Respiratory Colonization by *Kingella kingae*, Person-to-Person Transmission, and Pathogenesis of Invasive Infection

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Abstract: Increasing recognition of *Kingella kingae* as an important pathogen of early childhood in recent years has elicited interest in the study of the asymptomatic carriage of the organism, its dissemination in the human population, and the role played by colonization of the upper respiratory tract in the pathogenesis of *K. kingae* invasion of the skeletal system and the endocardium.

Research has revealed that *K. kingae* is a frequent component of the normal oropharyngeal microbiota, disclosed the subtle molecular mechanisms responsible for adherence of the bacterium to the pharyngeal mucosa, and revealed the presence of a potent RTX toxin, probably implicated in breaching the epithelial barrier, survival of the organism in the bloodstream, and damage to bone and joint tissues. Epidemiological studies have shown that carriage of *K. kingae* peaks in 6-30 month-old children, coinciding with the age of increased susceptibility to invasive disease, and daycare-center attendance represent a significant risk factor for pharyngeal colonization. The organism is transmitted from person-to-person by close contact between family members, playmaytes, and day-care center attendees. Carriage is characterized by frequent turnover of colonizing strains, similar to what has been described in other pathogens of respiratory origin.

Keywords: Invasive disease, *Kingella kingae*, pharyngeal colonization, risk factors, transmission, pathogenesis.

INTRODUCTION

For more than the 5 decades elapsed since the first characterization of *Kingella kingae,* this fastidious gram-negative b-hemolytic coccobacillus was considered a rare cause of human disease, rarely isolated from patients with endocarditis or skeletal system infections [1]. In the late 1980s, the serendipitous discovery that inoculation of synovial fluid and bone exudates into blood culture vials improves detection of the organism, resulted in increasing recognition of *K. kingae* as an invasive pathogen of children aged 6 months to 3 years [2, 3]. In recent years, development of sensitive nucleic acid amplification assays firmly established the role of *K. kingae* in cases of "culture-negative septic arthritis" and demonstrated that the bacterium is the leading etiology of joint and bone infections and hematogenous spondylodiscitis below 4 years of age [4-6]. In addition to skeletal system diseases, the organism causes bacteremia with no focal infection, lower respiratory infection, acute bacterial endocarditis and, more rarely, meningitis, peritonitis, cellulitis, and a variety of ocular and soft tissue infections [1].

Studies conducted by our research group have revealed that *K. kingae* is a normal component of the pharyngeal microbiota of young children and the colonized respiratory epithelium is the portal of entry of the organism to the bloodstream from which it disseminates to normally-sterile body sites and particularly to bones, joints, and endocardium, and elucidated many epidemiological aspects of *K. kingae* carriage in the population [7, 8].

The present review summarizes our current knowledge on *K. kingae* colonization, the mechanisms of adherence of the bacterium to mucosal surfaces, the epidemiology and dynamics of carriage, and the role of asymptomatic colonization in the person-to-person transmission of the organism and pathogenesis of invasive infections.

KINGELLA KINGAE COLONIZATION SITE

Asymptomatic colonization of the upper respiratory tract is the common strategy shared by many potentially virulent bacterial pathogens such as pneumococci, meningococci or *Haemophilus influenzae* to establish a foothold on the body's mucosal surfaces, persist, and disseminate within the human population. Based on anecdotal isolation of *K. kingae* from patients with pneumonia and children with respiratory symptoms [7], and the observation that other members of the *Neissseriaceae* family of bacteria are upper respiratory tract commensals, it was presumed that the organism could also be part of the residing respiratory microbiota. In a pioneering study, bi-weekly nasopharyngeal and oropharyngeal cultures were obtained from young attendees to a day-care center facility in southern Israel over an 11-month period. A total of 109 of 624 (17.5%) oropharyngeal cultures grew *K. kingae*, but the organism was not recovered from any of the nasopharyngeal cultures [7]. These results have been confirmed in a recent study in which 4,472 oropharyngeal and nasopharyngeal specimens were prospectively obtained

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from a cohort of 716 healthy children. A total of 388 (8.7%) oropharyngeal cultures, but only a single nasopharyngeal culture grew *K. kingae*, indicating that the organism occupies a rather narrow niche in the upper respiratory tract [Amit U, Flaishmakher S, Dagan R, Porat N, Yagupsky P. Age-dependent carriage of *Kingella kingae* in young children and turnover of colonizing strains. J Pediatr Infect Dis Soc 2013, (in press)].

DETECTION OF *K. KINGAE* IN THE RESPIRATORY TRACT

Because of the high density of the resident bacterial flora and the relative slow growth of *K. kingae*, detection of the organism in the pharynx by culture on routine media is difficult. A selective medium consisting of trypticase agar with 5% sheep hemoglobin and 2 mg/mL of vancomycin (BAV medium) has been developed to improve recognition of the organism [9]. The rationale behind this formulation is to facilitate detection of β-hemolytic *K. kingae* organisms by reducing the presence of competitive gram-positive bacteria. In a blinded evaluation, the BAV plate detected 43 of 44 (97.7%) pharyngeal cultures positive for *K. kingae* whereas only 10 of 44 (22.7%) cultures were positive by the routine blood-agar medium *(P<0.001)*. The original BAV and similar selective media [10] have been successfully employed in many epidemiological studies and made a substantial contribution to our understanding of the carriage of the organism by the healthy children's population.

Nucleic Acid Amplification

In recent years, novel molecular detection essays have enabled detection of *K. kingae* infections in patients in which cultures of synovial fluid on routine and BAV media have not disclosed presence of the organism [4-6, 11]. It was natural that this sensitive approach was consequently adopted to study the colonization of the respiratory tract by *K. kingae* and its connection to invasive disease. The RTX toxin, a putative virulence factor, is produced by all *K. kingae* strains examined so far and, therefore, the encoding RTX locus genes appear as pertinent targets for molecular diagnosis of invasive infections and mucosal colonization by the organism [10-13]. The nucleic acid amplification assays developed to amplify conserved segments of the RTX toxin endoding genes *rtxA* and/or *rtxB* are able to detect 30 colony forming units (c.f.u.) of the organism, exhibiting a higher sensitivity than that of broad range 16S rRNA [12, 14] or the *cpn*60 gene PCR tests [13], are highly specific, can be applied to a variety of clinical specimens, and enable detection of strains exhibiting RTX locus polymorphisms [10-14].

Using a real-time PCR assay that targets the *rtxA* and *rtxB* genes, Ceroni *et al*. detected *K. kingae* DNA sequences in the pharynx of 8.1% of 431 young asymptomatic Swiss children and in all 27 patients with positive culture and/or PCR-assay for *K. kingae* from a normally sterile body site (blood, synovial fluid or bone specimen) [15]. In a second study by the same group comprising 123 patients aged 6-48 months with skeletal system complaints, in all 30 children with *K. kingae*-proven arthritis or osteomyelitis (diagnosed either by culture and/or PCR), the oropharyngeal specimen was also positive, while 8 pharyngeal samples derived from 84 patients with microbiologically unconfirmed joint or bone infections or with skeletal infections caused by other bacteria were also positive for *K. kingae* DNA, reflecting the background respiratory carriage of the organism in the young pediatric population [Ceroni D, Dubois-Ferriere, Cherkaoui A, *et al*. Detection of *Kingella kingae* osteoarticular infections in children by oropharyngeal swab PCR. Pediatrics (in press)]. These results imply that failure to detect *K. kingae*-specific genomic sequences in a pharyngeal specimen may practically exclude the organism as the etiology of a concomitant joint or bone infection. However, because *K. kingae* is a frequent resident of the mucosal surfaces of children in the relevant age group, the predictive value of a positive respiratory PCR assay is limited.

In a refined study, Basmaci *et al*. grew *K. kingae* in the pharyngeal cultures of 8 of 12 PCR-positive/culture-negative synovial fluid specimens of young children with suppurative arthritis [10]. They succeeded in extracting and sequencing the *rtxA* gene amplicons from 6 PCR-positive synovial fluid samples and compared them with those of the pharyngeal isolates. The 6 paired pharyngeal-synovial fluid amplicons were found to contain identical sequences, establishing a firm link between *K. kingae* organisms colonizing the pharynx and those invading the skeletal tissues [10].

More recently, the real-time PCR assay targeting the toxin-encoding *rtxB* gene was employed to assess the bacterial load of asymptomatic pharyngeal carriers and children in whom *K. kingae* arthritis or osteomyelitis was confirmed by a positive PCR test performed in either blood and joint or bone exudates. The number of amplification cycles required to obtain a positive result was used as a surrogate for colonization density. Contrarily to what has been demonstrated in other respiratory pathogens such as pneumococcus or *H. influenzae*, no differences between healthy carriers and sick children were found, suggesting that factors other than colonizing bacterial density are more important for the development of an invasive *K. kingae* infection [Ceroni D, Dubois-Ferriere, Cherkaoui A, *et al*. Detection of *Kingella kingae* osteoarticular infections in children by oropharyngeal swab PCR. Pediatrics (in press)].

To the best of our knowledge, the sensitivity of cultures and nucleic acid techniques for detecting *K. kingae* colonization has been compared in a single study in which the yield of both approaches was assessed during the investigation of a large outbreak of invasive infections in a French day-care center facility [Bidet P, Collin E, Courroux, Ighmouracène, Dufour V, Bingen E, Grimpel E, Bonacorsi S. Investigation of an outbreak of culture-negative *Kingella kingae* osteoarticular infection in a day-care center by use of a specific PCR and multilocus sequence typing. Pediatr Infect Dis J (in press)]. Overall, 12 of 18 pharyngeal specimens were positive for *K. kingae* by real-time PCR compared to 6 of 18 positive by culture on modified BAV medium *(P<0.01),* suggesting that nucleic acid amplification assays are more sensitive than cultures to determine the true carriage rate. However, when multiple strains are found to be circulating in the population, the culture approach has the advantage of enabling a comprehensive genotypic comparison of isolates. In addition, when evaluating the efficacy of prophylactic antibiotics administration for eradicating *K. kingae* from colonized children, cultures have the advantage of detecting living bacteria, whereas the

viability of *K. kingae* organisms in PCR-positive/culture-negative specimens is questionable.

MECHANISMS OF COLONIZATION

Pili

To colonize the human mucosae, bacteria have to first adhere to epithelial surfaces. In a series of elegant studies, St. Geme *et al*. have shown that *K. kingae* expresses type IV pili and that these surfaced-exposed fibers are essential for the attachment of the organism to respiratory and synovial cells [16]. The investigators disclosed a chromosomal gene cluster homologous to that found in other gram-negative organisms consisting of a *pilA1* gene that encodes the major pilin subunit, and *pilA2* and *fimB* genes of unknown functions but that are dispensable for adherence and expression of pili [16]. As it is the case for other surface-exposed virulence factors, the PilA subunit exhibits significant strain-to-strain variation in sequence and antibody reactivity indicating that this component is subjected to intensive selective pressure by the immune system [17]. The expression of pili in *K. kingae* appears to be finely regulated by 3 genes (σ^{54}, *PilS* and *PilR*) [18], and the majority of colonizing strains and those isolated from individuals with bacteremia express piliation, whereas those derived from patients with bone, joint or endocardial infections are non-piliated [17]. This observation suggests that piliation offers a selective advantage in the colonization process and at the early stages of the infection, but is detrimental to the bacterium for the invasion of deep body tissues. Additional work by the same research group identified two other genes named *pilC1* and *pilC2* in physically separated chromosomal locations that encode homologs of the *Neisseria* PilC proteins that are essential for adherence and piliation [16, 19]. *Kingella kingae* PilC1 and PilC2 proteins have a low level of homology to each other, contain calcium-binding sites and are dispensable for pilus asssembly [16, 19]. While the PilC1 site is necessary for twitching motility and adherence, the PilC2 site has only a minor influence on motility and no influence on adherence [19]. Additional research indicated that a trimeric autotransporter protein called Knh is crucial for a firm adherence of *K. kingae* to the epithelium [20]. However, Knh is covered by the bacterial carbohydrate capsule rendering it inaccessible for attachment to the host's cell membrane receptor. The adherence process is initiated by attachment of the long type IV pili to their specific receptor on the epithelium. This is followed by strong retraction of the pili fibers that pulls the bacterium into close contact with the host cell membrane, physically displacing the capsule and unmasking the Knh element that can, then, anchor to the respiratory host cell [20].

Biofilm

Biofilm formation is the predominant mode of growth for bacteria in most environments and particularly on the human body surfaces. Colonizing bacteria buildup biofilms containing large quantities of tightly packed organisms encased in a polymeric matrix and attached to the epithelium. Life in such enclosed and crowded conditions protects the bacteria from the immune system, desiccation and antimicrobial drugs and, consequently, biofilms play an important role in the pathogenesis of persisting infections, such as chronic osteomyelitis or lung disease in cystic fibrosis patients [21, 22]. The cycle of biofilm establishment, growth and architectural remodeling is a precisely regulated and highly dynamic process. Periodic inhibition of biofilm is crucial in the life cycle of many pathogens because it makes possible the liberation of trapped bacterial cells, allowing dispersion and colonization of new body niches and hosts. In has been shown that two linear polysaccharides of *K. kingae* show potent anti-biofilm activity [22]. It is speculated that these compounds could play a role in the regulation of biofilm formation by *K. kingae* organisms colonizing the respiratory tract, enabling release and dissemination of the organism by intimate person-to-person contact, and/or inhibiting biofilm production by other bacterial species competing for a colonization site on the pharyngeal mucosa.

RTX Toxin

In a study carried-out by Kehl-Fie and St. Geme, it was demonstrated that the *K. kingae* genome harbors a genetic locus encoding a RTX (repeat in toxin) system [23]. The locus consists of 5 genes designated *rtxA, rtxB*, *rtxC, rtxD*, and *tolC*, is flanked by insertion elements, and has a reduced G+C content compared to the whole genome, suggesting than it has been horizontally transferred from another species [23]. Sequencing of the locus genes revealed that *rtxA*, *rtxB*, and *rtxC* genes encode proteins that share >70% identity with their homologs in *Moraxella bovis*, whereas *rtxD* and *tolC* genes encode proteins that share homology with their *Neisseria meningitidis* counterparts [23]. The *K. kingae* RTX toxin exhibits wide range of cytotoxic activity, especially to macrophage-like and synovial cells, and to a lesser degree to respiratory epithelial cells and appears to be secreted as a soluble protein in the extracellular environment as well as as a component of outer membrane vesicles that are internalized by host's cells [24]. These biological features suggest that the RTX toxin may play an important role in establishing colonization of the pharynx by disrupting the respiratory epithelium and perhaps, in the enhanced effect in the setting of a viral coinfection, causing mucosal erosions, and promoting survival of the bacterium in the bloodstream and invasion of skeletal system tissues [23].

Antibiotic Resistance

Because of the selective pressure exerted by the mounting use and abuse of antimicrobial drugs, many bacterial species have acquired antibiotic resistance as a mean to survive in the human host. Young children are particularly exposed to antibiotics and colonizing respiratory species such as pneumococci, *Staphylococcus aureus*, *Moraxella catarrhalis* or *H. influenzae* currently exhibit alarming rates of resistance to β-lactam drugs and other antimicrobials, causing difficult-to-treat infections. *Kingella kingae* has been traditionally considered exquisitely susceptible to antibiotics, and β-lactamase production has been described in only a few clinical isolates [25, 26]. When β-lactamase production and genomic clonality were studied in a large collection of *K. kingae* isolates from Israeli patients with a variety of clinical infections and asymptomatic pharyngeal carriers, the enzyme was detected in only 2 of 190 (1.1%) invasive isolates but in 68 of 428 (15.9%) randomly chosen carriage organisms *($P<0.001$)* (Yagupsky P, unpublished data). β-lactamase production was found to be limited to 6 distinct pulsed-field electrophoresis

(PFGE) clones, which were common among carriage strains but rare among invasive ones, suggesting that antibiotic resistance may confer a biological advantage to *K. kingae* organisms residing on the pharynx of young children, coinciding with the age of increased susceptibility to pharyngeal colonization and enhanced antimicrobial drugs consumption [1, 3, 27]. On the other hand, organisms expressing β-lactamase appear to be less capable of invading the bloodstream and deep host's tissues, suggesting that improved colonization ability does not necessarily imply enhanced virulence.

PREVALENCE OF *K. KINGAE* CARRIAGE

Because bacterial colonization of the respiratory mucosal surfaces is established at the interface between humans and their surroundings, the forces that shape the acquisition, composition, and elimination of residing organisms reflect a complex array of host, microbial, environmental, and socioeconomic determinants. Factors such as sampling season, population's age and health status, living quarters crowding, number of young children at home, day-care attendance, antibiotic consumption, or smoke exposure may profoundly influence the results of prevalence studies [28]. Monitoring the presence of individual components of the pharyngeal flora is also strongly dependent on technical and methodological aspects such as sampling site, specimen collection technique, quality of swabs, transport time to the laboratory, use of selective media, or number of bacterial colonies examined [28]. Not surprisingly, results of epidemiological investigations on *K. kingae* colonization have found discrepancies in the prevalence rate, although the overall picture indicates that the pharyngeal carriage is strongly age-dependent [Amit U, Flaishmakher S, Dagan R, Porat N, Yagupsky P. Age-dependent carriage of *Kingella kingae* in young children and turnover of colonizing strains. J Pediatr Infect Dis Soc 2013, (in press)].

In an early study carried-out in southern Israel, the organism was not detected in infants younger than 6 months attending a Well-Baby Care Clinic [7]. The prevalence of *K. kingae* was 10.0% in the 6-months to 4 years group, and decreased to 6.0% in children aged 4-14 years [7]. In a later study, oropharyngeal specimens submitted to a clinical microbiology laboratory for isolation of *Streptococcus pyogenes,* were also plated onto BAV medium [29]. *Kingella kingae* prevalence decreased with increasing age: the organism was detected in 22 of 694 (3.2%) samples obtained from children younger than 4 years, in 10 of 679 (1.5%) of those derived from patients aged 4-17 years, and in 5 of 671 (0.8%) cultures from adults *(P for trend <0.001)* [29]. In a longitudinal study in which the younger age group was targeted, a cohort of 716 healthy children living in southern Israel were repeatedly sampled between the ages of 2 months and 30 months, and *K. kingae* was not isolated below 6 months of age [Amit U, Flaishmakher S, Dagan R, Porat N, Yagupsky P. Age-dependent carriage of *Kingella kingae* in young children and turnover of colonizing strains. J Pediatr Infect Dis Soc 2013, (in press)]. The colonization rate was low at 6 months, increased in12 month-old children, remained relatively stable between 12-24 months of age, and decreased significantly at 30 months (*P<0.001*) (Fig. **1**). These figures are comparable to the prevalence rate found in a Swiss population of children aged less than 4 years studied by real-time PCR [15].

In a recent study aimed to identify risk factors for *K. kingae* colonization, age 6-29 months was strongly and independently associated with carriage in the multivariate analysis [Amit U, Dagan R, Yagupsky P. Prevalence of pharyngeal carriage of *Kingella kingae* in young children and risk factors for colonization. Pediatr Infect Dis J (in press)]. This age-dependent carriage rate overlaps remarkably with the epidemiological curve of invasive *K. kingae* disease found in a study comprising 291 previously healthy Israeli children with culture-proven infections (Fig. **2**) [30].

It has been demonstrated that most invasive *K. kingae* infections are diagnosed between July and December [1]. In a study aimed to investigate the temporal pattern of *K. kingae* carriage [29], throat cultures were obtained between February and May to represent the time of the year when only a small fraction of invasive *K. kingae* infections are

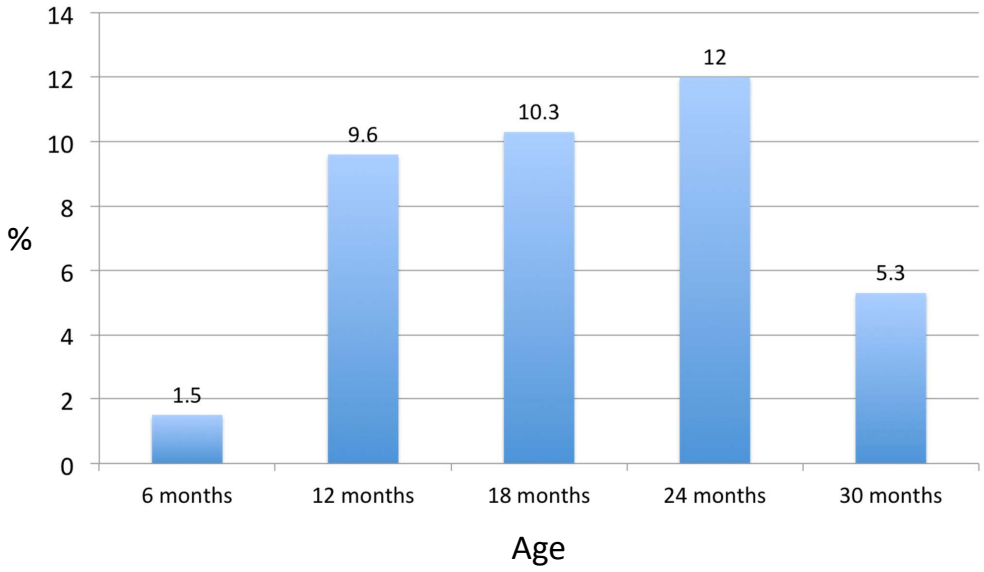

Fig. (1). Prevalence of pharyngeal *K. kingae* colonization among children aged 0-36 months.

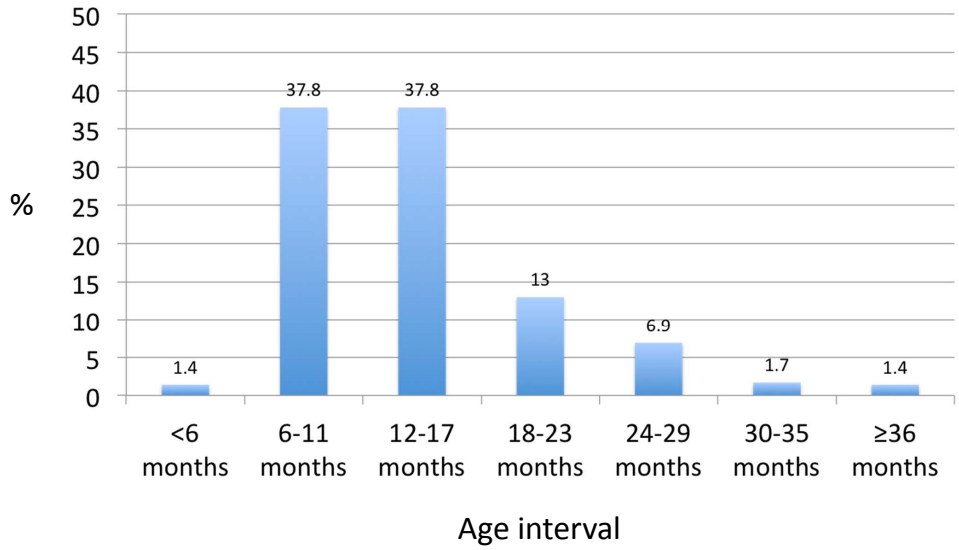

Fig. (2). Age distribution of 291 previously healthy children with invasive *K. kingae* infections.

diagnosed, and from October to December, coinciding with the annual peak attack rate. Overall 21 of 1,020 (2.1%) specimens cultured between February and May and 16 of 1,024 (1.6%) of those studied from October to December grew the organism *(P>0.4)*. The lack of seasonal *K. kingae* carriage was confirmed in a recent investigation of potential risk factors for colonization in children that showed no significant association between month of the year and carriage rate [Amit U, Dagan R, Yagupsky P. Prevalence of pharyngeal carriage of *Kingella kingae* in young children and risk factors for colonization. Pediatr Infect Dis J (in press)]. It appears, then, that the striking temporal distribution of invasive *K. kingae* disease cannot be explained solely on the basis of the characteristics of the pharyngeal carriage of the organism, implying interplay of mucosal colonization with still-unidentified cofactors, perhaps seasonal viral respiratory infections.

DAY-CARE CENTER ATTENDANCE AND *K. KINGAE* COLONIZATION

During the last decades, a growing proportion of children attend day-care outside the home. This trend has substantial public health significance because the risk of person-to-person transmission and occurrence of mucosal and invasive infection is significantly increased among day-care center attendees [31, 32]. In this close-community setting, respiratory pathogens easily spread horizontally by direct contact or through fomite transmission among young children with poor hygienic habits sharing toys coated with respiratory secretions or saliva [32]. Because of age stratification, day-care classes comprise children of approximately the same age and, therefore, with similar degrees of immunological immaturity and susceptibility to infectious agents. Under these circumstances, introduction of a virulent bacterium in a crowded day-care facility attended by immunologically naïve youngsters may result in prompt dissemination of the organism and cause outbreaks of disease.

In a prospective study conducted among 1,277 children younger than 5 years referred to a Pediatric Emergency Department in southern Israel, day-care attendance was strongly associated with *K. kingae* carriage after controlling for other variables (odds ratio: 9.66 [95% confidence intervals: 2.99-31.15], *P<0.001*) [Amit U, Dagan R, Yagupsky P. Prevalence of pharyngeal carriage of *Kingella kingae* in young children and risk factors for colonization. Pediatr Infect Dis J (in press)]. In an 11-month longitudinal study, 35 of 48 (72.9%) day-care center attendees carried *K. kingae* in the pharynx at least once and, on average, 27.5% of the children harbored the organism at any given time [7]. Molecular typing of the colonized attendees isolates showed genotypic similarities, demonstrating person-to-person transmission of the organism [33]. In an ongoing study, temporal clustering of PFGE clones was observed among *K. kingae* isolates of classmates attending 1st, 2nd, and 3rd elementary school grades, indicating that person-to-person transmission of *K. kingae* continues well beyond preschool age (Yagupsky P, unpublished data).

In recent years, clusters of invasive *K. kingae* infections, including the entire spectrum of the disease (septic arthritis, osteomyelitis, spondylodiscitis, endocarditis, and meningitis), have been reported from French, American, and Israeli day-care facilities [Bidet P, Collin E, Courroux, Ighmouracène, Dufour V, Bingen E, Grimpel E, Bonacorsi S. Investigation of an outbreak of culture-negative *Kingella kingae* osteoarticular infection in a day-care center by use of a specific PCR and multilocus sequence typing. Pediatr Infect Dis J (in press), 34-36]. The epidemiological investigation of these events revealed that the colonization rate among attendees to the classrooms where morbidity was detected was unusually high (up to 45% in a US cluster, as determined by culture [34], and 85% in the French outbreak, as demonstrated by the PCR method [Bidet P, Collin E, Courroux, Ighmouracène, Dufour V, Bingen E, Grimpel E, Bonacorsi S. Investigation of an outbreak of culture-negative *Kingella kingae* osteoarticular infection in a day-care center by use of a specific PCR and multilocus sequence typing. Pediatr Infect Dis J (in press)], and all pharyngeal isolates detected in the classrooms where disease occurred were genotypically identical and indistinguishable from the patients' clinical isolates, demonstrating that the outbreak

strains were both successful respiratory colonizers and highly virulent organisms.

COLONIZATION DYNAMICS AND TURNOVER OF STRAINS

The dynamics of carriage of respiratory bacteria can be studied in longitudinal surveys in which the population is repeatedly sampled over a prolonged period of time and isolates are analyzed by discriminative methods. In a prospective investigation in which pharyngeal swabs obtained from attendees to a day-care facility in southern Israel over an 11-month period, the *K. kingae* isolates detected were typed with a variety of methods to increase discriminative power [33]. Using a combination of PFGE with 3 restriction enzymes immunoblotting with rabbit immune serum, ribotyping with 2 restriction enzymes and stringent criteria for strain identity (full identity by all 3 typing methods) it was convincingly demonstrated that 2 distinct strains, characterized by specific typing profiles, represented 28.0% and 46.0% of the isolates typed and, once they got a foothold in the day-care center, disseminated effectively, displacing older strains [33].

In a large cohort study in which healthy children were periodically cultured between 2 and 30 months of age, the potential turnover of *K. kingae* strains over time was assessed by PFGE of isolates in the subset of colonized children in which the organism was recovered in >1 occasion [Amit U, Flaishmakher S, Dagan R, Porat N, Yagupsky P. Age-dependent carriage of *Kingella kingae* in young children and turnover of colonizing strains. J Pediatr Infect Dis Soc 2013, (in press)]. Overall, 283 of 716 (39.5%) children carried *K. kingae* at least once, of whom 64 were colonized twice, 13 had 3 positive visits, and 3 children had 4. Comparison of isolates showed that genotypic concordance was lost over time, and sequential carriage of as many as 4 different clones was detected. Carriage of *K. kingae* appears, then, as a dynamic process with frequent turnovers of colonizing strains that, after been carried continuously or intermittently for weeks or months, are replaced by newly acquired organisms, similar to observations made with other respiratory pathogens. Because in these 2 studies only a single *K. kingae* colony from each positive pharyngeal culture was typed, unrecognized colonization by multiple clones and persistence of "old" strains at a low level, rather than complete replacement, cannot be definitely excluded. It should be pointed-out, however, that re-isolation of a clone that was previously carried and lost was uncommon [Amit U, Flaishmakher S, Dagan R, Porat N, Yagupsky P. Age-dependent carriage of *Kingella kingae* in young children and turnover of colonizing strains. J Pediatr Infect Dis Soc 2013, (in press), 33]. This pattern, instead of a random temporal distribution of PFGE genotypes, suggests clearance or at least quantitative reduction in the colonizing density of individual strains. It is possible that prolonged carriage induces strain-specific immunity that facilitates elimination of the carried organism but does not prevent acquisition of an antigenically different strain. This possibility is supported by the demonstration of strain-to-strain variability of *K. kingae* outer-membrane proteins [37], the *PilA*1 gene encoding the major pilus subunit [17], and the RTX toxin [10, 13], suggesting that immunogenic surface-exposed bacterial components involved in pharyngeal colonization, undergo antigenic variation to evade the host's immune response.

IMMUNITY TO *K. KINGAE* COLONIZATION

It has been shown that the age-related incidence of invasive infection and the carriage rate of *K. kingae* are inversely related to the levels of antibodies to the organism, indicating that the immune response plays a regulatory role in the trafficking of *K. kingae* in the respiratory tract and the occurrence of clinical disease [38]. It has been recently demonstrated that *K. kingae* organisms are coated with a polysaccharide capsule, similarly to other pathogens of respiratory origin such as *N. meningitidis* and *Streptococcus pneumoniae* [20]. This important finding may cast light on the peculiar epidemiological curves of *K. kingae* carriage and disease. Synthesis of polysaccharide capsules is the result of convergent evolution of many important pediatric pathogens to survive in the mucosae, bloodstream and deep tissues, because the delayed maturation of the T-cell independent arm of the immune system results in an ineffective response to encapsulated organisms before the age of 2-3 years.

In a longitudinal serological study, IgG antibodies to *K. kingae* outer-membrane proteins were high at 2 months of age, reached nadir levels at 6-7 months, remained low until the age of 18 months, and increased in 24-month-old children. Serum IgA antibodies (which do not cross the placenta) were lowest at 2 months and slowly increased between the ages of 4 to 7 months. Further increment of both antibody types was observed in children aged ≥24 months [38]. The low attack rate of disease, absence of respiratory carriage, and high levels of IgG but no IgA antibodies detected in the first 6 months of life, indicate that the immunity to colonization and infection observed in young infants is conferred by maternally derived antibodies. Increased prevalence of *K. kingae* in the pharynx and enhanced susceptibility to invasive infections among 6 to 24-month-old children coincide with the age at which antibody levels are lowest. Increasing antibody levels found among older children probably represents immunological maturation and cumulative experience with *K. kingae* antigens or cross-reacting antibodies induced by related organisms, resulting in decline in the carriage rate and burden of invasive infection [38]. Because a substantial fraction of children harbor *K. kingae* in the pharynx, whereas invasive disease occurs infrequently, it is believed that asymptomatic respiratory colonization and not clinical infection is the immunizing event.

TRANSMISSION

In the last few decades, development of molecular typing techniques enabled for the first time distinction between genetically different strains of colonizing organisms, enabling deeper understanding of the geographic and temporal transmission of individual lineages in the population.

The dissemination of *K. kingae* in the open community was studied in two ethnic groups living side-by-side in southern Israel [39]. Organisms recovered from oropharyngeal cultures, obtained from healthy young Jewish and Bedouin children during a 12-month period, were typed by PFGE and compared. Isolates from Bedouin children

usually differed from those derived from Jews, confirming the relative social isolation of the two populations [39]. Typing of isolates also show significant spatial clustering of clones in the Bedouin towns' neighborhoods and households, indicating person-to-person transmission between family members and playmates, and confirming the importance of close mingling in the spread of *K. kingae.* However, no significant geographic clustering of *K. kingae* clones was detected the Jewish city quarters. Because the traditionally nomadic Bedouins have recently settled in towns but have kept the traditional tribal divisions, geographic clustering can be considered a surrogate for family ties and social intercourse. Bedouin youngsters do not usually attend day-care facilities and most of their social interaction takes place within their extended families and clans, explaining the similarity of *K. kingae* organisms isolated in households located within a short radius. Contrarily, Jewish children live in westernized urban conditions, attend day-care centers, and have multiple social contacts outside their immediate geographic environment. Under these circumstances, Jewish children living throughout the different city neighborhoods are connected by numerous and complex social networks, conspicuous and occult as well, through which respiratory bacteria may circulate. The multiplicity of potential sources of transmission in the Jewish community could, then, blur the connection between place of residence and distribution of *K. kingae* clones [39].

FROM COLONIZATION TO CLINICAL DISEASE

The link between colonization of the pharyngeal epithelium by *K. kingae* organisms and the development of an invasive infection was convincingly demonstrated in 3 bacteremic children in whom the organism was also recovered from an upper respiratory culture. Typing of paired blood and pharyngeal isolates by PFGE demonstrated genomic identity [8]. This finding was later supported by the results of two studies employing nucleic acid amplification technology. In a study in which 27 young children were diagnosed with PCR-proven *K. kingae* septic arthritis or osteomyelitis, the PCR assay performed on the pharyngeal swab was positive for *rtxB* gene DNA in all cases [15]. In a second and more definitive study, young children with arthritis and culture-negative/PCR-positive synovial fluid samples had *K. kingae* cultured from the pharynx. The *rtxA* gene sequences of the synovial exudate amplicons and those of the pharyngeal isolate were undistinguishable, suggesting that the oropharynx is likely the site from which virulent *K. kingae* organisms enter the bloodstream and disseminate, causing focal skeletal system, endocardial, and other deep site infections [10]. Because patients with invasive *K. kingae* disease frequently present with symptoms consistent with a viral respiratory infection, evidence of herpetic gingivostomatitis, oral varicella blisters, or concomitant buccal aphthous ulcers, it is plausible that viral induced damage to the respiratory mucosa facilitates penetration of colonizing *K. kingae* organisms and invasion of the bloodstream [1, 30].

Recent studies have revealed that *K. kingae* organisms display remarkable genomic variability [40, 41] and profound strain-to-strain differences in terms of invasive capability [42, 43]. Characterization of isolates by PFGE and multilocus sequence typing demonstrated that some clones are frequently isolated from healthy carriers but are seldom if ever detected in patients with invasive diseases, whereas others are common etiology of clinical infections and show significant association with syndromes such as bacteremia, skeletal system infections, or endocarditis [42, 43]. These results imply that strains that show enhanced colonization fitness are not necessarily able to penetrate or survive in the bloodstream or deep tissues, implying that invasion of these niches may require a different biological specialization and, conversely, carriage of particularly virulent strains entails increased risk for clinical disease and invasion of specific body sites.

ERADICATION OF COLONIZING ORGANISMS

As it is the case with other pathogens of respiratory origin, the population of asymptomatic carriers at any given time is huge compared to that of diseased individuals. Despite a background carriage rate as high as 5%-10%, the annual incidence of invasive *K. kingae* in Israeli children aged less than 5 years is 9.4/100,000 only [30], and the calculated annual risk of young Swiss carriers to develop osteomyelitis or septic arthritis was found to be low (<1%) [15]. Therefore, in the absence of symptoms, there is no indication to eradicate the organism from the colonized mucosal surfaces. However, the risk of acquisition of *K. kingae* and progression to a severe and even life-threatening infection appears to be greatly increased in day-care facilities. When data of the 4 clusters of invasive disease reported in day-care centers are pooled, a total of 14 of 75 (18.7%) classmates developed a proven or presumptive *K. kingae* infection, including fatal endocarditis, within a 1-month period [Bidet P, Collin E, Courroux, Ighmouracène, Dufour V, Bingen E, Grimpel E, Bonacorsi S. Investigation of an outbreak of culture-negative *Kingella kingae* osteoarticular infection in a day-care center by use of a specific PCR and multilocus sequence typing. Pediatr Infect Dis J (in press), 34-36]. This unusual attack rate, coupled with the finding that a large proportion of attendees to the classes where the index cases occurred carried the infecting strain, indicated that the causative organisms combined unusual colonization ability, transmissibility and virulence. Under these circumstances, administration of prophylactic antibiotics, aimed to eradicate pharyngeal colonization in contacts and prevent further cases of disease, was attempted. Either rifampin 10 mg/kg [Bidet P, Collin E, Courroux, Ighmouracène, Dufour V, Bingen E, Grimpel E, Bonacorsi S. Investigation of an outbreak of culture-negative *Kingella kingae* osteoarticular infection in a day-care center by use of a specific PCR and multilocus sequence typing. Pediatr Infect Dis J (in press)] or 20 mg/kg twice daily for 2 days alone [34] or in combination with amoxicillin (80 mg/kg per day) for 2 days [35] or 4 days [36] were used. The effectiveness of these regimens, however, was limited, and the success ranged between 47% [Bidet P, Collin E, Courroux, Ighmouracène, Dufour V, Bingen E, Grimpel E, Bonacorsi S. Investigation of an outbreak of culture-negative *Kingella kingae* osteoarticular infection in a day-care center by use of a specific PCR and multilocus sequence typing. Pediatr Infect Dis J (in press)] and 80% only [36], indicating that, despite the exquisite antibiotic susceptibility of the species, eradication of *K. kingae* from colonized mucosae is difficult to achieve.

CONCLUSIONS

Since the early 1990s there has been an impressive increment in the number of reports on invasive *K. kingae* infections from Western countries, reflecting the adoption of improved culture techniques, use of nucleic acid amplification assays, and increasing familiarity of clinical microbiology laboratories with its identification. Recognition of the organism as an important pediatric pathogen of early childhood has stimulated novel research on *K. kingae* carriage by healthy children, person-to-person transmission of the organism, and the role of colonization in the pathogenesis of infections such as bacteremia, arthritis, osteomyelitis, and endocarditis. Investigation of the epidemiological and molecular aspects of asymptomatic *K. kingae* colonization has elucidated the complex mechanisms involved in the persistence and dissemination of the bacterium in the population and showed striking similarities between *K. kingae* and other more traditional respiratory pathogens. It is to be expected that further research will reveal genomic traits among carriage strains responsible for colonization fitness, invasiveness, and tissue tropism.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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