ORIGINAL ARTICLE

Respiratory carriage of the novel *Kingella negevensis* species by young children

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Abstract

Kingella negevensis, a novel *Kingella* species implicated in a pediatric joint infection, has been recently characterized but its epidemiology remains largely unknown. The pharyngeal carriage of *K. negevensis* was studied by re-examining the results of a previous longitudinal study conducted in a cohort of healthy Israeli children from whom upper respiratory tract specimens were sequentially cultured between the ages of 2 and 36 months. Isolates were identified as *K. negevensis* by a species-specific nucleic amplification assay and genotyped by pulsed-field gel electrophoresis. β-lactamase production was determined by the nitrocephin test. *Kingella negevensis* was detected in 26 of 4,472 (0.58%) oropharyngeal cultures obtained from 24 of 716 children (3.35%) and was not isolated from any of 4,472 nasopharyngeal specimens. Following the first 6 months of life during which none of the children was colonized, the prevalence of carriage gradually increased reaching a peak of 1.09% at 24 months of age and decreased thereafter. *Kingella negevensis* strains showed genomic heterogeneity, and two clones represented 22 of 26 (84.62%) isolates. Twelve of the 26 (46.15%) isolates, belonging to two distinct clones, produced β-lactamase. *Kingella negevensis* shows remarkable similarities with *K. kingae* in terms of colonization site, age-related patterns of acquisition and carriage, and clonal distribution of β-lactamase production. Additional research is needed to investigate potential colonization sites of *K. negevensis* outside the respiratory tract, explore the mechanisms of pharyngeal colonization by the organism, and determine its role as an invasive human pathogen.

Keywords: β-Lactamase production, acquisition, genomics, *Kingella negevensis*, pharyngeal carriage, young children
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Article Summary Line: The novel *Kingella negevensis* species shows remarkable similarities with *Kingella kingae* in terms of colonization site, patterns of acquisition and carriage, and clonal distribution of β-lactamase production.

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Introduction

The genus *Kingella* in the family *Nesseriaceae* traditionally comprised four distinct species, of which three, namely *Kingella potus*, *Kingella denitrificans* and *Kingella oralis*, cause opportunistic infections in adults but are a rare aetiology in paediatric disease, and *Kingella kingae*, which is a common pathogen of young children and the prime cause of skeletal system infections between the ages of 6 and 48 months [1,2]. A decade ago, we conducted a longitudinal study to investigate the age-related carriage of *K. kingae* and other respiratory tract bacteria in the healthy paediatric population of the Negev desert region of southern Israel [3]. Pharyngeal specimens were plated onto a selective vancomycin-containing medium (BAV agar) and incubated in aerobic conditions to inhibit the competing Gram-positive and anaerobic flora, and facilitate the recognition of the haemolytic *K. kingae* colonies [2]. A few isolates showed an atypical phenotype consisting of long chains of coccobacilli, poor growth as pinpoint β-haemolytic colonies on blood-agar plates but excellent growth on GC-base medium, early autolysis and production of acid from glucose but not from maltose, and were initially considered as small-colony variants (SCV) of

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K. kingae [2]. In-depth analysis of the SCV isolates demonstrated that they belong to a novel *Kingella* species that shows a digital DNA–DNA hybridization of only 19.9% with *K. kingae* and which was recently named *K. negevensis* sp. nov. [4]. Although the clinical importance of this species is still unknown, the organism elaborates an RTX toxin identical to that produced by *K. kingae*, which is an important virulence factor in an infant rat model of invasive *K. kingae* infection [5–7]. Recently, *K. negevensis*-specific DNA sequences have been detected in the joint aspirate of an 8-month-old infant with culture-negative septic arthritis, suggesting that, similar to *K. kingae*, *K. negevensis* could be an invasive pathogen of the skeletal system in early childhood [5].

The unexpected discovery of the novel species prompted us to re-examine the results of the original study [3] to investigate the colonization niche of *K. negevensis* in the upper respiratory tract and the age-related acquisition and prevalence of the organism in the healthy young paediatric population.

Materials and methods

The details of the original study have been published elsewhere [3]. In brief, after obtaining written parental consent, a cohort of 716 healthy children was gradually enrolled over a 12-month period starting on August 2005. Oropharyngeal and nasopharyngeal specimens were obtained at scheduled visits at 2, 4, 6, 7, 12, 13, 18, 19, 24 and 30 months of age, and plated on BAV agar to isolate *K. kingae*. The study was approved by the Ethics Committee of the Soroka University Medical Centre, Beer-Sheva, as well as by the Israel Ministry of Health [3]. By the time the first specimens were processed for *K. kingae* detection, the study had already been running for almost a year and, therefore, many samples obtained during the first visits were not examined for the presence of the organism.

As many presumptive *Kingella* colonies as possible were collected from the primary plates and isolated separately, and particular efforts were made to harvest colonies exhibiting dissimilar morphologies. All isolates exhibiting the SCV phenotype detected in the original investigation were kept frozen at −70°C in a 15% glycerol-containing medium and re-examined for the purposes of the present study. To confirm the *K. negevensis* identification of the SCV isolates, frozen vials were thawed, subcultured, and the recovered organisms were subjected to a species-specific quantitative PCR assay that targets the *K. negevensis groEL* gene [5].

Kingella negevensis isolates thus identified were genotyped by pulsed-field gel electrophoresis (PFGE) employing the *Eag*I restriction enzyme, as described elsewhere [3]. To estimate the genetic relatedness among strains, PFGE restriction patterns were interpreted according to the criteria proposed by Tenover et al. [8]. Isolates exhibiting similar (indistinguishable and closely related) profiles were considered to belong to the same clone. Isolates differing from each other by one to three DNA bands were considered to belong to different subclones within the same PFGE clone.

Production of β-lactamase by the isolates was determined by the nitrocephin test.

Statistical analysis

Proportions were compared using the chi-squared test. A p-value <0.05 was considered statistically significant for all calculations.

Results

Colonies exhibiting the SCV morphology were identified in 26 of 4472 (0.58%) oropharyngeal cultures obtained from 24 of 716 children (3.35%), but were not isolated from any of the 4472 nasopharyngeal specimens ($p < 0.01$). All 26 SCV isolates were confirmed as *K. negevensis* by the molecular assay. Two children were colonized by *K. negevensis* in two separate visits (one child at the ages of 12 and 24 months and the second child at 8 and 9 months). The point prevalence of *K. negevensis* in each visit is shown in Fig. 1. Because a single culture per child was obtained at each scheduled visit, the prevalence of positive cultures (no. of *K. negevensis* cultures/no. of cultures obtained in that particular visit) equals the age-related prevalence of the organism among the population of children at each visit (no. of *K. negevensis* carriers identified at a given visit/no. of children sampled in that visit).

None of the children was colonized in the first 6 months of life, the prevalence of carriage commenced in the second life

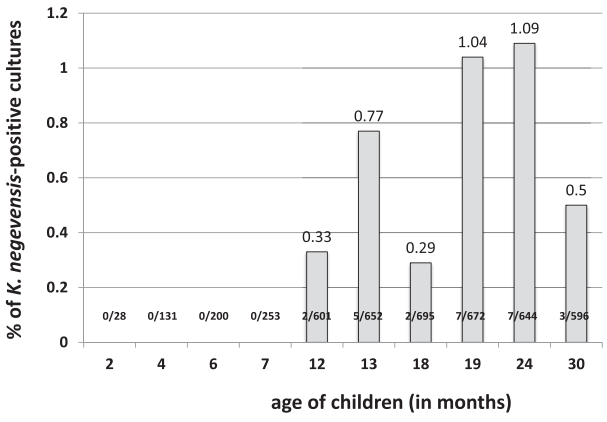

FIG. 1. Longitudinal detection of *Kingella negevensis* in oropharyngeal cultures.

TABLE 1. Genotyping results of 26 *Kingella negevensis* isolates derived from 24 oropharyngeal carriers

PFGE clones	Subclones	Isolates *n*	Children *n*
b	b1	5	5
	b2	4	4
	b3	1	1
	b4	2	2
T	T1	9	8
	T2	1	1
$unique_1$		1	1
$unique_2$		2	1
untypable		1	1
Total		26	24

semester, increased gradually in the second year, and decreased thereafter. By the age of 12 months, 2 of the 24 (8.33%) children had been colonized, and the number of carriers grew subsequently: 7 (29.17%) at 13 months of age, 9 (37.50%) at 18 months, 16 (66.67%) at 19 months, and by 24 months, 21 of 24 (87.50%) children had carried *K. negevensis* at least once. In the three remaining children the organism was first detected in the pharyngeal culture obtained at the age of 30 months.

Among the 24 colonized children, a total of four distinct PFGE clones were identified, whereas one *K. negevensis* strain, carried by a single child, could not be typed by the *Eag*I restriction enzyme. Two clones, namely b and T, predominated in the sample and were found in 12 (46.15%) and 10 (38.46%) of the 26 isolates, respectively (Table 1). The two children who were colonized twice, carried the same PFGE clone strain in the two separate visits (clone T_1 and $unique_2$ clones, respectively).

The two isolates belonging to the b_4 subclone, as well as all ten isolates belonging to clone T, produced β-lactamase.

Discussion

The results of the present study show that, similar to the other species of the genus *Kingella*, *K. negevensis* is a commensal member of the upper respiratory tract microbiota of healthy young children [2,3,9]. The habitat of the organism in the respiratory tract appears to be restricted to the oropharynx and, as observed in *K. kingae*, *K. negevensis* does not colonize the nasopharyngeal mucosa [2,9]. The carriage rate of *K. negevensis*, however, is comparatively low and roughly represents only one-tenth of that of *K. kingae* found in the same paediatric population [2,3,10]. It should be noted that in a recent publication, *K. negevensis* was isolated from a vaginal specimen of an adult patient with bacterial vaginosis [11], suggesting that, similar to other *Neisseriaceae*, the novel species could also colonize the genital mucosal surfaces [2].

Although the number of children sampled during the first months of life is too small to allow for definitive conclusions, it appears that, similar to other respiratory organisms, *K. negevensis* is not usually carried by infants younger than 6 months [12]. The rate of *K. negevensis* colonization substantially increases in the second life semester, reaching a zenith during the second year, and diminishes in older children. It is speculated that in early infancy, maternally derived antibodies protect the child from acquiring the bacterium and becoming colonized, whereas limited social mingling reduces the risk of contact with potential sources of transmission. Vanishing vertically acquired immunity with increasing age renders 12- to 24-month-old children susceptible to colonization, whereas growing social interaction and, especially, daycare facility attendance facilitate person-to-person acquisition of the organism by close contact. In older children, maturation of the immune system induced by cumulative exposure to *K. negevensis* and, possibly, to other related bacterial species, could result in the decreasing carriage rate observed at the age of 30 months.

The *K. negevensis* strains detected in the study exhibited substantial genomic heterogeneity, although two clones (T and b) showed clear predominance. Remarkably, β-lactamase production was detected in almost one-half of the isolates and was clonally distributed and restricted to two distinct PFGE subpopulations. It is suggested that β-lactamase resistance may confer a biological advantage to *K. negevensis* organisms carried by young children, coinciding with the period in life of highest antimicrobial drug exposure [13].

The present investigation has the limitation of being based on cultivation of a fastidious bacterial species and, hence, it is plausible that low concentrations of the organism on the pharyngeal epithelium could have been overlooked by the sampling and/or culture procedures. It is, then, possible that the detection of *K. negevensis* in the original oropharyngeal specimens could have been improved by subjecting them to a more sensitive molecular diagnostic method [5]. In addition, a substantial fraction of children missed the initial surveillance cultures and the population was sampled discontinuously, resulting in full 6-month gaps in the later stages of the study. Obtaining cultures at such prolonged intervals could have overlooked periods of short-term carriage and, therefore, the figures found in the present study should be considered only a minimum estimate.

In summary, the results of this pioneering investigation show that the novel *K. negevensis* species shares remarkable similarities with *K. kingae*, including colonization of the oropharyngeal niche, patterns of acquisition and carriage, as well as clonal distribution of β-lactamase production [2]. Additional studies are clearly needed to search for potential carriage sites of *K. negevensis* outside the respiratory tract, elucidate the mechanisms of mucosal colonization by the organism, and determine the role of the species as an invasive human pathogen.

Conflict of interest

The corresponding author declares no potential conflict of interest on behalf of his coauthors.

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