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Original article

Genomics of the new species *Kingella negevensis*: diagnostic issues and identification of a locus encoding a RTX toxin

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Abstract

Kingella kingae, producing the cytotoxic RTX protein, is a causative agent of serious infections in humans such as bacteremia, endocarditis and osteoarticular infection, especially in young children. Recently, *Kingella negevensis,* a related species, has been isolated from the oral cavity of healthy children. In this study, we report the isolation of *K. negevensis* strain *eburonensis*, initially misidentified as *K. kingae* with MALDI-TOF MS, from a vaginal specimen of a patient suffering of vaginosis. The genome sequencing and analysis of this strain together with comparative genomics of the *Kingella* genus revealed that *K. negevensis* possesses a full homolog of the *rtx* operon of *K. kingae* involved in the synthesis of the RTX toxin. We report that a *K. kingae* specific diagnostic PCR, based on the *rtxA* gene, was positive when tested on *K. negevensis* strain *eburonensis* DNA. This cross-amplification, and risk of misidentification, was confirmed by in silico analysis of the target gene sequence. To overcome this major diagnostic issue we developed a duplex real-time PCR to detect and distinguish *K. kingae* and *K. negevensis*. In addition to this, the identification of *K. negevensis* raises a clinical issue in term of pathogenic potential given the production of a RTX hemolysin.

Keywords: *Kingella kingae*; Hemolysin proteins; Genomics; Real-time polymerase chain reaction; Infection; Matrix-assisted laser desorption-ionization mass spectrometry

1. Introduction

The genus *Kingella* belongs to the Neisseriaceae family in the *Betaproteobacteria* subclass. Members of this genus are Gram negative anaerobic facultative rods and it currently includes 5 species: *Kingella kingae, Kingella denitrificans*, *Kingella oralis, Kingella potus* and *Kingella negevensis* which has only recently been characterized [1–3]. *K. kingae*, which is part of the human oropharyngeal flora, is associated with serious invasive infections such as bacteremia, endocarditis and osteoarticular infections, especially in young children [4,5]. *K. kingae* can colonize and adhere to the oropharyngeal mucosa thanks to its pili. Then, relying on the production of the cytotoxin RTX (repeats in toxin), a secreted protein characterized by C-terminus glycine and aspartate-rich repeats, *K. kingae* can damage the epithelial barrier, allowing the bacterium to reach the bloodstream [6]. The synthesis of a

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polysaccharide capsule contributes to the host immune response escape, allowing the bacteria to avoid phagocytosis [1]. The RTX toxin is also linked to the bacterial immune escape through the lysis of macrophages and polynuclear cells.

K. denitrificans, which is also commonly found in the human nasopharynx, has been associated with invasive infections in humans including bacteremia and endocarditis [5,7–9]. *K. oralis*, identified from human dental plaque has been essentially associated with periodontitis [10,11]. *K. potus* was identified from an infected wound resulting from a bite from a kinkajou, an arboreal mammal of the rain forest of Central and South-America [12]. Finally, *K. negevensis* has been isolated from the oropharynx of healthy children [3]. All the *Kingella* species described so far have been associated with infections in humans; however, *K. kingae* is by far the most virulent bacterium of this genus and its pathogenesis largely relies on the production of the RTX hemolysin.

In this study we report the identification a beta-hemolytic bacterium isolated from a vaginal swab, related to *K. negevensis*. The bacterium was initially wrongly identified as *K. kingae* based on matrix-assisted laser desorption/ionization time of flight (MALDI-TOF MS) analysis. Because of the unusual body site for the recovery of *K. kingae,* additional characterizations were performed. Mass-spectrometry analysis with a distinct instrument and 16S rRNA gene sequencing both failed to confirm the identification as *K. kingae*; nevertheless, the 16S rRNA gene suggested that this bacterium belongs to the genus *Kingella*. To further characterize this *K. kingae*-like organism, we sequenced and analysed its genome. Based on the taxogenomics data and the comparative genomics study presented herein we conclude that this strain belongs to the new species *K. negevensis* in which we identified several virulence factors found in *K. kingae,* including the full *rtx* operon described for the first time in a *Kingella* species other than *K. kingae*. This strain was named *K. negevensis* strain "*eburonensis*" because it was isolated in the city named Yverdon (latin name: Eburodunum), Switzerland. The data presented herein – taxogenomics, virulence factor analysis and real-time PCR identification – raise important clinical and diagnostic issue of the identification of *K. negevensis*.

2. Materials and methods

2.1. Sample, culture and microbial identification

The strain *K. negevensis eburonensis* has been recovered from the vaginal swab of a 22 years old patient. The strain grew within 24 h on blood agar plate incubated at 37 °C in the presence of 5% of CO_2 as beta-hemolytic colonies identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), initially with the Vitek MS instrument (bioMérieux, Lyon France) and then with the Microflex instrument (Bruker Daltonics, Leipzig, Germany). Minimum inhibitory concentrations (MICs) were determined using the E-test method (bioMérieux, Lyon, France) and their interpretations were achieved using the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://eucast.org/). The biochemical assays were performed with the Vitek 2 instrument (bioMérieux, Lyon France) and the apiCoryne (bioMérieux, Lyon France). Identification by a broad-spectrum bacterial polymerase chain reaction (PCR) consisted in the amplification and sequencing of a partial sequence of the 16S rRNA gene, as previously described [13,14]. The resulting sequence was queried against the GenBank database using BLAST.

2.2. Development of a new K. kingae specific duplex real-time PCR

The *K. kingae* specific duplex real-time PCR was developed to meet the criteria of our automated molecular diagnostic platform that allows to perform reactions for the detection and identification of viruses, bacteria, fungi and pathogens on the same 384-well plate using the Taqman probe technology (Applied Biosystems) as described in Greub et al. [14]. This duplex PCR targeted two different genes, the *rtxA* gene, part of the *rtx* operon and encoding the RTX toxin, and the *cpn60* gene, encoding the chaperone Cpn60. The PCR targeting the *rtxA* gene was adapted from Lehours and colleagues [15] (forward primers F2-KK-rtxA 5′-GCGCACAAGCAGGTGTACAA-3′, reverse primer R2-KK-rtxA 5′-ACCTGCTGCTACTGTACCTGTTTTAG-3′ and the probe KK-rtxA2 5′-FAM-TTGAACAAAGCTGGACACG-MGB-NFQ-3′) at respective primers/probe concentrations 0.5/0.1 μM. The PCR targeting the *cpn60* gene was adapted from Levy and colleagues [16] (forward primers KKing1_F 5′-CCGATTTGAAACGCGGTATT-3′, reverse primer KKing1_R 5′-TTTGCCAACTTGCTCGTCAG-3′ and the probe KKing1_P 5′–VIC-AGTGGCGGCTTTGGTTGGCG-TAMRA-3′) at respective primers/probe concentrations 0.3/0.1 μM. Details of the development of this *K. kingae* specific duplex PCR can be found in the Supplementary Materials. The specificity of the PCRs was assessed in silico using BLAST, and also in vitro. Both monoplex PCRs were tested on DNA from closely related organisms and from unrelated organisms that can colonize or infect the same body sites as *K. kingae.* No amplification resulted from organisms other than *K. kingae*, with neither the *rtxA* PCR nor the *cpn60* PCR (Table S1). The sensitivity of the duplex PCR was then tested on 13 samples previously tested positive for *K. kingae* which gave positive results for all the samples, with both PCRs and with similar cycle threshold value (Ct) (Table S2).

2.3. Genome sequencing and analysis

The genomic DNA was extracted and purified using a Wizard Genomic DNA purification kit (Promega, Duebendorf, Switzerland). The purified genomic DNA was subjected to whole-genome shotgun sequencing using 2 × 150 bp paired-end sequencing on a MiSeq sequencer (Illumina, San Diego CA) from a single library. The quality of the reads was checked using fastQC [17] and they were filtered using trimmomatic [18]. We trimmed of the 5′ and the 3′ ends of the

reads the bases whose quality score was below 32. The remaining reads were assembled using SPAdes v3.10.1 [19] using kmer values from 55 to 77. The quality of the assembly returned by SPAdes was assessed using quast v4.4 [20].

The genome was annotated using Prokka 1.11 [21]. Orthogroups were inferred using orthofinder v1.1.4 [22] with the proteomes of *K. negevensis* strain *eburonensis*, *K. kingae ATCC 23330* (NCBI assembly accession GCA_000213535.1), *K. kingae KWG1* (GCA_001458475.1), *K. denitrificans* (GCA_000190695.1), *K. oralis* (GCA_000160435.1) and *K. negevensis* strain *Sch538* (GCA_000751855.1). For *K. potus*, raw reads of the type strain DSM 18304 were downloaded from the Short Read Archive (SRA) database (run number SRR3503442). They were assembled using Spades and the genome annotated with Prokka in order to include this species in our comparative analyses. The proteome of *Neisseria gonorrhoeae* (GCA_000006845.1) was used as outgroup. Orthogroups with a single gene in each of the genomes were aligned with MAFFT [23] and concatenated to calculate the average nucleotide identity (ANI), excluding the gaps from the pairwise alignments. 16S rRNA sequences from *K. kingae, K. denitrificans, K. oralis, K. potus, K. negevensis* strain *Sch538* and *N. gonorrhoeae* were extracted from GenBank and aligned with MAFFT with the genomic 16S rRNA sequence from *K. negevensis* strain *eburonensis*. The phylogeny was calculated and bootstrapped using RAxML [24] with the GTRCAT model.

A phylogeny of the *Kingella* genus was reconstructed based on the concatenated amino-acid alignments of 878 one to one orthologs with RAxML, a Gamma model of rate heterogeneity and the Le & Gascuel model of amino-acid substitution [25]. We searched for previously studied *K. kingae* specific virulence factors [1] in the other species of *Kingella:* using blastp [26], we selected the best reciprocal matches between the virulence factor proteins and the proteomes of each species and excluded the matches with an e-value higher than 10^{-10}. Conserved protein domains on the virulence proteins were identified using InterproScan [27]. Resistance genes were searched using ResFinder [28].

3. Results

3.1. Isolation and identification of K. negevensis *strain eburonensis*

Bacterial culture was achieved from a vaginal swab of a woman suffering from vaginosis. The culture was positive with *Gardnerella vaginalis* (moderate quantity), *Mycoplasma hominis* (strong quantity) and a hemolytic Gram negative bacterium initially identified as *K. kingae* with the MALDI-TOF Vitek MS instrument with a high confidence score (99.9%). Beta-hemolytic colonies of this bacterium grew within 24 h on blood agar plate incubated a 37 °C in the presence of 5% of CO_2 and were oxydase-positive.

Because of the unusual body site from which the bacterium was identified, a second analysis was performed using the Microflex MS instrument which did not confirm the identification. However, the best match was a *K. kingae* spectrum with a score below 1.7, which is not reliable for identification according to the manufacturer identification algorithm.

The 16S rRNA gene fragment of the *Kingella*-like bacterium, obtained by broad-range eubacterial 16S rDNA PCR and Sanger sequencing, exhibited a maximum of 93.9% sequence identity with sequences from *K. kingae*. Given the relatively low sequence similarity, we hypothesized that the *Kingella*-like organism might be a new species within the genus *Kingella*. Interestingly the 16S rRNA gene fragment exhibited 99.6% of identity with sequence corresponding to the newly

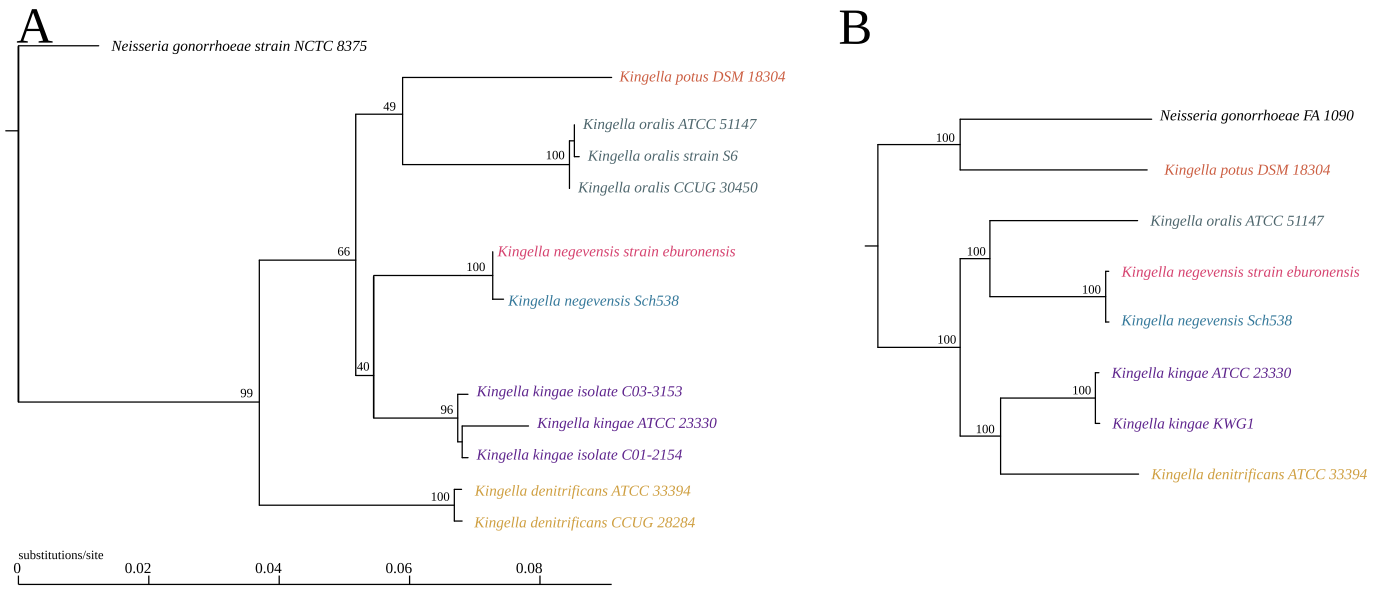

Fig. 1. Phylogenetic relationships of *Kingella* species reconstructed by maximum likelihood. The values shown on the nodes represent the bootstrap values. A. Reconstruction based on the 16S rRNA gene sequence rooted with *Neisseria gonorrhoeae.* B. Reconstruction based on the concatenated amino-acid sequence of 878 orthologous proteins rooted with *Neisseria gonorrhoeae.*

identified species *K. negevensis*. We thus named this new isolate *K. negevensis* strain *eburonensis*, isolated in the city named Yverdon (Switzerland).

We then tested this isolate with the *K. kingae* specific duplex real-time PCR targeting *rtxA* and *cpn60*. In silico and in vitro evaluations had demonstrated that both PCRs are expected to be positive for *K. kingae* with a detection limit between 100 and 1000 DNA copies per reaction (Supplementary Material, Tables S1 and S2). Surprisingly, the duplex PCR was positive for the *rtxA* gene but negative for the *cpn60* gene. So far this result had never been observed neither for a *K. kingae* strain nor for any organism other than *K. kingae*. This suggested a cross-identification of the *rtxA* PCR between *K. kingae* and *K. negevensis*. This finding was further confirmed with the genomic sequences from the *Kingella*-like organism further named *K. negevensis* strain *eburonensis*. Indeed, primers targeting *K. kingae rtxA* gene displayed a perfect match with the *rtxA* gene of *K. negevensis* strain *eburonensis* (Fig. S1) whereas the *cpn60* gene showed respectively 2, 4 and 4 mismatches on the target sequences of the forward primer, the reverse primer and the probe (Fig. S1).

Altogether these data suggested that the beta-hemolytic bacterium identified from a vaginal swab was a *K. negevensis* strain. The main phenotypic characteristics of *K. negevensis* strain *eburonensis* are presented in Table S3. *K. negevensis* strain *eburonensis* displays a complete hemolysis observed around colonies grown on blood-agar plate, a characteristic that was so far unique to *K. kingae* within the *Kingella* genus and related to the pathogenesis of this bacterium (Fig. 2, panel C).

3.2. *Genome analysis of* K. negevensis *strain eburonensis, comparative genomics and phylogeny of the* Kingella *species*

In order to extend our knowledge on *K. negevensis* and to determine the phylogenetic relationship of this species with other members of the *Kingella* genus, we sequenced and annotated the genome of *K. negevensis* strain *eburonensis*. The assembly of the genome resulted in 108 contigs longer than 500 base pairs (bp), which corresponded to an estimated genome size of 2,109,050 bp. Its GC content is of 45.54% (Table 1). The N50 was 44,725 bp. *K. negevensis* strain *eburonensis* encodes 2168 genes, among which 2112 are protein-coding genes. Less than 50% of the genome of *K. negevensis* strain *eburonensis* could be aligned with other genomes of the genus *Kingella*. In addition, the genome of *K. negevensis* strain *eburonensis* exhibited only 76.4% of average nucleotide identity with *K. kingae* (Table 1). This is much lower than the 95% ANI threshold commonly used to delineate species [29], which confirms the results from the comparison of the 16S rRNA genes. Moreover, *K. negevensis* strain *eburonensis* strain exhibited an overall 98.6% ANI with the *K. negevensis* strain *Sch538* genome, proving that those

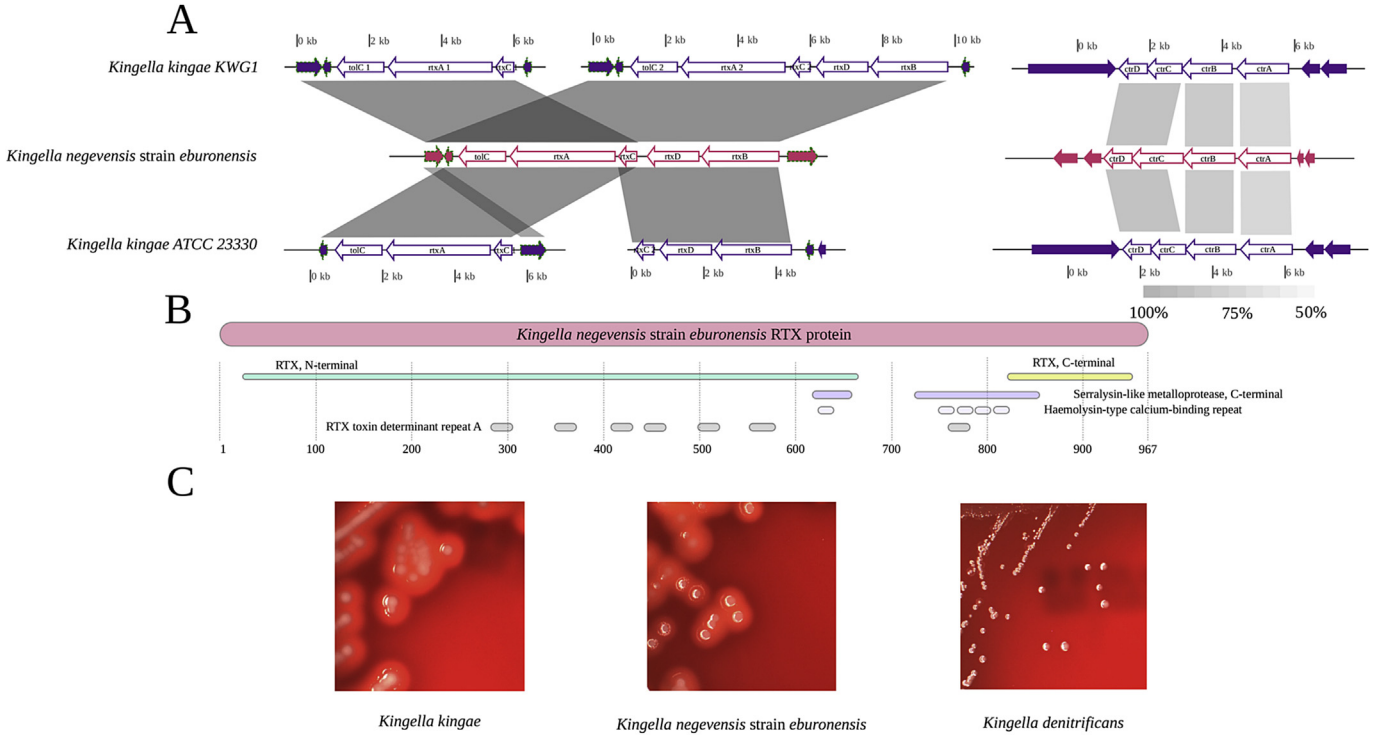

Fig. 2. Virulence factors of *K. negevensis* strain *eburonensis*. A. Comparison between the *rtx* and *ctr* operons of *K. negevensis* strain *eburonensis* and *K. kingae KWG1*. Dashed bordered arrows represent transposase genes. B. Domain analysis of the predicted RTX protein sequence of *K. negevensis* strain *eburonensis*. C. Growth on blood agar plate showing a complete hemolysis (beta-hemolysis) for *K. kingae* and *K. negevensis* strain *eburonensis* and an absence of hemolysis for *K. denitrificans*.

Table 1
General features of the *Kingella negevensis* strain *eburonensis* genome and of the genomes from other species studied.

	Kingella negevensis strain *eburonensis*	*Kingella kingae ATCC 23330*	*Kingella oralis ATCC 51147*	*Kingella denitrificans ATCC 33394*	*Kingella potus DSM18304*	*Neisseria gonorrhoeae FA1090*
Genome length (Mb)	2.11	1.95	2.41	2.22	2.35	2.15
Gene number	2168	1923	2367	2174	2495	1953
% coding sequences	86.9	87.7	83.5	86.3	84.0	78.0
% GC	45.5	46.6	54.3	54.1	57.9	52.7
tRNA genes	53	48	48	50	60	55
Plasmid	No	No	No	No	No	No
Transposases	15	8	9	12	1	21
Average nucleotide identity with *K. negevensis* strain *eburonensis* (%)	NA	76.4	72.9	71.0	64.6	65.5
rtx operon	Present	Present	Absent	Absent	Absent	Absent

two strains belong to the same species. Surprisingly, *K. potus* exhibited a much lower nucleotide identity with *K. negevensis* than other *Kingella* species, even lower than *N. gonorrhoeae*. The phylogenetic divergence of *K. potus* from other *Kingella* species (Fig. 1, panel B), together with the very low identity strongly suggests that *K. potus* belongs to a different genus. The phylogeny based on the 16S rRNA genes supported with low confidence (bootstrap of 40%) a grouping of *K. kingae* and *K. negevensis*. However, the phylogeny over the core genome, based on the protein alignments of 878 one to one orthologs over the *Kingella* species and *N. gonorrhoeae*, supported that *K. negevensis* was most closely related to *K. oralis* whereas *K. kingae* was closer to *K. denitrificans* (Fig. 1, panel B). We hypothesized that a relatively high rate of recombination in the *Kingella* genus, as observed in *Neisseria* [30,31], could be responsible for blurring the real relationships between the species.

3.3. Virulence factors of K. negevensis and identification of the rtx operon

The full *rtx* operon, composed of 5 genes (*tolC*, *rtxA/B/C/D*), found in the genome of *K. kingae* strain *KWG1* and homologous to the locus encoding *E. coli* hemolysin was also found in the genome of *K. negevensis*, with a high level of identity (99%) between the two *Kingella* species (Fig. 2, panel A, Tables S3–S5). In the genome of *K. kingae* strain *KWG1,* in addition to the full *rtx* operon, another locus encoding for *tolC, rtxA* and *rtxC* only was identified. However, in *K. kingae* strain *ATCC 23330,* the *rtx* genes do not constitute an operon and were found in two different genomic locations: one locus encoded for *tolC, rtxA* and *rtxC* and another locus for *rtxC*, *rtxD* and *rtxB* (Fig. 2, panel A). In both *K. negevensis* strains *eburonensis* and *Sch538*, all RTX specific domains were conserved on the *rtxA* encoded protein, indicating that the function of the toxin is probably conserved in *K. negevensis* (Fig. 2, panel B). This result is consistent to the complete hemolysis of the colonies observed on blood-agar plates (Fig. 2, panel C).

The *ctr* operon involved in the synthesis of the polysaccharide capsule was also found in *K. negevensis*, but it exhibited lower nucleotide identity (from 70% to 80%) to *K. kingae* (Fig. 2 panel A, Tables S4 and S5) with a 125 amino-acid insertion at the beginning of the CtrC sequence.

The *knh* gene, encoding a protein involved in cell adhesion in *K. kingae* and homologous to *nhhA* in *Neisseria meningitidis* [32] was also found in *K. negevensis*, although with lower identity (52%, Table S4). Moreover, a type V secretion system loci, absent in the *K. kingae* genome was found in *K. negevensis.* This loci is composed of genes of the *fha* family coding for a large filamentous hemagglutinin and for an outer membrane pore protein for which homologs are found in *N. meningitidis* [33] and in *Bordetella pertusis* [34]. Except for the genes encoding for pili synthesis proteins, most of the virulence factors of *K. kingae* were not found in *K. denitrificans, K. oralis* or *K. potus* (Table S4).

No known antibiotic resistance genes were found using ResFinder. However *K. negevensis* strain *eburonensis* was resistant to the macrolides erythromycin and clarithromycin and to the lincosamide clindamycin according to the EUCAST breakpoints (Table S6).

4. Discussion

4.1. Evidence of a new Kingella species and diagnosis issues

In the last decades, novel molecular and mass-spectrometry methods revolutionized diagnostic microbiology by both accelerating the identification of microorganisms from clinical samples and improving the sensitivity and the specificity of the detection [35–37]. In particular, the increased discriminatory power of these new technologies allowed the discovery of new organisms. A solid microbiology background together with a precise knowledge on the performance and limits of new methods, especially of their discrimination limits, is nevertheless required to prevent misidentification.

In this study we report the isolation of a strain of the genus *Kingella* belonging to the new species *K. negevensis.* This strain was first wrongly identified by MALDI-TOF MS as *K. kingae*. We hypothesize that this misidentification could stem either from a wrongly annotated spectrum in the database of

this instrument or from a problem of the algorithm calculating confidence scores. However, an alternative MALDI-TOF instrument did not confirm the identification. The amplification and sequencing of a partial region of the 16S rRNA gene using a broad range eubacterial PCR suggested a species closely related to *K. kingae*. Whole genome comparisons as well as phylogenetic reconstructions based on the core genome of *Kingella* and *Neisseria* support the classification of this strain as *K. negevensis*.

The in vitro analyses demonstrated that a *K. kingae* real-time PCR targeting the *rtxA* gene may also detect *K. negevensis*. The genomic analysis confirmed the presence of the full *rtx* operon. The *rtx* locus and in particular the *rtxA* and *rtxB* genes are commonly used as targets for the molecular detection (PCR) and for identification of *K. kingae* from clinical samples to investigate the carriage or infection due to this organism [15]. Our study reveals that misidentification may occur when relying only on monoplex PCR targeting the *rtxA* gene. The retrospective analysis of the samples previously tested in our institution as positive for *K. kingae* did not detect any species that could potentially be *K. negevensis*. With the duplex real-time PCR described here it is possible to i) overcome any possible polymorphisms within one of the targets and to ii) reduce the probability of misidentification due to non-specific PCR targets. Indeed, the first PCR targeting the *rtxA* gene is expected to detect both *K. kingae* and *K. negevensis* whereas the second PCR targeting the *cpn60* gene is expected to be positive for *K. kingae* only, being negative for *K. negevensis*. While many laboratories rely only on monoplex PCRs for the detection of *K. kingae* from clinical samples, in particular PCRs targeting the *rtx* operon, there is a high risk of misidentification of *K. kingae* and *K. negevensis*. Clinical microbiologists should now be rapidly informed of the discovery of this new species. In addition, MALDI-TOF databases should be implemented with the spectrum of *K. negevensis*.

4.2. Clinical relevance and pathogenic potential

Following the study of El Houmami et al. which identified *K. negevensis* in the oropharynx of healthy children [3], our study is the first identification of *K. negevensis* outside the oral cavity, namely in the female genital region. Additional studies are needed to establish the tissue tropism, prevalence and pathogenic potential of this organism. However, analysis of the virulome of *K. negevensis* identified the presence of a complete *rtx* operon encoding for the RTX hemolysin. Together with the hemolytic activity observed in vitro on blood-agar plates, this suggests a pathogenic potential of *K. negevensis*. *K. negevensis* strain *eburonensis* has been identified from the vaginal swab of a woman suffering from vaginosis. Future studies will help to determine the tissue tropism, the epidemiology and the clinical relevance of this new bacterial species. It is neither common nor rare to find commensal of the oropharyngeal mucosa as colonizers of the urogenital mucosa. For instance *K. denitrificans* has already been identified in the setting of gynecologic infections [38,39]. However, the presence of the full *rtx* locus associated with a complete hemolysis of the colonies visible on blood-agar plate is puzzling as the RTX toxin was thought to be restricted to *K. kingae* in the *Kingella* genus. *K. negevensis* also contains genes involved in the production of a type IV pilus and in the biosynthesis of a polysaccharide capsule, two components contributing to tissue invasion and immune system escape of *K. kingae* [1], further supporting the idea that *K. negevensis* might represent a true pathogen. Finally, future work will also help defining new diagnosis methods to detect this new bacterial species.

In conclusion our taxogenomics study confirms the characterization of *K. negevensis* as a new species in the genus *Kingella* and raises two major issues. The first one is the diagnostic problem caused by the relatedness of *K. negevensis* and *K. kingae* and the high risk of misidentification when using methods lacking discriminatory power. The second issue is the pathogenic potential given the presence in *K. negevensis* of some of the major known virulence factor of *K. kingae*, including the locus encoding the RTX hemolysin. A particular attention should be made on the identification of *K. kingae*-like organisms that do not fulfill all clinical, phenotypic or genetic criteria usually required for the identification.

5. Sequence accession

The genome assembly of *K. negevensis* strain *eburonensis* was deposited to the European Nucleotide Archive, under the accession ID GCA_900182485.

Competing interests

The authors report no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2017.08.001.

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