**Investigation of *Kingella kingae* invasive infections outbreaks in day-care facilities: assessment of a rapid genotyping tool targeting the DNA-uptake-sequence**

**Philippe Bidet1, Violaine Tran Quang1, Pablo Yagusky2, André Birgy1, Stéphane Bonacorsi1, Romain Basmaci1**#

1IAME, UMR 1137, INSERM, Université Paris Diderot, Sorbonne Paris Cité, Service de Microbiologie, Hôpital Robert-Debré, AP-HP, Centre National de Référence associé *Escherichia coli*, Paris, France

2Clinical Microbiology Laboratory, Soroka University Medical Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Running Title: *K. kingae* infection clusters investigated by DUS PCR

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# Address correspondence to Romain Basmaci, romain.basmaci@aphp.fr romain.basmaci@aphp.fr

**Abstract**

*Objectives*. Outbreaks of *Kingella kingae* invasive infections have recently been reported in day-care centers. Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) revealed that although the invasive strains had a widespread dissemination in the day-care population, less virulent strains were also found to be circulating in the facility. However, these typing tools are costly, time-consuming and labor-intensive, and provide delayed results. A study was conducted to assess the performance of a rapid and cost-effective genotyping tool targeting theDNA uptake sequence (DUS) in the investigation of outbreaks of *K. kingae* disease.

*Methods*. DUS typing (DUST) patterns of each strain from 7 different clusters were compared to distinguish genotypically-linked strains from others. PFGE and, when available, MLST results were used as gold standard.

*Results*. DUST was assessed on 80 *K. kingae* isolates from: Nir-Itzhak (n=14); Tel-Nof (n=14); Palmahim (n=5); Umm-al-Fahm (n=7); Eilat (n = 8); Nevatim (n = 15); and Paris (n=17). A unique DUST pattern was involved in the Nir-Itzhak, Palmahim, Umm-al-Fahm and Paris episodes. Two DUST patterns were found in Eilat, whereas at least 3 were identified in the Tel-Nof and Nevatim episodes. In total, 11 (13.8%) children carried a *K. kingae* isolate that differed from the outbreak strain. These results were concordant with those obtained with the traditional PFGE and MLST methods.

*Conclusions*. DUST appears sensitive and specific to distinguish the invasive outbreak strain from others in asymptomatic carriers, and could be useful to limit the unnecessary exposure of the entire day-care population to selective antibiotic pressure.

**Introduction**

As the result of increasing use of improved detection methods, *Kingella kingae* is being increasingly recognized as an important pediatric pathogen and the most common etiology of skeletal system infections in children aged 6 to 48 months in countries where sensitive molecular diagnostic methods are routinely used for processing joint aspirates and bone exudates (1-3). The organism is carried in the oropharynx without clinical symptoms and is transmitted from child-to-child by close contact among siblings and playmates (4-7). The colonized mucosal surface is also the portal of entry of *K. kingae* to the bloodstream from which it may invade the skeletal system and the endocardium for which the species exhibits a particular tropism (8-10). Carried *K. kingae* strains differ in their virulence. Some strains belonging to a few distinct genotypic clones and characterized by polysaccharide capsules “a” or “b” are responsible for the vast majority of clinical diseases (11). On the other hand, other clones that elaborate capsule types “c” or “d” are usually found as mere respiratory colonizers, and are rarely isolated from invasive infections (11). The age-related prevalence of *K. kingae* colonization parallels that of invasive disease reaching 10-12% during the second year of life and decreasing in older children (4, 5). Similarly to other pathogens of respiratory origin, the colonization rate is substantially increased among children attending day-care facilities, and out-of-home care is significantly and independently associated with *K. kingae* carriage (6).

Although the majority of invasive *K. kingae* infections are sporadic, 11 outbreaks of invasive disease have been reported in US, French and Israeli day-care centers (12-21), as well as one still unpublished event in Spain (Varela Martínez C, personal communication). Clinical isolates from ill children and *K. kingae* organisms detected in the pharynx of attendees to the affected facility were characterized by the traditional pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) methods, and widespread dissemination of the invasive strain in the day-care population was usually demonstrated although, in some cases, other less virulent strains were also found to be circulating in the facility (17).

We have recently developed a PCR-based genotyping tool that targets *K. kingae* DNA uptake sequence (DUS) (10), using the sequence published by Frye et al. (22). Initial evaluation of the method demonstrated that it was reproducible and capable of detecting strains belonging to the same MLST sequence-type complex (STc) (10) at a cost of less than 5 USD per strain, and results were available on the same day (10). In the present study we employed the DUS typing (DUST) method in the molecular characterization of *K. kingae* organisms isolated in seven of the reported outbreaks of disease and compared the results with those originally obtained with the traditional PFGE and MLST genotyping schemes.

**Results**

We collected 80 *K. kingae* isolates from 7 outbreaks of invasive *K. kingae* infections detected in the following day-care facilities: Nir-Itzhak (Israel, 2014, n=14); Tel-Nof (Israel, 2005, n=14); Palmahim (Israel, 2014, n=5); Umm-al-Fahm (Israel, 2013, n=7); Eilat (Israel, 2012, n = 8); Nevatim (Israel, 2014, n = 15); and Paris (France, 2011, n=17) (Table 1). Clinical origins of the strains as well as the sample origin are summarized in Table 1.

Each outbreak was analyzed independently to determine if one or several DUST patterns could be identified. The different DUST patterns were named with the number of the STc exhibiting the same pattern, if applicable, or with a lower case letter followed by a number if not (see Methods). Overall, the DUST pattern assignments for each strain of each cluster were identical between the 4 authors.

Among the 7 clusters of *K. kingae* invasive infections, DUST allowed to rapidly determine that a unique clone was involved in 4 episodes (Nir-Itzhak, Palmahim, Umm-al-Fahm and Paris; Table 1 and Figure 1) and the STc involved in each one of these outbreaks could be accurately inferred based on the DUST patterns. Strains from Nir-Itzhak and Umm-al-Fahm outbreaks seemed to be related to STc-6, those from the Paris cluster to STc-23/25, and the DUST-p1 pattern obtained for the Palmahim strains appeared close to STc-6. These results are concordant with those obtained with PFGE and MLST (Table 1) (14, 20).

Of interest, 2 distinct DUST patterns were identified in the Eilat outbreak (Table 1, Figure 2). The 2 affected children, as well as 3 healthy carriers, exhibited the DUST-6 pattern and belonged to STc-6, while 3 other attendees carried a different isolate, DUST-e1, belonging to ST-8 (not including in a major STc) (Table 1).

The 2 other outbreaks (Tel-Nof and Nevatim) exhibited the coexistence of several different *K. kingae* genotypes. In the Tel-Nof cluster, 3 different DUST patterns (DUST-6, DUST-14, and DUST-23/25) could be identified (Figure 3). Of interest, MLST results were available for 2 strains and were concordant with those obtained by DUST (Table 1). Overall, PFGE identified 4 different clones in this cluster (TNA, TNB, TNC and TND clones). Isolates belonging to TNA and TNB clones were indistinguishable by DUST, while PFGE clone TNC was associated with DUST-23/25 and clone TND with DUST-14 (Table 1). Finally, the invasive Tel-Nof strain, which belonged to DUST-6 (lane 14), was also present in 11 asymptomatic contacts.

Concerning the Nevatim outbreak, due to the high number of available strains (n=15) and high number of potential different patterns (Figure 4A), we decided to confirm our DUST prognoses with Bionumerics software in a single gel electrophoresis step. Thus, we excluded 4 strains (Figure 4A; lanes 4, 7, 9 and 11) exhibiting obviously the same DUST pattern than 5 other strains that we selected (Figure 4A; lines 5, 6, 8, 10 and 14), in addition with the 6 remaining strains, for a new migration. This new migration suggested the coexistence of 5 different DUST patterns (Table 1) that was confirmed by the dendrogram graph analysis (Figure 4B). While 2 strains exhibited a DUST-6 pattern, the 4 other patterns could not be clearly affiliated to a major DUST profile. However, DUST-n2 (n=9, PFGE H) was close to DUST-14, that was concordant with the fact that clone H is known to be associated with STc-14 (9). Finally, DUST was able to determine that the invasive strain responsible for the clinical cases in Nevatim (DUST-n2, lanes 9 and 10) was also asymptomatically carried by 7 children, whereas 6 other children carried a different strain.

**Discussion**

In recent years, the development of genotypic tools has made a profound impact on the understanding of the epidemiology of infectious diseases. The ability to discriminate between subpopulations of the same species enables the study of the virulence and other biological properties of individual bacterial strains and to firmly establish chains of transmission of communicable diseases in the context of outbreak investigations (23).

Since the first cluster of *K. kingae* osteoarthritis was detected in a Minnesota day-care center in 2005, similar outbreaks are being increasingly reported (12-21). These events are characterized by a sudden onset and simultaneous or successive occurrence of multiple cases of invasive disease, including skeletal infections, meningitis, and fatal endocarditis, within less than one month. The alarming severity of these infections and the high attack rate, indicated enhanced transmission of a particularly virulent strain and prompted a meticulous epidemiological investigation employing PFGE (24) or MLST typing (10, 25). These investigations revealed that the outbreaks were usually caused by organisms belonging to the highly virulent MLST STc-6/PFGE clone K or to its E and c variants (n=45; 56.3%). These *K. kingae* strains also exhibited enhanced colonization fitness and transmissibility and, once introduced in a crowded day-care center attended by susceptible young children, easily disseminated, colonizing multiple attendees as exemplified by the Palmahim, Nir Itzhak, and Umm-al-Fahm outbreaks. The investigations also confirmed that *K. kingae* colonization is a dynamic process at the individual and population levels, as previously suggested (26). Indeed, although the concomitant colonization by multiple *K. kingae* clones has not yet been fully explored, the re-isolation of a *K. kingae* clone that was previously carried and lost is uncommon (26). This pattern, instead of a random temporal distribution of genotypes, suggested clearance or at least quantitative reduction in the colonizing density over time. New strains are repeatedly introduced in the facility, gradually displacing “old strains” after weeks or months and, thus, at any given time, a variety of *K. kingae* strains exhibiting different levels of virulence may be found circulating in the day-care centers, as shown in the Nevatim, Tel-Nof and Eilat outbreaks.

The two traditional typing methods employed in the original investigations of these outbreaks (PFGE and MLST) have the drawbacks of being costly, labor-intensive, and results are delayed by several days. In addition, the MLST method, which is based on sequencing of housekeeping genes that encode crucial metabolic functions and, thus, evolve at a slow pace, is not sensitive enough to detect subtle genomic changes that alter virulence and the recent emergence of new strains (23). While awaiting for an inexpensive and definitive whole-genome sequencing typing tool, we developed a promising *K. kingae*-specific typing method that enables characterization of strains and comparison of multiple isolates in a single PCR run. Similar to other members of the *Neisseriaceae* family, *K. kingae* is naturally transformable and the uptake of exogenous DNA is finely regulated by DUS, which facilitate incorporation of homologous DNA and discriminate against horizontal transfer of heterologous and possibly harmful genomic sequences (22). Given that DUS should be randomly repeated, on average, every 500 to 1,000 bp in the 2 Mb *K. kingae* genome (10, 22, 27, 28) with a variable chromosomal location between the strains, a PCR assay that targets this highly ubiquitous sequence and amplifies sequences located between two consecutive DUS, produces multiple DNA bands that differ in length and, thus, can be separated in an electrophoretic gel. It is suggested that a similar strategy based on DUS could be also developed for rapid typing the other pathogenic members of the *Neisseriaceae* family such as *N. meningitidis* and *N. gonorrhoeae.*

After the development of the DUST method, the demonstration of its reproducibility in different experiments using the same strain, and its initial evaluation with a limited number of strains chosen to belong to the major STcs (10), the present is the first large-scale assessment of the performance of the method in a blind investigation of actual *K. kingae* outbreaks of invasive disease. Our results were remarkably congruent with those obtained with PFGE and MLST, strongly suggesting that DUST could be useful as a first-line genotyping method, enabling to discriminate epidemiologically linked strains. Despite the fact that results depend on visual inspection of the electrophoretic gels, as the gold-standard PFGE method but in contrast to the robust sequence analysis by MLST, the results were easy to interpret with an excellent inter-observer concordance by four different investigators, and the use of an expensive bioinformatics software appeared dispensable. This method appears accurate to identify asymptomatic day-care carriers of a potentially invasive strain and, therefore, at an increased risk for developing clinical disease. Carriers thus identified can be offered prophylactic antibiotics to avoid occurrence of additional cases of clinical disease, while children carrying other strains could be dispensed with them. This strategy could have reduced the antibiotic prescription in 3/6 (50%), 2/13 (15.4%) and 6/13 (46.2%) healthy contacts in Eilat, Tel-Nof and Nevatim clusters, respectively.

However, as for traditional genotyping methods (PFGE and MLST), DUST needs to obtain the culture of the isolates. Since the first description of a specific medium for primary isolation of *K. kingae* from upper respiratory tract specimens (29), a more simple medium was described based on Columbia blood agar with added vancomycin (2mg/L) (30), allowing to isolate *K. kingae* with a up to 85% sensitivity (31). Once the isolates are obtained, DUST results could be available on the same day, in contrast to PFGE or MLST. However, because the sensitivity of the pharyngeal culture to detect *K. kingae* is suboptimal, molecular detection methods appear to be necessary to conduct a thoughtful investigation of clusters of invasive disease in day-care facilities, as recently published (19). Antibiotic prophylaxis could be restricted to classmates with a positive culture and carrying the outbreak strain, as rapidly determined by DUST, and if no positive culture results were obtained antimicrobial therapy could be limited to day-care center attendees with a positive species-specific real-time PCR assay, and withheld from those with negative PCR results or carrying other strains.

In conclusion, the DUS typing procedure timely and accurately differentiate carriers of *K. kingae* strains responsible for clinical disease from attendees colonized by strains of lesser invasiveness which may be circulating among the day-care population, enabling rapid completion of the epidemiological investigation of clusters of infections and limiting the unnecessary exposure of all day-care attendees to selective antibiotic pressure.

**Materials and methods**

*Bacterial strains*

All the bacterial strains involved in the Israeli and the Parisian *K. kingae* invasive infection outbreaks, published to date, were included in the study. Bacterial strains were isolated either from a sterile site (e.g. blood, bone sample) or from the pharynx of the patients or from the pharyngeal specimen of asymptomatic close contacts, as previously described (1, 29, 30, 32). Israeli strains were sent to Paris for further examination and were stored at -80° after subculture. DNA was extracted from specimens and stored at -80°C, as previously described (25).

*DNA uptake sequence typing*

The DUS typing (DUST) was performed for all strains in the Parisian center as previously described (10). Briefly, DNA PCR amplification was performed with the primer king3DUS (5’-AAGCAGCCTGCA-3’) (22) in a 50 µL reaction mixture that contained 25 µL of multiplex PCR master mix (Qiagen), 1 µL of primer stock solution (50 µM), and 2 µL of DNA (5 ng/µL) in optimal amplification conditions and annealing temperatures. Then the amplification products were separated by gel electrophoresis, stained with ethidium bromide and visualized under UV light. Each outbreak was separately studied, and all the strains belonging to each outbreak were concomitantly studied within the same DUS PCR experiment and migration gel. Patterns of the strains belonging to the same outbreak were visually compared to identify similar or different clones. Moreover, patterns were compared to the patterns of reference strains, belonging to the 4 major sequence type complexes (STcs), i.e. STc-6, STc-14, STc-23/25 and STc-35, in order to prognosticate which major STc each strain belongs to. Thus, we attributed a prognostic DUST pattern number (DUST-), related to the number of the corresponding STc, i.e. a strain exhibiting a similar DUST pattern than one of the STc-6 would be called DUST-6. If no similarity was observed, a lower case letter would be attributed, corresponding to the name of city where the outbreak occurred followed by a number (e.g. DUST-a1). Analysis was blindly performed by 4 different authors (PB, VTQ, SB, and RB); of note, the clinical origins of the strains, and, if available, their belonging ST and PFGE clone were not known by investigators. Knowing that each pattern can fairly associate one strain to one major STc, and that the reproducibility of the method was previously assessed (10), it was assumed that 2 isolates exhibiting the same DUST pattern were considered as identical.

In case of a complex outbreak, due to a large number of different DUST patterns, the initial hypothesis of genetic relationship between the strains was assessed by comparing fingerprint profiles using the curve based Pearson correlation (optimization, 0.5%; curve smoothing, 0%). A dendrogram was constructed by using the unweighted-pair group method using average linkages (UPGMA) method (branch quality, cophenetic correlation) with BioNumerics software (version 7.1; Applied-Maths, Belgium).

*Pulse field gel electrophoresis (PFGE)*

In order to evaluate the efficiency of the DUST method to identify similar and different clones, we used PFGE typing as a gold standard. Bacterial cells were harvested and chromosomal DNA was prepared as previously described (9). After restriction with EagI (New England Biolabs, Inc, Ipswich, Massachusetts), DNA fragments were separated in a CHEF DRII PFGE system (Bio-Rad Laboratories, Hercules, California). PFGE clones of the Israeli strains were determined in Israel, while the PFGE analysis for the Parisian outbreak had been already performed (14).

*Multilocus Sequence Typing (MLST)*

Finally, the sequence types of the Parisian as well as for certain Israeli strains were available (10, 14, 20, 25), and were also used as comparator.

**Transparency Declaration**

The authors declare no conflicts of interest

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**Legend to Figures.**

**Figure 1. Gel electrophoresis of the PCR targeting the *Kingella kingae* DNA uptake sequence. Patterns were identical for each strain belonging to one cluster**

(A) Nir-Itzhak cluster; lane 1, KK447; lane 2, UT; lane 3, GA; lane 4, TG; lane 5, AT; lane 6, DY; lane 7, GN; lane 8, NAG; lane 9, CN; lane 10, MA; lane 11, NA; lane 12, SN; lane 13, DS; lane 14 FL.

(B) Palmahim cluster; lane 1, Pal 4; lane 2, Pal 5; lane 3, Pal 6; lane 4, Pal 9; lane 5, Pal 10.

(C) Umm-al-Fahm cluster; lane 1, KK438; lane 2, KK439; lane 3, OEF1; lane 4, OEF7; lane 5, OEF10; lane 6, OEF11; lane 7, OEF12.

(D) Paris cluster; lane 1, STF12A; lane 2, STF13A; lane 3, STF13B; lane 4, STF14B; lane 5, STF15A; lane 6, STF15B; lane 7, STF16A; lane 8, STF16B; lane 9, STF19B; lane10, STF2B; lane 11, STF35B; lane 12, STF3B; lane 13, STF41B; lane 14, STF4A; lane 15, STF5A; lane 16, STF6B; lane 17, STF7B.

Lad, DNA ladder.

**Figure 2. Gel electrophoresis of the PCR targeting the *Kingella kingae* DNA uptake sequence in the strains of the Eilat outbreak**

Lane 1, Pan 24.9; lane 2, Pan 14.10; lane 3, Nam 29.9; lane 4, Nam 14.10; lane 5, Bit 24.9; lane 6, Ben 24.9; lane 7, Oha 24.9 ph; lane 8, KK431; Lad, DNA ladder; \*, DUST-6 pattern; \*\*, DUST-e1 pattern

**Figure 3. Gel electrophoresis of the PCR targeting the *Kingella kingae* DNA uptake sequence in the strains of the Tel-Nof outbreak**

Lane 1, P8; lane 2, P14; lane 3, P5; lane 4, P12; lane 5, P13a; lane 6, Ofi H53; lane 7, O5a; lane 8, O8a; lane 9, O8b; lane10, O6a; lane 11, O6b; lane 12, O9; lane 13, KK224; lane 14, KK223; Lad, DNA ladder; line above lane number, DUST-6 pattern; \*, DUST-23/25 pattern; \*\*, DUST-14 pattern

**Figure 4. Investigation of the Nevatim cluster using the DNA uptake sequence typing method by gel electrophoresis (A) and a dendrogram constructed by using the unweighted-pair group method using average linkages (UPGMA) method with BioNumerics software (B)**

Lane 1, MANE; lane 2, HRNE; lane 3, BRNE; lane 4, SENE; lane 5, BSNE; lane 6, ANNE; lane 7, LDNE; lane 8, GONE; lane 9, YNNE; lane10, HENE; lane 11, ZSNE; lane 12, SSNE; lane 13, NTNE; lane 14, AJNE; lane 15, MONE; Lad, DNA ladder; \*, DUST-6 pattern; †, DUST-n1 pattern; line above lane number, DUST-n2 pattern; ‡, DUST-n3 pattern; \*\*, DUST-n4 pattern; vertical dashed black line (B) represents 99% of similarity between patterns

**Table 1. Demographic, clinical origin and genotyping data of the strains isolated during *Kingella kingae* invasive infections outbreaks**

PFGE, pulsed-field gel electrophoresis; ST, sequence type; STc, sequence type complex; DUST, DNA uptake sequence typing.

\*DUST-p1 was close to STc-6; †DUST-n2 was close to STc-14; ‡DUST-n1, -n3, and –n4 were close to DUST-23/25.