Microbiology, Genomics, and Population Structure

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Microbiology

K. kingae appears as pairs or chains of 4–8 plump (0.6–1 μm by 1–3 μm) gram-negative coccobacilli, resembling "a pile of bricks" (Fig. 1a). The organism is facultatively anaerobic and grows best in a 5 % CO_2-enriched atmosphere, similar to other bacterial species that reside in the respiratory tract [1]. *K. kingae* is *β*-hemolytic and is associated with a faint ring of hemolysis around colonies, resembling the hemolytic properties of *Streptococcus agalactiae* and *Listeria monocytogenes*. All strains of *K. kingae* are non-motile and non-spore-forming and exhibit negative catalase, urease, and indole reactions, and almost all strains possess oxidase activity. *K. kingae* produces acid from glucose and usually from maltose [2, 3], hydrolyzes indoxyl phosphate and L-prolyl-*β*-naphthylamide, and yields positive alkaline and acid phosphatase. Its fatty acid content is characterized by a high concentration of myristic acid and lower concentrations of palmitic, lauric, palmitoleic, linoleic, oleic, 3-hydroxylauric, 3-hydroxymyristic, and cis-vaccenic acids [4, 5].

K. kingae grows on routine laboratory media such as trypticase soy agar with 5 % added hemoglobin (the familiar blood agar plates), chocolate agar, and GC-based media as gray colonies producing noticeable pitting of the agar surface, which is most evident after the removal of the colony [6]. The vast majority of strains grow well on Mueller–Hinton agar with 5 % added defibrinated horse blood

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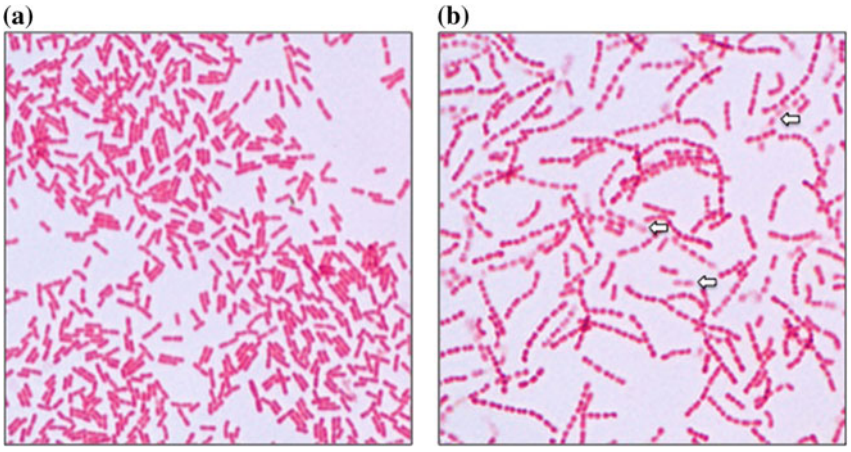


Fig. 1 **a** Gram staining of a typical *Kingella kingae* isolate depicting plump gram-negative coccobacilli arranged in pairs or short chains; **b** small-colony variant morphology exhibiting long chains of coccobacilli and early autolysis (*arrows*)

and 20 mg/L of β-NAD and on Thayer–Martin agar. In contrast, the organism fails to grow on MacConkey agar or Kligler agar.

Typical *K. kingae* strains produce three different colony types that correlate with the density of pilus expression: a spreading/corroding type consisting of a small central colony surrounded by a wide fringe, a non-spreading/non-corroding type characterized by a flat colony encircled by a narrow fringe, and a dome-shaped type with no fringe. Spreading/corroding colonies are associated with dense type IV pili, non-spreading/non-corroding colonies are associated with sparse type IV pili, and domed colonies are associated with the absence of type IV pili [7–10]. The spreading/corroding morphology type can be irreversibly lost after repeated subculture [3]. Most respiratory tract and non-endocarditis blood isolates grow as spreading/corroding or non-spreading/non-corroding colonies, whereas isolates from joint fluid and bone aspirates generally grow as domed colonies [10].

In addition to the colony morphologies of typical *K. kingae* strains, distinctive small-colony variants (SCV) have been described and are characterized by growth as pinpoint colonies on blood agar and chocolate agar plates, but do not develop on Thayer–Martin medium [11, 12]. On blood agar plates, colonies are surrounded by a wide halo of β-hemolysis that facilitates their recognition. SCV strains are isolated in approximately 10 % of healthy pediatric carriers [11] but are rarely found in patients with invasive disease, suggesting decreased virulence. These strains form long chains of coccobacilli, indicating defective cell separation (Fig. 1b). Reversion to a rapidly growing phenotype but without modification of the long-chain configuration can be obtained by seeding *K. kingae* SCVs on GC-based medium but not on chocolate agar, implying a defect in electron transport, menadione metabolism, or thymidine uptake [13]. Many SCV strains have a 16S rRNA gene sequence that is ≥98.85 % homologous with the 16S rRNA gene sequence of the

ATCC 23330 type strain [12], indicating that they are genuine members of the *K. kingae* species. In contrast, other SCV strains show <97.12 % similarity with strain ATCC 23330, suggesting that they might represent a different *Kingella* species (Yagupsky P, unpublished data). Both typical *K. kingae* strains and SCV strains tend to undergo early autolysis (Fig. 1b) and thus should be subcultured frequently (every 2–3 days) on fresh medium to ensure ongoing viability.

Kingella kingae should be suspected on the basis of the characteristic Gram stain morphology, β-hemolysis, and a negative catalase test. Confirmation as *K. kingae* can be achieved with commercial systems and technologies such as the quadFERM +kit [14], the API NH card, the VITEK 2 instrument [15, 16], matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) [16, 17], and sequencing of the 16S rRNA gene [18]. Of note, the Remel RapID NH kit consistently misidentifies *K. kingae* [16].

Genomics

Comparison of the genomes of 22 *K. kingae* isolates from asymptomatic carriers and 28 *K. kingae* isolates from patients with different invasive infections and diverse geographic origins demonstrated that the genome size of the species ranges between 1,990,794 base pairs (bp) and 2,140,065 bp, contains 1981–2300 protein-encoding genes and between 43 and 54 RNA genes, and has a GC content between 46.5 and 46.9 % [12, 19–21] (Rouli L, unpublished data).

Similar to the other members of the Neisseriaceae family, *K. kingae* is naturally competent, making acquisition of DNA from other organisms by horizontal gene transfer an important source of genetic diversity. As with other Neisseriaceae, the uptake of exogenous DNA by *K. kingae* is finely regulated by DNA uptake sequences (DUS), which facilitate the transformation with homologous DNA and discriminate against acquisition of heterologous and potentially deleterious genomic sequences [22]. *K. kingae* DUS are short DNA sequences (12 nucleotides) consisting of a conserved 5′-CTG-3′ core flanked by variable sequences, resulting in DUS variants called "dialects," which are present in the bacterial genome of strain ATCC 23330 in 2787 copies [22]. Integrity of the core is strictly required for transformation, and the degree of genetic similarity between the dialects of the donor and recipient organisms influences the efficiency of the process [22]. Thus, DUS are competent obstacles to between-species recombination, contributing to the genetic stability and sexual isolation of the species.

Typing of K. kingae Isolates

With the development of improved diagnostic methods for *K. kingae* and the recognition of *K. kingae* as an important cause of sporadic pediatric disease and an

increasingly common cause of outbreaks of invasive disease among children in daycare centers, the need for accurate genotyping methods has become imperative [1, 23].

Pulsed-Field Gel Electrophoresis

The first genotyping technology applied to *K. kingae* was pulsed-field gel electrophoresis (PFGE). Using this method, over 70 different clones designated by arbitrarily selected capital letters have been identified so far, including some associated commonly with invasive diseases, others associated rarely with invasive diseases [11], others associated with specific diseases (e.g., osteoarticular infections, occult bacteremia, or endocarditis) [24], and others associated with specific populations of people (e.g., urban or seminomadic Israeli Bedouin children) [11, 25].

Similar to observations with other bacterial species, strain-to-strain genomic heterogeneity is especially noticeable among *K. kingae* carrier isolates. In contrast, a limited number of *K. kingae* strains are responsible for most cases of invasive diseases. Although colonization of the oropharyngeal mucosa is a prerequisite for invasive disease, not all colonizing strains are able to breach the epithelial barrier, survive in the bloodstream, and invade host tissues. The hypothesis that *K. kingae* strains differ in their invasive capabilities has been confirmed by Basmaci et al. employing an experimental animal model [26]. When 5-day-old albino Sprague–Dawley rats were inoculated intraperitoneally with 10^7 colony-forming units of *K. kingae*, the ATCC 23330 type strain (a respiratory isolate) was avirulent. In contrast, two strains recovered from children with invasive disease (one with bacteremia and the other with septic arthritis) were able to establish infection but showed significant differences in terms of animal mortality [26]. In Israel, 4 of 32 PFGE clones (namely clones B, H, K, and N) were collectively responsible for 120 of 181 (66.3 %) invasive infections over a two-decade period [24]. In Barcelona, 32 invasive isolates belonged to only 6 distinct clones, including a clone resembling the Israeli clone K [26]. PFGE clone K appears to combine optimal colonization fitness, transmissibility, and invasiveness. On the one hand, it is frequently found among asymptomatic carriers, was the predominant strain detected as early as 1993 among attendees to a daycare facility in southern Israel (persisting in the pharynx of colonized children for up to 4 months [27]), and ranked second among strains carried by healthy Jewish children in a study carried out in 2006–2007 [11]. On the other hand, clone K or variants of clone K represented 41.7 % of all invasive strains isolated in southern Israel during the period from 1991 through 2012, were also responsible for the excess of *K. kingae* morbidity observed in the Jewish population of the region [36], and caused outbreaks of invasive infections among daycare center attendees [23, 28, 29].

To investigate whether certain *K. kingae* genotypes are associated with specific clinical diseases, 181 invasive isolates from Israeli patients were characterized by

PFGE typing [24]. Of the 32 genotypically distinct clones, three were significantly associated with specific clinical syndromes (namely clones K, N, and P). Clone K was positively correlated with bacteremia and negatively correlated with osteoarticular infection, clone N was positively correlated with osteoarticular infection and negatively correlated with bacteremia, and clone P was strongly associated with bacterial endocarditis [24]. These statistical associations were subsequently confirmed employing the MLST typing method [26]. The observation that clone N and clone P strains are infrequently recovered from the oropharynx of healthy *K. kingae* carriers [11] despite their common association with invasive disease suggests that they may be rapidly cleared from the respiratory tract, implying that colonization and persistence on the mucosal surfaces and invasion of bloodstream and deep body niches require different biologic properties.

Multilocus Sequence Typing

Despite the success of PFGE typing in elucidating the chain of *K. kingae* child-to-child transmission and differentiating between invasive strains and carrier isolates, this method suffers from subjective interpretation of DNA band profiles and suboptimal interlaboratory reproducibility. To gain greater insight into the global population structure of *K. kingae*, the development of more robust genotyping tools based on genomic sequencing was clearly needed. Recent studies have demonstrated that several virulence genes (such as type IV pili [30], the RTX toxin [26, 31], the porin-encoding *por* gene [32], and the ribosomal RNA (*rrn)* operon [33]) display a remarkable degree of genomic diversity within the species. However, as *K. kingae* is a naturally competent organism subject to transformation, examination of a single gene is not sufficient for robust phylogenetic analysis. In recent years, multilocus sequence typing (MLST) has been proposed as a portable, universal, and definitive method for characterizing bacteria and studying their pathogenicity, evolution, and population structure [34]. In addition to providing a standardized approach to data collection by examining the nucleotide sequences of multiple loci encoding 6 or 7 housekeeping genes, MLST data are highly reproducible and transportable and are freely available over the Internet, ensuring that a uniform strain nomenclature is readily available [34]. The development of a MLST scheme was an important contributor to our current understanding of the epidemiology and population structure of *K. kingae*, facilitating large-scale international studies [26]. Although the 6 genes of this typing scheme were chosen before a *K. kingae* complete genome sequence was available, the 6 genes are physically dispersed across the chromosome and exhibit substantial polymorphism, thus providing significant discriminatory power. Based on the whole-genome sequence of *K. kingae* strain KWG1, the position of these 6 genes along the circular chromosome (from 0′ to 60′) is as follows: *rec*A: 18′, *gdh*: 23′, *cpn*60: 30′, *abc*Z: 34′, *adk*: 48′, and *aro*E: 49′. In order to facilitate the data analysis, a different allele number was assigned to each distinct sequence within a locus, and a different

sequence-type (ST) number was assigned to each distinct combination of alleles [26]. Isolates were grouped into ST complexes (STcs) if they differed at no more than one locus [26]. Founder genotypes were defined as the ST of the STc with the highest number of neighboring STs [26].

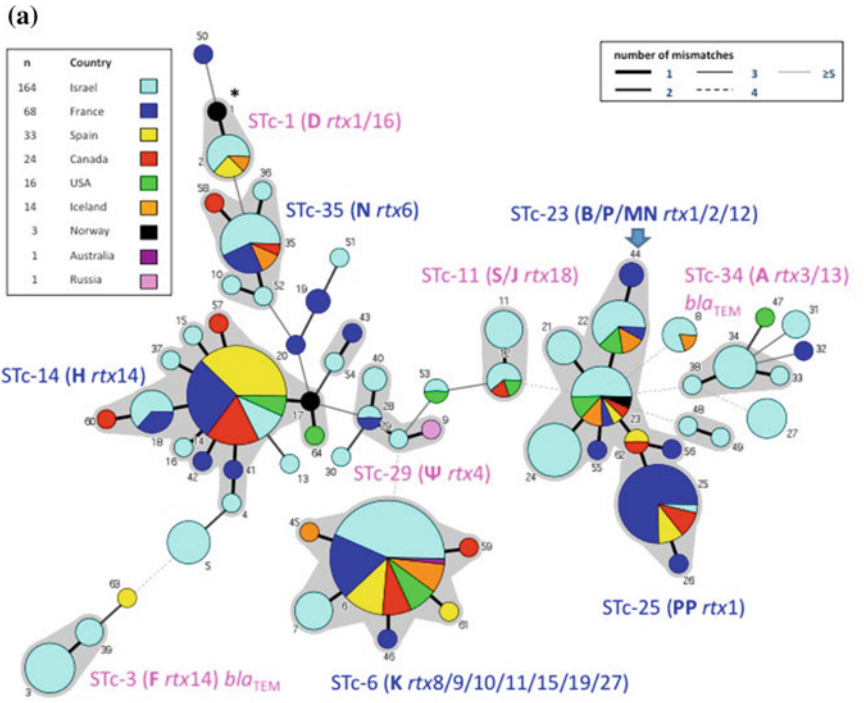
In an important study, a collection of 324 *K. kingae* isolates from Israel, Europe, North America, and Australia was investigated by the novel MLST method. Most European and American isolates were associated with invasive infections, while Israeli isolates came from both invasive infections and asymptomatic carriage [26]. Overall, strains segregated into more than 60 different STs and 12 STcs. Interestingly, *K. kingae* type strain ATCC 23330 (marked by an asterisk in Fig. 2a, b), a pharyngeal isolate from Norway, was the sole member of ST-1 identified and thus appears to be a poor representative of the main *K. kingae* population.

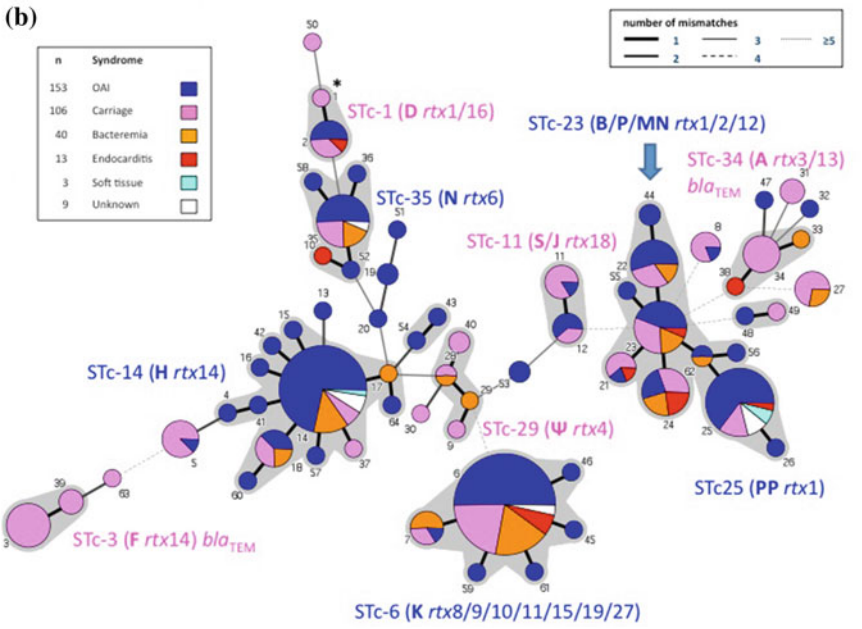
Population Structure

Despite the natural competence of *K. kingae,* almost perfect congruency has been found between results obtained with the different genotyping methods, enabling integration of multiple data sets to infer the population structure of the species [26, 33, 35] (Fig. 2a, b). Individual *K. kingae* strains are characterized by distinct combinations of PFGE typing profiles and consistent allele content of a variety of housekeeping genes, *rtx*A genes, and *por* genes, disregarding the associated clinical syndrome or the temporal or geographic source of the isolates [26, 33, 35]. For instance, all PFGE clone A isolates studied so far belong to MLST STc-34 and share *rtx*A allele 3 or 13 and the *por*4 allele; PFGE clone ψ isolates belong to MLST STc-29 and harbor the *rtx*A4 and *por*12 alleles; PFGE clone K isolates belong to MLST STc-6 and possess the closely related *rtx*A8 or *rtx*A9 allele and the *por*1 allele [26, 33, 35].

It should be noted that the diverse genotyping methods probe different sections of the bacterial chromosome. The PFGE method cuts the entire genome into 10–20 DNA pieces, enabling the analysis of approximately 90 % of the entire genome content [36, 37]. MLST explores 6 core genes that are responsible for key metabolic

Fig. 2 Minimum spanning tree analysis, using BioNumerics version 7.1, of 324 *Kingella kingae* ▶ isolates based on allelic profiles of 6 housekeeping genes. Each *circle* corresponds to a sequence type (ST). The ST number is given beside the circle, and the size of the *circle* is related to the number of isolates found with that ST (from 1 for *small circles* [e.g., ST-1] to 60 [e.g., ST-6]). The color scheme represents the geographic origin (**a**) or the associated clinical syndrome (**b**). *Gray zones* surrounding groups of *circles* indicate that these STs belong to the same ST complex. Weight of the line joining two neighboring STs indicates the number of differing alleles. Each major ST complex (STc) mainly associated with either invasive infection (*blue font*) or commensalism (*pink font*) is indicated by a legend with STc number and *within parentheses* the associated PFGE clones (*capital letters*) and *rtx*A allele numbers. STcs composed of a majority of β-lactamase producers are marked bla_{TEM}. *: Type strain of *K. kingae* (ATCC 23330) sole isolate of ST-1. OAI: osteoarticular infection


(a)
n Country
164 Israel
68 France
33 Spain
24 Canada
16 USA
14 Iceland
3 Norway
1 Australia
1 Russia
number of mismatches
1
2
3
4
≥5
STc-1 (D rtx1/16)
STc-35 (N rtx6)
STc-23 (B/P/MN rtx1/2/12)
STc-11 (S/J rtx18)
STc-34 (A rtx3/13)
bla TEM
STc-14 (H rtx14)
STc-29 (Ψ rtx4)
STc-25 (PP rtx1)
STc-3 (F rtx14) bla TEM
STc-6 (K rtx8/9/10/11/15/19/27)


(b)
n Syndrome
153 OAI
106 Carriage
40 Bacteremia
13 Endocarditis
3 Soft tissue
9 Unknown
number of mismatches
1
2
3
4
≥5
STc-1 (D rtx1/16)
STc-23 (B/P/MN rtx1/2/12)
STc-34 (A rtx3/13)
bla TEM
STc-35 (N rtx6)
STc-11 (S/J rtx18)
STc-14 (H rtx14)
STc-29 (Ψ rtx4)
STc25 (PP rtx1)
STc-3 (F rtx14) bla TEM
STc-6 (K rtx8/9/10/11/15/19/27)

functions and thus evolve at a slow pace. Sequencing of *rtx*A and *por* investigates *K. kingae* genes that encode surface-exposed and virulence-associated factors, resulting in the immune selective pressure and frequent genetic change [38]. The remarkable linkage disequilibrium and genetic stability of the different *K. kingae* strains over time and space revealed by the different typing methods indicate that they represent distinct and relatively homogeneous bacterial populations. The persistence of defined combinations of PFGE profiles, MLST genotypes, *por* alleles, and *rtx*A alleles despite the disruptive effect of horizontal gene transfer suggests that these fixed combinations are maintained because they amalgamate an array of advantageous traits. It is postulated that biologically successful *K. kingae* strains are subjected to positive selection that enables them to resist the disruptive effects of transformation. These highly fit strains experience clonal expansion, resulting in the wide geographic dispersion and persistence over prolonged periods, while less competitive strains do not spread and are promptly eradicated from the mucosal surfaces and purged from the human population [39].

Geographic Distribution

Although the five main STcs and many STs are internationally distributed, regional specificities are evident. For example, ST-25 is the most common ST in France and accounts for one-third of French invasive isolates but is rarely found outside of France, while most Spanish invasive isolates belong to ST-14. Both ST-25 and ST-14 are underrepresented in Israel [35]. Conversely, ST-6 is an invasive clone with a worldwide distribution.

Clonal Evolution

Some of the predominant STcs, such as STc-23 and STc-14, have been circulating since the 1960s and the 1970s. In contrast, other STcs such as STc-25 have only been isolated in recent years, suggesting that they are new and emerging clones [35]. *K. kingae* STcs also display different evolutionary histories. STc-6 is mainly composed of one ST (ST-6), distantly related to the rest of the population with few neighboring STs. As this intercontinental invasive ST is also frequently associated with oropharyngeal carriage [35] and has been associated with clusters of infections in daycare centers [23], it can be considered as a successful clone that has reached an optimal equilibrium between enhanced colonization fitness, high transmissibility, and virulence. In contrast, STc-23 and STc-25 are linked together by a complex net of multiple STs, suggesting a more chaotic evolution, and the ST-25 clonal group has probably emerged from the STc-23 clonal group in recent times [26] (Fig. 2a, b).

Population Structure and Virulence

Among the invasive isolates, five major clonal groups (identified by blue font in Fig. 2a, b) have been distinguished [26, 35]. While STc-23 and STc-25 appear linked, sharing the ST-62 clonal group, the other three predominant STcs (STc-6, STc-14, and STc-35) appear to be only distantly related. Interestingly, all outbreaks of *K. kingae* infections among daycare center attendees have been caused by strains belonging to four of these invasive clones, indicating that they are also associated with high transmissibility [23, 28, 29, 40–44]. Although pharyngeal isolates from asymptomatic children are also found among these five major groups, they generally belong to other clonal groups that are more rarely associated with invasive infections (in pink font in Fig. 2b) [11, 24, 35]. However, it is important to emphasize that this observation has the limitation that most commensal isolates studied were recovered from Israel only; accordingly, these findings should be confirmed by additional global research.

The noticeable virulence of certain *K. kingae* strains and their predilection for invading specific host tissues can be also inferred from the clusters of disease occurring in daycare facilities. Although bone infections represent less than 7 % of all cases of *K. kingae* disease [45], a ST-25 strain caused osteomyelitis in 4 of 5 attendees in a daycare center [40] and a later sporadic case [46] in Marseille, France, and a ST-6 strain caused osteomyelitis in a cluster of three children in an Israeli facility [41], suggesting that these strains have a high propensity for invading bone tissue. Of note, although certain *K. kingae* strains exhibit tissue tropism at the population level, all of the individual clinical syndromes can be caused by any of the invasive *K. kingae* clones (Fig. 2b).

Association with the RtxA Gene Alleles

The RTX toxin responsible for *K. kingae* β-hemolysis and broader cytotoxicity appears to be an important *K. kingae* virulence factor and is absent from other *Kingella* species [47]. The sequence encoding the *rtx*A gene is 72 % homologous with the hemolysin gene of *Moraxella bovis* and has a G + C % that is lower than the G + C % of the core *K. kingae* genome, suggesting that it has been horizontally acquired from a donor species. This gene displays a high degree of polymorphism, with more than 20 different alleles, making it a potential target for strain typing and suggesting that it is subject to high selective pressure by the immune system [26, 31]. Alleles of the *rtx*A gene have a close correlation with PFGE clones and MLST STcs (Fig. 2a, b), with the exception of a few alleles (*rtx*A1 and *rtx*A14) that are distributed among unrelated STcs [26]. This suggests that the *rtx*A gene has experienced rearrangements or horizontal DNA transfer between *K. kingae* strains belonging to different clonal groups. Interestingly, among the intercontinental ST-6 and the Israeli commensal ST-5 clonal groups, some *rtx*A alleles have a duplication

(in alleles 8, 10, 11, 17, 19, and 27) or a triplication (in allele 9) of a 33-bp sequence [26]. As the *rtx*A DNA sequences flanking the duplication or triplication sites differ between ST-6 and ST-5 strains, it appears that this particular trait has arisen through convergent evolution and probably not by horizontal gene transfer. Whether the duplication or triplication of a part of the gene encoding a virulence factor confers any selective advantage or translates into increased strain invasiveness remains to be determined.

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