within the genomes of *Acinetobacter lwoffii* ZS207 (GenBank accession no. CP019143), *Acidovorax* sp. strain RAC01 (GenBank accession no. CP016447), *Neisseria* sp. strain KEM232 (Gen-

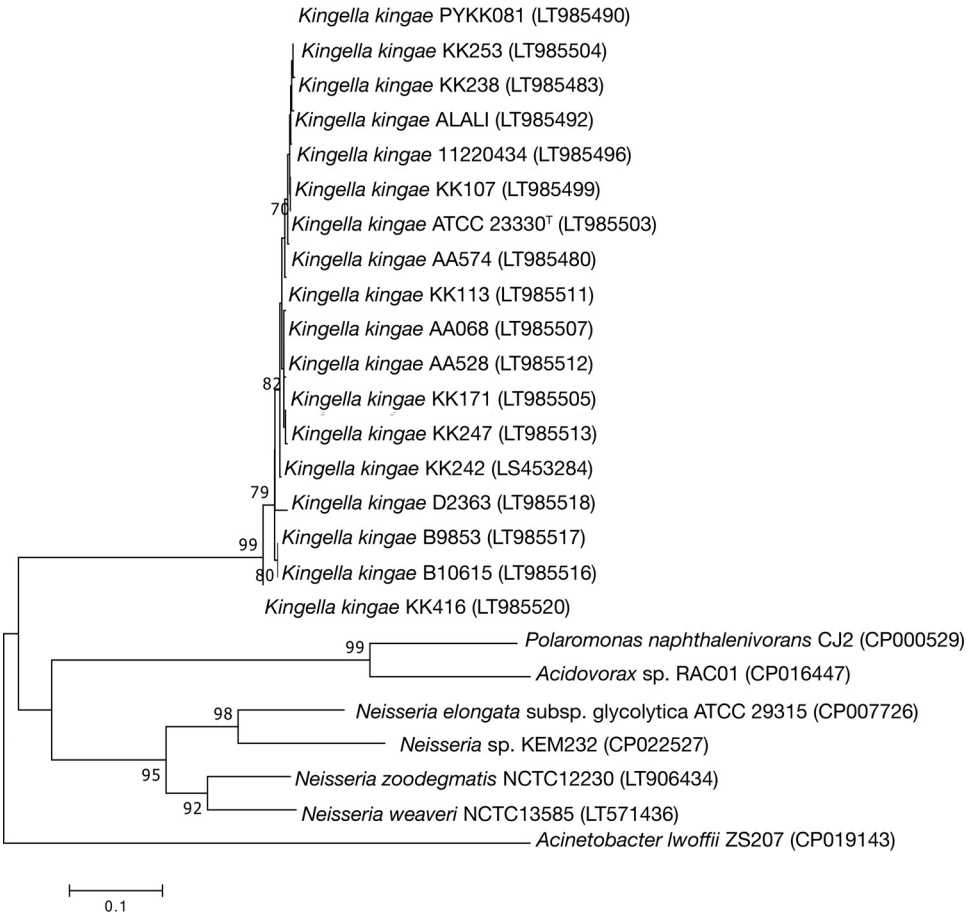

FIG 2 Neighbor-joining tree (40) based on the comparison of *mdh* nucleotide sequences from 18 genetic variants of *Kingella kingae* and their closest orthologs in *Acinetobacter*, *Acidovorax*, *Polaromonas*, and *Neisseria* species. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (41) and are in the units of the number of base substitutions per site. The scale bar indicates a 10% nucleotide sequence divergence. Bootstrap values (expressed as percentages of 1,000 replications) are showed next to the branches. Only bootstrap values greater than or equal to 70% were displayed. All positions containing gaps and missing data were eliminated. There was a total of 978 positions in the final data set. Numbers in parentheses next to the organism names are accession numbers.

TABLE 1 Results of the real-time PCR assays targeting the *groEL* and *mdh* genes of *K. kingae* that were tested on 20 genotypically distinct *K. kingae* isolates and 201 pediatric specimens

Isolate or specimen group (*n*)	No. of positive isolates by real-time PCR	
	Kkimdh	***KkigroEL***
K. kingae specimens (20)	20	20
Other bacterial species (86)	0	0
Clinical specimens (201)[a]		
Initially *KkigroEL*-positive specimens (96)	96	96
Initially *KkigroEL*-negative specimens (105)	8	0

[a]Patients were tested for a suspected *K. kingae* invasive infection.

Bank accession no. CP022527.1), *Neisseria elongata* subsp. *glycolytica* ATCC 29315 (GenBank accession no. CP007726.1), *Neisseria weaveri* NCTC13585 (GenBank accession no. LT571436.1), *Neisseria zoodegmatis* NCTC12230 (GenBank accession no. LN869922.1), and *Polaromonas naphthalenivorans* CJ2 (GenBank accession no. CP000529). The MAFFT alignment of the nucleotide sequences of these *mdh* orthologous genes showed that the Kkimdh-F and Kkimdh-R primers and the Kkimdh-P probe displayed a total of 13 to 15 mismatches between the *mdh* genes from *K. kingae* ATCC 23330^T and those from the *Neisseria* species, which are the closest orthologous genes related to *Kkimdh*, showing nucleotide sequences identities ranging from 77.1 to 79.1% (Fig. S1 and Table S5). These data thereby demonstrate a high index of *in silico* specificity of *Kkimdh* RT-PCR assay.

Validation of the *Kkimdh*-specific RT-PCR assay. The detection threshold of the *Kkimdh* RT-PCR assay was determined to be 10 CFU/ml. The assay was positive for all 20 *K. kingae* strains belonging to 20 distinct STs, whereas no amplification was obtained for *K. negevensis*, *K. denitrificans*, *K. oralis*, and *K. potus* strains (Table 1). Similarly, no amplification was obtained from any of the other 82 tested bacterial species. As expected, *Kkimdh*-specific RT-PCR testing was positive in the 96 *KkigroEL*-positive specimens. Unexpectedly, eight joint fluid samples that initially tested *KkigroEL* negative were detected to be *Kkimdh* positive, whereas the remaining 97 *KkigroEL*-negative pediatric specimens were both *Kkimdh* and *KkigroEL* negative. Triplicate assays were carried out on the eight discrepant samples. All eight were repeatedly *Kkimdh* positive, with cycle threshold values ranging from 30 to 34. Of these eight *Kkimdh*-positive clinical specimens, six specimens were derived from children with septic arthritis between age 7 and 29 months, one specimen was sampled from the oropharynx of a 13-month-old boy, and one specimen was identified in the joint fluid of a 7-year-old boy (Table S3).

DISCUSSION

This study reports a novel *K. kingae*-specific RT-PCR assay targeting the *Kkimdh* gene, a housekeeping gene encoding the malate dehydrogenase that was identified in 45 distinct *K. kingae* genomes and firmly detected in 20 various clinical isolates and 104 clinical specimens from infants and young children originally from Europe, South America, Africa, and the South Pacific. The high specificity of this PCR system was demonstrated by the absence of any *mdh* gene in the genomes of all other *Kingella* species. In addition, orthologous *mdh* genes were found in only a few bacterial species, which exhibited low levels of nucleotide identity with *Kkimdh*, as demonstrated by the presence of 13 to 15 mismatches between the nucleotide sequences of primers Fwd-Kkimdh, Rev-Kkimdh, and probe P-Kkimdh and orthologous *mdh* genes from *Neisseria* species.

In contrast to the *Kingella* sp. RTX locus, *Kkimdh* is only present in *K. kingae*, is located in a genomic region that presents a conserved synteny and a GC content of only 2 to 3% greater than that the whole genome of *K. kingae*, and is not surrounded by transposable elements. For all these reasons, it appeared particularly pertinent to

target *Kkimdh* for the development of a new *K. kingae*-specific molecular tool in clinical microbiology.

To the best of our knowledge, this new *K. kingae*-specific RT-PCR assay is currently the only molecular tool showing optimal sensitivity and specificity for the diagnosis of *K. kingae* infection and carriage compared to all those previously reported. Although *K. kingae*-specific RT-PCR assays targeting the *groEL* gene may be considered the gold standard for the detection of *K. kingae* (15, 22), primers and probes reported by Ilharreborde et al. and Levy et al. may present between one and three mismatches with *KkigroEL* nucleotide sequences from *K. kingae* isolates belonging to sequence type complex 6 (STc-6) and STc-35 (data not shown), which are two STcs previously shown to be responsible for invasive infections in pediatrics (35) and which may impact the degree of sensitivity of these RT-PCR tests (15, 23). Although mismatches in primer regions may have a limited effect on the quality of the amplification curves, they may result in a significant increase in cycle threshold values (36, 37). Two mismatches can delay amplification by three to five cycles, while three mismatches can delay amplification by seven to 13 cycles (37). In addition, single mismatches in the minor groove-binding-modified probes may result in no or weak amplification curves, leading to the risk of being interpreted as negative (38). Because *K. kingae*-positive clinical specimens commonly contain a low bacterial load, a highly sensitive molecular tool is therefore of significant importance to maximize the detection yield of the organism from paucibacillary specimens.

Regarding the RT-PCR assays targeting the RTX locus, it was recently demonstrated that such molecular tools are not valid to formally confirm the diagnosis of *K. kingae* infection because of cross-detection with *K. negevensis* (15). In addition, given the numerous uncharacterized microbes colonizing humans (39) and the multiple genomic factors indicating that the *Kingella* sp. RTX locus was horizontally acquired, such as IS*Kne1*, a *Kingella* sp. RTX locus-related transposable element found in multiple copies in both *K. negevensis* and *K. kingae* genomes (15), it cannot be entirely ruled out that a similar *Kingella* sp. RTX locus may have been transferred to other as-yet-uncharacterized *Kingella* species. Consequently, this implies that the numerous studies conducted over the past decade to calculate the prevalence rate of *K. kingae* infection and carriage using *Kingella* sp. RTX-related molecular methods are likely to have unintentionally overestimated the results.

To overcome the lack of specificity of RT-PCRs assays targeting the *Kingella* sp. RTX locus and to distinguish *K. kingae* from *K. negevensis* in clinical samples, Opota et al. recently proposed a strategy which consists of targeting both *KkigroEL* and *Kingella* sp. *rtxA* by using a duplex RT-PCR assay for diagnosing *K. kingae* infection (25). Such a diagnostic strategy is strongly debatable and has serious limitations for its use in the clinical diagnostic setting. Indeed, the genomic nature of the RTX locus from *Kingella* spp. makes its lateral transfer in uncharacterized *Kingella* species possible. More importantly, while *K. kingae* and *K. negevensis* share the same oropharyngeal niche and are potentially involved in pediatric osteoarticular infections, this duplex RT-PCR assay does not make it possible to diagnose potential dual infections or carriage caused by both *K. kingae* and *K. negevensis* (P. Yagupsky, unpublished data).

Recently, de Knegt et al. developed a similar approach to diagnose osteoarticular infections caused by *K. kingae* in a Danish pediatric population, after designing new primers and probes against the *rtxA* gene to maximize sensitivity and optimizing the *KkigroEL*-specific RT-PCR assay reported by Ilharreborde et al. (23). Interestingly, 12 specimens were *Kingella* sp. *rtxA* positive, and only 10 specimens were positive for both *rtxA* and *groEL*. The authors suggested that the two *rtxA*-positive and *KkigroEL*-negative specimens may be explained by a positive result near the limit of detection of their two RT-PCR tests, a sampling error, or decreased sensitivity due to mismatches of either primers or probes in the *KkigroEL* gene. Nevertheless, because it was previously demonstrated that *K. negevensis*, which is *rtxA* and *rtxB* positive and *KkigroEL* negative, may occasionally induce joint infections in infancy, such findings in Denmark may be

consistent with infections caused by *K. negevensis*, as previously observed in France (15).

Therefore, as there are currently a lack of clinical data regarding *K. negevensis*, being able to formally discriminate *K. kingae* from *K. negevensis* is important. As a consequence, the development of highly species-specific and sensitive RT-PCR assays emerges as the most effective and reliable diagnostic strategy in clinical microbiology. Recently, the development of a *K. negevensis*-specific RT-PCR testing targeting the *K. negevensis groEL* gene enabled identification of the first arthritis caused by *K. negevensis* in an 8-month-old boy (15). To diagnose infections and carriage caused by *K. kingae*, the new *Kkimdh*-specific RT-PCR assay that we describe herein thereby appears to be an optimal molecular tool that could be used either alone or in combination with *K. negevensis*-specific RT-PCR assays. However, it should be emphasized that such RT-PCR assays remain costly, and that dual-target PCR is particularly advantageous to compensate for the potentially decreased sensitivities of assays applying minor groove-binding probes (38). Given that no target variation in the *Kkimdh* gene was detected in a large and diverse collection of *K. kingae* strains, the *Kkimdh*-related primers and probe designed in the present study appear to be robust enough to be applied alone in the clinical diagnostic setting.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00505-18.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 4, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.
SUPPLEMENTAL FILE 6, XLSX file, 0.1 MB.

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