Title:

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

Running title (52<54 characters and spaces):

A new real-time PCR targeting the *K. kingae mdh* gene

Authors:

Nawal El Houmami[1#], MD, PhD; Guillaume André Durand[1], MD, MSc; Janek Bzdrenga[2], PhD; Anne Darmon[1], MD, MSc; Philippe Minodier[3], MD, MSc; Hervé Seligmann[1], PhD; Didier Raoult[1], MD, PhD; Pierre-Edouard Fournier[1#], MD, PhD

Authors' affiliations :

[1]UMR VITROME, Aix-Marseille Univ, IRD, Service de Santé des Armées, Assistance Publique-Hôpitaux de Marseille, Institut Hospitalo-Universitaire Méditerranée-Infection, Marseille, France.

[2]Univ. Grenoble-Alpes, CEA, CNRS, IBS, F-38000, Grenoble, France.

[3]Department of Pediatric Emergency Medicine, North Hospital, Marseille, France.

Corresponding authors :

[#]Dr. Nawal El Houmami, UMR VITROME, Institut Hospitalo-Universitaire Méditerranée Infection, Assistance Publique-Hôpitaux de Marseille, 19-21 Boulevard Jean Moulin, 13005 Marseille, France. [nawal.el-houmami@etu.univ-amu.fr].

[#]Prof. Pierre-Edouard Fournier, UMR VITROME, Institut Hospitalo-Universitaire Méditerranée-Infection, 19-21 Boulevard Jean Moulin, 13005 Marseille, France. [pierre-edouard.fournier@univ-amu.fr].

Telephone: + (33) 413 732 401, Fax: + (33) 413 732 402.

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 by culture and broad-range 16S rRNA gene polymerase chain reaction (PCR) assays from clinical specimens have proven unsatisfactory and were gradually let out for the benefit of specific real-time PCR tests targeting the *groEL* gene and RTX locus of *K. kingae* by the late 2000s. 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 of specificity because they could not distinguish between *K. kingae* and the recently described *K. 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 a 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 against 18 variants of the *K. kingae mdh* gene from 20 distinct sequences types of *K. kingae*. This novel *K. kingae*-specific RT-PCR assay demonstrated a high specificity and sensitivity and was successfully used to diagnose *K. kingae* infections and carriage in 104 clinical specimens from children aged between 7 months and 7 years old.

INTRODUCTION

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 in 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 aged between six and 36 months in countries where *K. kingae*-specific real-time PCR assays are routinely employed [4-7]. This contributed to significantly improve 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 contributed to a better understanding of the epidemiology of *K. kingae* carriage among healthy carriers and ill children [10-12]. Although polymerase chain reaction (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 for the development of *K. kingae*-specific RT-PCR assays [4,5,16,17]. Comprehensively studied, the *groEL* gene encodes a chaperone protein that is considered as 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 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 mobilome, Kehl-Fie *et al*. 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 oropharynxes 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 both species when used alone [15]. *Kingella negevensis* has also been identified in the hip of an 8-month-old boy with a specific 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 a 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 aged between six 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 (Table S1). 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 between 24 and 36 hours 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 specificity 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 swab (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 that 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 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 *K. kingae* ATCC 23330^T (FOJK01000000) [26], *K. kingae* KKWG1 (LN869922) [29],

K. kingae PYKK081 (NZ_JH621344), *K. kingae* 11220434 (JH768595) [30] and *K. kingae* KK247 (CCJT01000000) [31], was performed using the Geneious R11.0.5 software (http://www.geneious.com) [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*) coding the malate dehydrogenase met the above 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), as well as 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. (a) 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- 6-carboxyfluorescein [FAM]- CATCATCACGCCCTGAACGGCTT -3) 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. Primers and probe specificity was confirmed in silico using the BLAST tool (http://blast.ncbi.nlm.nih.gov).

(b) *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 NoRox 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 two minutes and 95°C for three minutes, followed by 45 cycles of a two-stage temperature profile of 95°C for three seconds and 60°C for 30 seconds.

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 LT985524 (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 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 a *mdh* gene within the genomes of *Acinetobacter iwoffii* ZS207 (CP019143), *Acidovorax* sp. RAC01 (CP016447), *Neisseria* sp. KEM232 (CP022527.1), *Neisseria elongata* subsp. *glycolytica* ATCC 29315 (CP007726.1), *Neisseria weaveri* NCTC13585 (LT90643.1), *Neisseria zoodegmatis* NCTC12230 (LN869922.1), and *Polaromonas naphthalenivorans* CJ2 (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* by showing nucleotide sequences identities ranging from 77.1 to 79.1% (Fig. S1, 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 initially tested *KkigroEL*-negative were

detected *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 were derived from children with septic arthritis aged between seven and 29 months, one was sampled from the oropharynx of a 13-month-old boy, and one was identified in the joint fluid of a seven-year-old boy (Table S3).

DISCUSSION

This study reports a novel *K. kingae*-specific RT-PCR assay targeting the *Kkimdh* gene, a housekeeping gene coding 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 and probe Fwd-Kkimdh, Rev-Kkimdh, 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 when compared to all those previously reported. Although *K. kingae*-specific RT-PCR assays

targeting the *groEL* gene may be considered as 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 STc-6 and -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 the 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 *ISKne1*, 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 in 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 comprises serious limitations for its use in the clinical diagnostic setting. Indeed, the genomic nature of the RTX locus from *Kingella* sp. makes its lateral transfer in uncharacterized *Kingella* species possible. More importantly, while *K. kingae* and *K. negevensis* share the same oropharyngeal niche and may potentially be 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* [Pablo 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 were positive for both *rtxA* and *groEL*. The authors suggested that the two *rtxA*-positive and *KkigroEL*-negative specimens may be explained by a positivity 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 is 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 eight-month boy [15]. To diagnose infections and carriage caused by *K. kingae*, the new *Kkimdh*-specific RT-PCR assay that we describe herein thereby appears as 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 sensitivity 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.

ACKNOWLEDGEMENTS

The study was funded by the Foundation Mediterranée Infection and the French National Research Agency under the program "Investissements d'avenir", reference ANR-10-IAHU-03. The authors would like to gratefully acknowledge Prof. Pablo Yagupsky, microbiologist at the Clinical Microbiology Laboratory, Soroka Medical Center, Beer-Sheva, Israel, for providing numerous *K. kingae* isolates, and for having critically reviewed this manuscript.

300 **REFERENCES**

301 1. Yagupsky P. 2015. *Kingella kingae*: carriage, transmission, and disease. Clin Microbiol Rev
302 28:54–79.

303 2. Yagupsky P, El Houmami N, Fournier PE. 2017. Outbreaks of Invasive *Kingella kingae* Infections in
304 Daycare Facilities: Approach to Investigation and Management. J Pediatr 182:14–20.

305 3. Fournier PE, Drancourt M, Colson P, Rolain JM, La Scola B, Raoult D. 2013. Modern clinical
306 microbiology: new challenges and solutions. Nat Rev Microbiol 11:574–585.

307 4. Ilharreborde B, Bidet P, Lorrot M, Even J, Mariani-Kurkdjian P, Liguori S, Vitoux C, Lefevre Y, Doit C,
308 Fitoussi F, Penneçot G, Bingen E, Mazda K, Bonacorsi S. 2009. New real-time PCR-based method
309 for *Kingella kingae* DNA detection: application to samples collected from 89 children with acute
310 arthritis. J Clin Microbiol 47:1837–1841. Erratum in: 2009. J Clin Microbiol 47:3071.

311 5. Levy PY, Fournier PE, Fenollar F, Raoult D. 2013. Systematic PCR detection in culture-negative
312 osteoarticular infections. Am J Med 126:1143.e25–33.

313 6. Chometon S, Benito Y, Chaker M, Boisset S, Ploton C, Bérard J, Vandenesch F, Freydiere AM. 2007.
314 Specific real-time polymerase chain reaction places *Kingella kingae* as the most common cause of
315 osteoarticular infections in young children. Pediatr Infect Dis J 26:377–381.

316 7. El Houmami N, Yagupsky P, Ceroni D. 2018. *Kingella kingae* hand and wrist tenosynovitis in young
317 children. J Hand Surg Eur Vol. Jan 1:1753193418764818.

318 8. Juchler C, Spyropoulou V, Wagner N, Merlini L, Dhouib A, Manzano S, Tabard-Fougère A, Samara E,
319 Ceroni D. 2018. The Contemporary Bacteriologic Epidemiology of Osteoarticular Infections in
320 Children in Switzerland. J Pediatr 194:190-196.e1.

321 9. Ceroni D, Belaieff W, Cherkaoui A, Lascombes P, Schrenzel J, de Coulon G, Dubois-Ferrière V, Dayer
322 R. 2014. Primary epiphyseal or apophyseal subacute osteomyelitis in the pediatric population: a
323 report of fourteen cases and a systematic review of the literature. J Bone Joint Surg Am
324 96:1570–1575.

10. Ceroni D, Dubois-Ferrière V, Anderson R, Combescure C, Lamah L, Cherkaoui A, Schrenzel J. 2012. Small risk of osteoarticular infections in children with asymptomatic oropharyngeal carriage of *Kingella kingae*. Pediatr Infect Dis J 31:983–985.

11. Ceroni D, Dubois-Ferriere V, Cherkaoui A, Gesuele R, Combescure C, Lamah L, Manzano S, Hibbs J, Schrenzel J. 2013. Detection of *Kingella kingae* osteoarticular infections in children by oropharyngeal swab PCR. Pediatrics 131:e230–235.

12. Ceroni D, Llana RA, Kherad O, Dubois-Ferriere V, Lascombes P, Renzi G, Lamah L, Manzano S, Cherkaoui A, Schrenzel J. 2013. Comparing the oropharyngeal colonization density of *Kingella kingae* between asymptomatic carriers and children with invasive osteoarticular infections. Pediatr Infect Dis J 32:412–414.

13. Verdier I, Gayet-Ageron A, Ploton C, Taylor P, Benito Y, Freydiere AM, Chotel F, Bérard J, Vanhems P, Vandenesch F. 2005. Contribution of a broad range polymerase chain reaction to the diagnosis of osteoarticular infections caused by *Kingella kingae*: description of twenty-four recent pediatric diagnoses. Pediatr Infect Dis J 24:692–696.

14. Morel AS, Dubourg G, Prudent E, Edouard S, Gouriet F, Casalta JP, Fenollar F, Fournier PE, Drancourt M, Raoult D. 2015. Complementarity between targeted real-time specific PCR and conventional broad-range 16S rDNA PCR in the syndrome-driven diagnosis of infectious diseases. Eur J Clin Microbiol Infect Dis 34:561–570.

15. El Houmami N, Bzdrenga J, Durand GA, Minodier P, Seligmann H, Prudent E, Bakour S, Bonacorsi S, Raoult D, Yagupsky P, Fournier PE. 2017. Molecular Tests That Target the RTX Locus Do Not Distinguish between *Kingella kingae* and the Recently Described *Kingella negevensis* Species. J Clin Microbiol 55:3113–3122.

16. Cherkaoui A, Ceroni D, Emonet S, Lefevre Y, Schrenzel J. 2009. Molecular diagnosis of *Kingella kingae* osteoarticular infections by specific real-time PCR assay. J Med Microbiol 58:65–68.

17. Lehours P, Freydière AM, Richer O, Burucoa C, Boisset S, Lanotte P, Prère MF, Ferroni A, Lafuente C, Vandenesch F, Mégraud F, Ménard A. 2011. The *rtxA* toxin gene of *Kingella kingae*: a pertinent target for molecular diagnosis of osteoarticular infections. J Clin Microbiol 49:1245–1250.

18. Links MG, Dumonceaux TJ, Hemmingsen SM, Hill JE. 2012. The chaperonin-60 universal target is a barcode for bacteria that enables de novo assembly of metagenomics data. Plos One 7:e49755.

19. Chaban B, Hill JE. 2012. A 'universal' type II chaperonin PCR detection system for the investigation of Archae in complex microbial communities. ISME J 6:430–439.

20. Olson AB, Sibley CD, Schmidt L, Wilcox MA, Surette MG, Corbett CR. 2010. Development of real-time PCR assays for detection of the *Streptococcus milleri* group from cystic fibrosis clinical specimens by targeting the *cpn60* and 16S rRNA genes. J Clin Microbiol 48:1150–1160.

21. Chaban B, Musil KM, Himsworth CG, Hill JE. 2009. Development of *cpn60*-based real-time quantitative PCR assays for the detection of 14 *Campylobacter* species and application to screening of canine fecal samples. Appl Environ Microbiol 75:3055–3061.

22. El Houmami N, Fournier PE, Ceroni D. 2017. Targeting the *Kingella Kingae* groEL gene is a reliable method for the molecular diagnosis of *K. Kingae* infection and carriage. J Paediatr Child Health 53:1030–1031.

23. de Knegt VE, Kristiansen GQ, Schønning K. 2017. Evaluation of dual target-specific real-time PCR for the detection of *Kingella kingae* in a Danish paediatric population. Infect Dis (Lond) 15:1–7.

24. Kehl-Fie TE, St Geme JW 3rd. 2007. Identification and characterization of an RTX toxin in the emerging pathogen *Kingella kingae*. J Bacteriol 189:430–436.

25. Opota O, Laurent S, Pillonel T, Léger M, Trachsel S, Prod'hom G, Jaton K, Greub G. 2017. Genomics of the new species *Kingella negevensis*: diagnostic issues and identification of a locus encoding a RTX toxin. Microbes Infect 19:546–552.

26. El Houmami N, Bakour S, Bzdrenga J, Rathored J, Seligmann H, Robert C, Armstrong N, Schrenzel J, Raoult D, Yagupsky P, Fournier PE. 2017. Isolation and characterization of *Kingella negevensis* sp.

nov., a novel *Kingella* species detected in a healthy paediatric population. Int J Syst Evol Microbiol 67:2370–2376.

27. El Houmami N, Schrenzel J, Yagupsky P, Robert C, Ceroni D, Raoult D, Fournier PE. 2017. Draft Genome Sequence of *Kingella negevensis* SW7208426, the First European Strain of *K. negevensis* Isolated from a Healthy Child in Switzerland. Genome Announc 5(26).

28. Yagupsky P, Merires M, Bahar J, Dagan R. 1995. Evaluation of novel vancomycin-containing medium for primary isolation of *Kingella kingae* from upper respiratory tract specimens. J Clin Microbiol 33:1426–1427.

29. Bidet P, Basmaci R, Guglielmini J, Doit C, Jost C, Birgy A, Bonacorsi S. 2015. Genome Analysis of *Kingella kingae* Strain KWG1 Reveals How a β-Lactamase Gene Inserted in the Chromosome of This Species. Antimicrob Agents Chemother 60:703–708.

30. Fournier PE, Rouli L, El Karkouri K, Nguyen TT, Yagupsky P, Raoult D. 2012. Genomic comparison of *Kingella kingae* strains. J Bacteriol 194:5972.

31. Rouli L, Robert C, Raoult D, Yagupsky P. 2014. *Kingella kingae* KK247, an Atypical Pulsed-Field Gel Electrophoresis Clone A Strain. Genome Announc 2(6).

32. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649.

33. Katoh K, Misawa K, Kuma K, Miyata T. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res 30:3059–3066.

34. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874.

35. Amit U, Porat N, Basmaci R, Bidet P, Bonacorsi S, Dagan R, Yagupsky P. 2012. Genotyping of invasive *Kingella kingae* isolates reveals predominant clones and association with specific clinical syndromes. Clin Infect Dis 55:1074–1079.

401 36. Bourhy P, Bremont S, Zinini F, Giry C, Picardeau M. 2011. Comparison of real-time PCR assays for
402 detection of pathogenic Leptospira spp. in blood and identification of variations in target sequences.
403 J Clin Microbiol 49:2154–2160.
404 37. Whiley DM, Sloots TP. 2005. Sequence variation in primer targets affects the accuracy of viral
405 quantitative PCR. J Clin Virol 34:104–107.
406 38. Kamau E, Agoti CN, Lewa CS, Oketch J, Owor BE, Otieno GP, Bett A, Cane PA, Nokes DJ. 2017.
407 Recent sequence variation in probe binding site affected detection of respiratory syncytial
408 virus group B by real-time RT-PCR. J Clin Virol 88:21–25.
409 39. Kowarsky M, Camunas-Soler J, Kertesz M, De Vlaminck I, Koh W, Pan W, Martin L, Neff
410 NF, Okamoto J, Wong RJ, Kharbanda S, El-Sayed Y, Blumenfeld Y, Stevenson DK, Shaw GM, Wolfe
411 ND, Quake SR. 2017. Numerous uncharacterized and highly divergent microbes which colonize
412 humans are revealed by circulating cell-free DNA. Proc Natl Acad Sci USA 114:9623–9628.
413 40. Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing
414 phylogenetic trees. Mol Biol and Evol 4:406-425.
415 41. Tamura K, Nei M, and Kumar S. 2004. Prospects for inferring very large phylogenies by using the
416 neighbor-joining method. Proc Natl Acad Sci USA 101:11030–11035.
417
418 **FIGURE LEGENDS**
419 **Figure 1.** Genomic architecture of the region carrying the *mdh* gene of *Kingella kingae* (*Kkimdh*). The
420 genomic elements are not drawn to scale. The dashed lines indicate that the sensor histidine kinase
421 may be coded by either a single gene or two-component genes.
422 **Figure 2.** Neighbor-joining tree [40] based on the comparison of *mdh* nucleotide sequences from 18
423 genetic variants of *Kingella kingae* and its closest orthologs in *Acinetobacter*, *Acidovorax*,
424 *Polaromonas*, and *Neisseria* species. The tree is drawn to scale, with branch lengths in the same units
425 as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances
426 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 dataset.

TABLE LEGENDS

Table 1. Results of the real-time PCR assays targeting the *groEL* and *mdh* genes of *K. kingae* (*KkigroEL* and *Kkimdh*) that were tested on 20 genotypically distinct *K. kingae* isolates, and 201 paediatric specimens.

(1) ribosomal small subunit-dependent GTPase
(2) sensor histidine kinase
(3) GTP cyclohydrolase FolE2

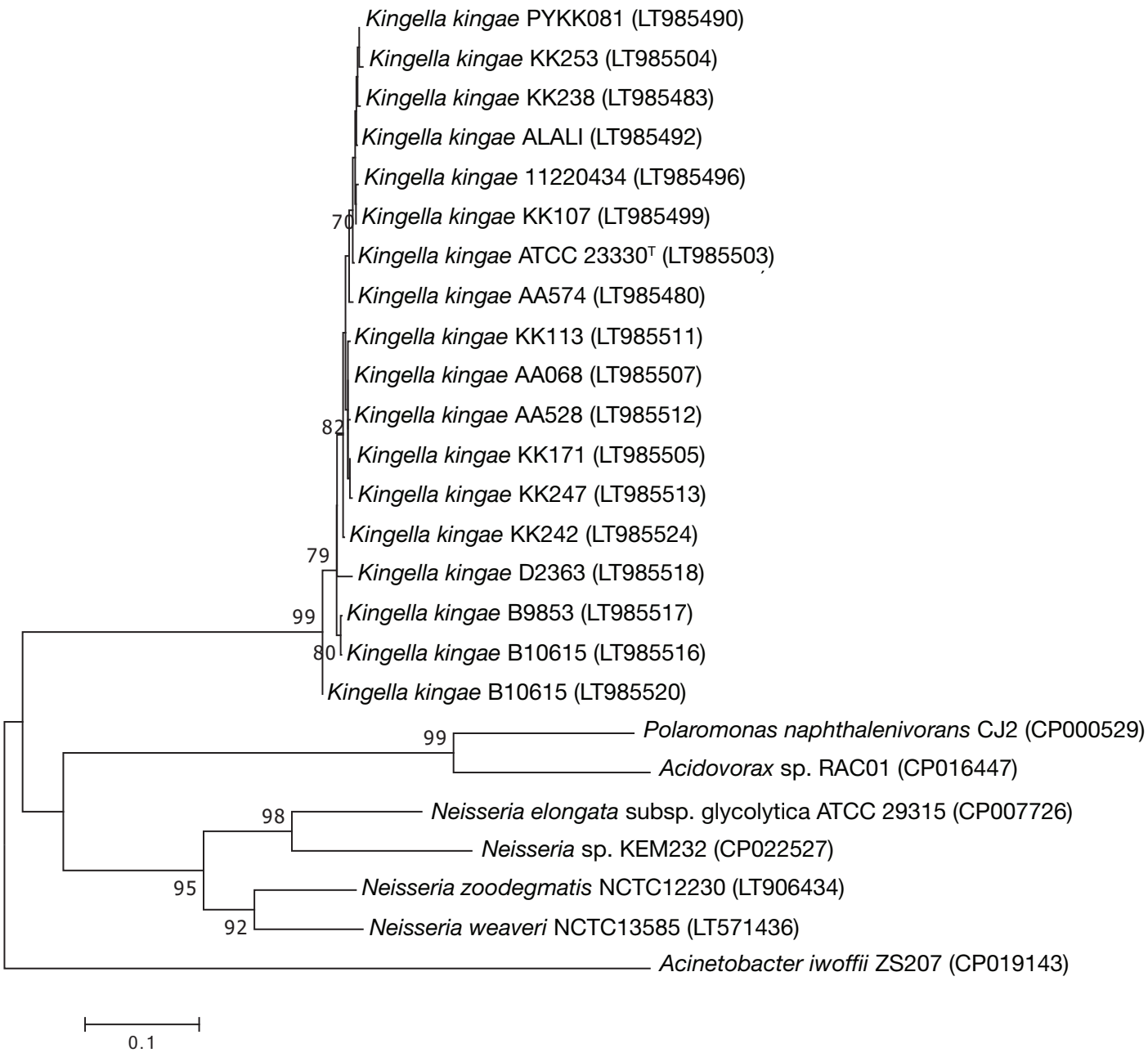
Kingella kingae PYKK081 (LT985490)
Kingella kingae KK253 (LT985504)
Kingella kingae KK238 (LT985483)
Kingella kingae ALALI (LT985492)
Kingella kingae 11220434 (LT985496)
70
Kingella kingae KK107 (LT985499)
Kingella kingae ATCC 23330^{T} (LT985503)
Kingella kingae AA574 (LT985480)
Kingella kingae KK113 (LT985511)
Kingella kingae AA068 (LT985507)
82
Kingella kingae AA528 (LT985512)
Kingella kingae KK171 (LT985505)
Kingella kingae KK247 (LT985513)
Kingella kingae KK242 (LT985524)
79
Kingella kingae D2363 (LT985518)
99
Kingella kingae B9853 (LT985517)
80
Kingella kingae B10615 (LT985516)
Kingella kingae B10615 (LT985520)
99
Polaromonas naphthalenivorans CJ2 (CP000529)
Acidovorax sp. RAC01 (CP016447)
98
Neisseria elongata subsp. glycolytica ATCC 29315 (CP007726)
Neisseria sp. KEM232 (CP022527)
95
Neisseria zoodegmatis NCTC12230 (LT906434)
92
Neisseria weaveri NCTC13585 (LT571436)
Acinetobacter iwoffii ZS207 (CP019143)
0.1

Table 1. Results of the real-time PCR assays targeting the *groEL* and *mdh* genes of *K. kingae* (*KkigroEL* and *Kkimdh*) that were tested on 20 genotypically distinct *K. kingae* isolates, and 201 paediatric specimens.

	***Kkimdh*-positive**	***KkigroEL*-positive**
***K. kingae* isolates (n=20)**	20	20
Other bacterial species (n=86)	0	0
Clinical specimens (n=201)*		
Initially *KkigroEL*-positive (n=96)	96	96
Initially *KkigroEL*-negative (n=105)	8	0

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