Genotyping of Invasive *Kingella kingae* Isolates Reveals Predominant Clones and Association With Specific Clinical Syndromes

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**Background.** Despite the increasing recognition of *Kingella kingae* as an important pathogen of early childhood, the relative frequency and invasiveness of different strains of the organism has not been investigated. A study was conducted to determine the association of *K. kingae* genotypes with specific clinical syndromes and the temporal and geographic distribution of invasive clones.

**Methods.** A collection of 181 invasive *K. kingae* strains, isolated between 1991 and 2012 from Israeli patients with bacteremia, skeletal system infections, or endocarditis, were typed by pulsed-field gel electrophoresis (PFGE). In addition, the correspondence between PFGE, multilocus sequence types (MLSTs), and *rtxA* gene sequencing results was also examined for organisms belonging to the predominant PFGE clones isolated from asymptomatic carriers and patients with invasive infections.

**Results.** A total of 32 different *K. kingae* clones were identified by PFGE, of which 5 (B, H, K, N, and P) caused 72.9% of all invasive infections, and were recovered during the 21-year period from different regions of the country. Clone K was significantly associated with bacteremia, clone N with skeletal system infections, and clone P with bacterial endocarditis. Strains belonging to the same PFGE clone, either carried asymptotically or causing different invasive infections, shared MLST complexes and exhibited identical or closely related *rtxA* alleles.

**Conclusions.** Although *K. kingae* exhibits noteworthy genetic heterogeneity, a limited number of distinct clones cause the majority of invasive infections in Israel, exhibiting genetic stability, long-term persistence, and wide geographic dispersal. *K. kingae* strains also show significant association with specific clinical syndromes.

As the result of inoculation of synovial fluid and bone exudates from children with arthritis into blood culture vials and use of nucleic acid amplification methods, *Kingella kingae* is increasingly being recognized as a common etiology of bacteremia, septic arthritis, and osteomyelitis in children aged <3 years [1–7]. Less frequently, the organism causes endocarditis, spondylodiscitis, and meningitis in pediatric and adult patients [8].

*K. kingae* is carried asymptptomatically in the pharynx of young children, and this phenomenon has important implications for organism transmission and the pathogenesis of invasive disease [9]. Research has shown that the bacterium spreads from child to child through close contact within families and among day care attendees [10–13]. In addition, in patients with *K. kingae* disease, genotypically identical isolates are recovered from the pharynx and the bloodstream, supporting the concept that upper respiratory tract colonization represents an essential step in the pathogenesis of invasive infections [14, 15]. It is currently unknown, however, whether all *K. kingae* strains have similar capability of causing human disease and whether certain clones are associated with particular clinical syndromes.

A large collection of invasive *K. kingae* organisms, isolated in different regions of Israel over a 21-year period,
was studied to investigate the frequency of the different *K. kingae* clones, their distribution by time and place of isolation, and their potential association with specific clinical diseases.

**MATERIALS AND METHODS**

**Source of Invasive *K. kingae* Strains**

The Clinical Microbiology Laboratory of the Soroka University Medical Center (CML-SUMC), located in southern Israel, has been a pioneer in the use of the blood culture vial method for culturing synovial fluid exudates from children with presumptive septic arthritis, resulting in the diagnosis of >130 invasive *K. kingae* infections since 1988 [1, 2]. Over the years, this bacteriological practice has been gradually adopted in other Israeli hospitals, improving the culture detection of the organism [2]. Because the CML-SUMC serves as the national reference center for identifying *K. kingae* organisms, a large number of invasive isolates from Israeli patients from other regions of the country were also available for typing.

**Patient Data**

Demographic and clinical data of patients with invasive *K. kingae* disease, defined as isolation of the organism from a normally sterile body site, have been prospectively collected at the CML-SUMC since the late 1980s. Laboratory records were consulted, and data on isolation date and patients’ clinical syndrome were extracted. This information was also available for the majority of isolates derived from other regions of the country. Clinical syndromes were divided into 3 mutually exclusive categories: (1) bacteremia (including bacteremia with no focus and bacteremia associated with respiratory symptoms but no focal infection involving joints, bones, tendons, or the endocardium), (2) skeletal system infections (including osteomyelitis, septic arthritis, abortive osteoarthritis [8], and tenosynovitis), and (3) endocarditis.

**Source of Colonizing *K. kingae* Strains**

To investigate the relationship between organisms that colonize the respiratory tract and those isolated from patients with invasive disease, a collection of >450 strains isolated from healthy carriers, already characterized by pulsed-field gel electrophoresis (PFGE), was available. These organisms were gathered in different studies on the carriage of *K. kingae* conducted among children living in southern and central Israel during the last 2 decades. Details of the population from which these strains derived and the culture protocols used are provided elsewhere [9–11, 13].

**Identification of *K. kingae***

Isolates were identified on the basis of the typical morphologic and cultural characteristics of the species [16] and confirmed by the API NH kit (bioMérieux, Marcy-l’Etoile, France). Isolates were kept frozen at −70°C in a 15% glycerol-containing broth until further testing.

**PFGE Analysis**

Chromosomal DNA of *K. kingae* isolates was purified by the method of Maslow et al [17]. Enzymatic digestion was performed with * Eagl* according to the manufacturers’ guidelines (New England Biolabs, Ipswich, MA), and the resulting restriction fragments were separated in a counterclamped homogenous electric field CHEF DRIII apparatus (Bio-Rad Laboratories, Hercules, CA). Electrophoretic conditions used were 6 V/cm, 14°C, ramp 5–35 seconds, and 23 hours. Restriction patterns were visualized by ethidium bromide fluorescence and photographed. An American Type Culture Collection *K. kingae* organism (ATCC 23330), characterized by genotyping methods in previous studies [11–14], was used as the reference strain.

**Interpretation of PFGE Results**

To estimate the genetic relatedness among strains, restriction patterns were interpreted according to the criteria proposed by Tenover et al [18]. Isolates exhibiting similar (ie, indistinguishable and closely related) PFGE profiles were considered to belong to the same clone.

**MLST and rtxA Gene Sequencing Typing**

A random sample of *K. kingae* isolates belonging to the predominant PFGE clones, derived from patients with a variety of invasive diseases and from healthy carriers, was further typed by multilocus sequence type (MLST) and rtxA gene sequencing. The sequences of the 6 housekeeping genes *abcZ, adk, aroE, cpn60, gdh,* and *recA* used for MLST, as well as those of the rtxA gene, have been described elsewhere [7, 19]. A different allele number was given to each distinct sequence within a locus, and a sequence type (ST) number was attributed to each distinct alleles combination. Isolates were grouped into ST complexes (STCs) if they differed at no more than 1 locus from at least one other member of the group. The ST of the examined strains is available on the Pasteur Institute of Paris Web site [http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kingellakingae.html](http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kingellakingae.html) and rtxA alleles 1, 6, 8, 9, 10, 12, 14, and 19 are deposited in Genbank under the accession numbers JQ340459, JQ340464, JQ340466, QJ340467, JQ340468, JQ340470, JQ340472, and JQ801376, respectively.

**Statistical Analysis**

To assess whether some PFGE clones were overrepresented in the population (ie, whether the number of isolates found to belong to a given clone significantly differed from what should have been expected from a random distribution), the observed/expected ratio for each clone was calculated using the $\chi^2$ goodness-of-fit test. The expected number of isolates was
Table 1. Distribution of 3 Predominant *Kingella kingae* Clones, by Clinical Syndrome, and Statistical Significance of Their Association With Specific Diseases

<table>
<thead>
<tr>
<th>PFGE clone</th>
<th>Bacteremia (n = 69)</th>
<th>Skeletal Infection (n = 97)</th>
<th>Endocarditis (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K (n = 50)</td>
<td>28</td>
<td>.0041</td>
<td>NS</td>
</tr>
<tr>
<td>N (n = 23)</td>
<td>4</td>
<td>.0302</td>
<td>NS</td>
</tr>
<tr>
<td>P (n = 8)</td>
<td>3</td>
<td>NS</td>
<td>.0022</td>
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</table>

Abbreviations: CI, confidence interval; NS, not significant; OR, odds ratio; PFGE, pulsed-field gel electrophoresis.

the average resulting from the division of the total number of isolates by the number of different PFGE clones identified, and the observed number was the actual number of isolates found to belong to a given clone.

The distribution of the different PFGE clones by period of isolation (before or after 1 January 2001), geographic location (southern, central, eastern, or northern Israel), and clinical syndrome (bacteremia, skeletal system infection, or endocarditis) was also determined. The χ² test was used to assess the statistical significance between proportions. A P value of < .05 was considered statistically significant for all calculations.

**Estimation of Site-Specific Invasiveness of *K. kingae* Clones**

The potential of individual *K. kingae* clones to cause skeletal system infections was assessed using the method described by Brueggemann et al [20], with appropriate modifications. An empirical odds ratio (OR) was calculated for the most common invasive *K. kingae* clones by reference to all the other clones as follows: OR for clone π = (ad)/(bc), where a is the number of isolates belonging to clone π causing skeletal infections, b is the number of clone π isolates causing other syndromes, c is the number of nonclone π isolates causing skeletal system infections, and d is the number of nonclone π isolates causing other syndromes. For instance, an OR >1 indicated an increased probability for a given clone to cause skeletal system infection, and an OR <1 indicated a reduced probability for the clone to invade skeletal tissues. By use of a similar method, the ORs for the association of invasive clones with bacteremia or endocarditis were also calculated. We computed 95% confidence intervals (CIs) by means of MedCalc software, version 12.1.1 (Mariakerke, Belgium). When the 95% CI did not include the unity, the observed OR was considered statistically significant. For purposes of the calculation, isolates for which the associated clinical syndrome was unknown were ignored.

**RESULTS**

A total of 181 invasive *K. kingae* strains isolated between 1991 and 2012 could be retrieved and studied. One hundred and thirteen strains (62.4%) were isolated in southern Israel, 22 (12.1%) in central Israel, 22 (12.1%) in eastern Israel, and 24 (13.3%) in the northern region (Supplementary Table 1). Sixty-nine strains (38.1%) were isolated from children with bacteremia, 97 (53.6%) were derived from children with skeletal system infections, and 11 (6.1%) were from adult and pediatric patients with endocarditis. The associated clinical disease was unknown for 4 isolates (2.2%). Overall, 32 distinct PFGE clones were identified, of which 18 (56.3%) were found in multiple patients and 14 were unique. The 5 predominant clones, namely B, H, K, N, and P, collectively comprised 132 (72.9%) isolates. Clone B was found in 21 (11.6%) isolates, clone H in 27 (14.9%), clone K in 51 (28.2%), clone N in 25 (13.8%), and clone P in 8 (4.4%). The overrepresentation of clones B, H, K, and N reached high statistical significance (P < .005).

Clone N represented 18 of 77 (23.4%) invasive *K. kingae* organisms isolated in Israel between 1991 and 2000, but only 7 of 104 (6.7%) isolated in the 2001–2012 period (P = .003). No significant differences in the temporal distribution of other clones were detected, and no geographic clustering of clones was observed.

Three clones, namely K, N, and P, were significantly associated with specific clinical syndromes (Table 1). Clone K isolates were positively associated with bacteremia and negatively with skeletal system invasion, the opposite trend was observed for clone N organisms, and clone P was strongly associated with bacterial endocarditis. The remaining predominant clones B and H did not show significant association with any particular clinical syndrome (data not shown). The 4 isolates for which the associated clinical syndrome was unknown belonged to clones H (n = 1), K (n = 1), and N (n = 2).

Table 2 shows the association of the predominant PFGE clones with MLST ST and STCs and with rtxA gene sequencing. Results exhibited remarkable congruence between the 3 different typing methods, disregarding the clinical source (asymptomatic carriage or different invasive infections), and time or place of isolation. Strains belonging to the same PFGE clone shared MLST STCs and showed identical or closely related rtxA gene alleles. A single exception was observed, with isolate KK180 (ST 10) belonging to PFGE clone N.
However, it should be noted that ST10 is closely related to STC 35 [19], and therefore this minor discrepancy did not alter the global congruence between PFGE and MLST.

Of note, STs allowed us to distinguish several subclones. For example, among the 10 typeable clone H strains, 6 different STs were observed (Table 2). Moreover, although STC 23 was shared by clones B and P, MLST showed that each PFGE

<table>
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<tr>
<th>PFGE clone</th>
<th>Healthy Carriers</th>
<th>Invasive Disease</th>
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<tr>
<td></td>
<td>Strain (Time, Region)</td>
<td>St</td>
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<tr>
<td>B</td>
<td>BB728 (2006, S)</td>
<td>22</td>
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<td></td>
<td>C1563 (2006, S)</td>
<td>22</td>
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<td>C1724 (2007, S)</td>
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<td>H</td>
<td>KK12 (1994, S)</td>
<td>14</td>
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<td></td>
<td>PV1572 (2006, S)</td>
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<td></td>
<td>KK156 (2002, S)</td>
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Abbreviations: C, central Israel; E, eastern Israel; MLST, multilocus sequence type; N, northern Israel; PFGE, pulsed-field gel electrophoresis; S, southern Israel; ST, sequence type; STC, sequence type complex; UT, untypeable.
clone was characterized by different STs. Interestingly, these closely related clones harbored 2 distantly related rtxA alleles (allele 1 in clone B strains and allele 12 in most clone P strains) [19].

**DISCUSSION**

The results of the present study demonstrate that, although *K. kingae* organisms causing a variety of invasive diseases in Israel show considerable genetic diversity, some clones are significantly overrepresented in the sample and exhibit wide geographic dissemination, as well as temporal stability.

Because MLST examines relatively conserved portions of the core genome that encode central metabolic enzymes, PFGE makes a random exploration of the whole genome affecting core and accessory genes, and *rtxA* sequencing studies a putative virulence factor, it should have been expected to find considerable dissociation between results obtained by these 3 different typing methods [21]. In addition, the sequences examined by MLST analysis evolve at a slow pace, whereas accessory genes encoding virulence factors and surface-exposed antigens are subjected to high selective pressure and, thus, tend to change rapidly to escape the immune response. Having said that, the remarkable congruence of multiple typing results is striking and implies that the predominant *K. kingae* clones comprise distinct populations of genetically homogeneous bacteria.

It might be suggested that these predominant clones have emerged recently because the different accumulation pace of allelic diversity in core and accessory genes over a prolonged time should have resulted in gradual loss of typing results congruence [22]. However, the fact that the predominant clones have maintained an identical genetic configuration for the past 2 decades at least and disseminated countrywide does not support this possibility. Alternatively, the linkage disequilibrium observed might suggest that these strains exhibit a genomic profile that is strongly favored by selection, resisting the homogenizing effects of recombination and mutation. Enhanced colonization fitness would have resulted in clonal expansion, explaining the extensive circulation of epidemic clones among the Israeli pediatric population.

*K. kingae* PFGE clones A, C, G, J, M, R, T, and U, which collectively represented 38.8% of all strains recovered from 240 healthy carriers in a community survey [11], comprised only 4.4% of invasive isolates in the present study. This discrepancy suggests a trade-off between transmissibility and virulence because by invading deep organs, bacteria lose access to human body surfaces and, therefore, cannot propagate further. Moreover, sick individuals are isolated from other members of the community, are treated with antimicrobial drugs, and may even succumb to the disease. Obviously, all of these possible scenarios interrupt the chain of person-to-person transmission, resulting in extinction of the pathogen. Conversely, clone N, which was a common etiologic agent of invasive *K. kingae* disease in Israel in the 1990s, is almost never found as a pharyngeal colonizer in healthy children [11], was negatively associated with bacteremia, but showed significant affinity for bone and joint infections. These observations indicate that strains that show remarkable tissue invasiveness may be rapidly cleared from the respiratory tract and the bloodstream, implying that persistence in these niches may require a different biological specialization. Consistent with this concept is the observation that the organism was recovered from the blood from only 11 of 69 children (15.9%) with culture-proven *K. kingae* arthritis or osteomyelitis diagnosed in southern Israel over the years, suggesting that bacteria that invade skeletal tissues are not able to survive in the bloodstream for long (unpublished data). Clone *P* organisms, which are also exceptionally carried asymptomatically [11], were strongly associated with bacterial endocarditis but were rare in other infected sites.

Clone K organisms, on the other hand, appear to exhibit an optimal balance between transmissibility and invasiveness. This PFGE clone was the predominant strain detected as early as 1993 in a southern Israeli day care, lasting in the pharynx of colonized attendees for up to 4 months [10], and ranked second among strains carried by healthy Jewish children in a 2006–2007 study [11]. Clone K organisms, which represented 41.7% of all invasive strains isolated in southern Israel over the last 2 decades and were responsible for the excess of *K. kingae* morbidity observed in the Jewish population of the region [23], were also found to be frequent etiologic agents of childhood bacteremia countrywide in the present study. Interestingly, all clone K strains harbor a 33–base pair duplication or triplication of the *rtxA* sequence, which is exceptionally found in other clones [19]. Whether this repetition in a gene encoding for a putative virulence factor contributes to the epidemic success of the clone remains speculative.

The present results demonstrate that, although a variety of *K. kingae* strains circulate within the pediatric population of Israel, a small subset is responsible for most clinical infections, and a few exhibit significant association with particular clinical diseases. The study, however, has the limitation of being based on data originating from a single country and needs to be confirmed by additional research involving other populations. Determining which bacterial factors are most closely associated with colonization fitness, invasiveness, and proliferation in normally sterile environments such as the bloodstream and skeletal or endocardial tissues will require use of experimental animal models and whole-genome comparisons [19]. The present results, in which certain *K. kingae* clones were found at the population level to be mostly restricted to asymptomatic
carriage or associated with specific clinical syndromes, should help guide the design of such studies and the selection of strains to investigate [20, 24].

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We are grateful to Michael Friger, for his assistance with statistical analysis, and to the directors of the clinical microbiology laboratories of Israel, for providing their K. kingae isolates over the years.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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