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Advances in Understanding *Kingella kingae*

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Preface

Kingella kingae was first isolated in the 1960s from blood, joint fluid, bone exudates, and respiratory secretions by Elizabeth O. King, a bacteriologist at the US Centers for Disease Control. Dr. King's initial studies suggested to her that this organism was a novel *Moraxella* species. Analysis by S.D. Henriksen and K. Bovre at the University of Oslo, Norway, confirmed Dr. King's initial impressions and resulted in a publication describing the new species, which they named *Moraxella kingii* in honor of King. Additional studies examining biochemical properties, fatty acid composition, and genetic characteristics established significant differences between *M. kingii* and other members of the *Moraxella* genus and led to reclassification as a novel genus in the Neisseriaceae family and renaming as *K. kingae* in 1976.

Over the next two decades, *K. kingae* was largely ignored as a human pathogen because of its uncommon recovery from patients with disease. However, in recent years *K. kingae* has been increasingly recognized as a clinically important pathogen in young children, reflecting improvements in culture techniques and DNA-based detection methods that have resulted in more frequent identification of this organism. Currently, *K. kingae* is recognized as the leading cause of osteoarticular infections in young children in a growing number of countries. As sensitive culture techniques and molecular methods to identify this fastidious organism are adopted more routinely, the clinical spectrum of *K. kingae* disease will likely continue to evolve. Research into this organism has grown tremendously over the past 15 years, resulting in a better appreciation of the importance of *K. kingae* in pediatric patients and of the molecular mechanisms of disease.

This book describes the growing body of information on the epidemiology, clinical manifestations, transmission, pathogenesis, diagnosis, and treatment of *K. kingae* infections in young children. In addition, it covers experimental methods that have been developed to study the microbiology, genetics, and virulence factors of *K. kingae*, information that provides the foundation for new approaches to treatment and prevention of *K. kingae* disease. With this content in mind, excerpts from the book will hopefully have relevance for clinicians who care for pediatric

patients, for clinical microbiologists who are involved in detecting organisms in clinical specimens, and for scientists who are studying *K. kingae* in an effort to develop novel targets for antimicrobial therapy and new approaches to prevention.

Philadelphia, PA, USA Joseph W. St. Geme, III

Contents

Microbiology, Genomics, and Population Structure

Stéphane Bonacorsi, Philippe Bidet and Pablo Yagupsky

Microbiology

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

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

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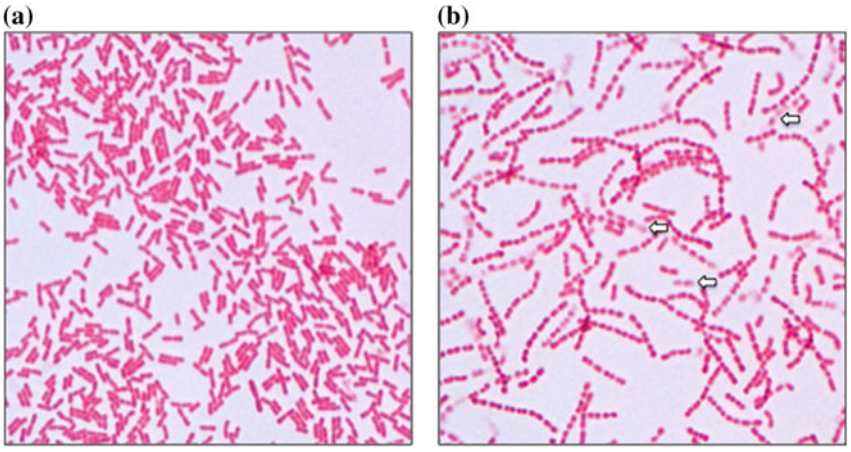


Fig. 1 **a** Gram staining of a typical *Kingella kingae* isolate depicting plump gram-negative coccobacilli arranged in pairs or short chains; **b** small-colony variant morphology exhibiting long chains of coccobacilli and early autolysis (*arrows*)

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

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

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

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

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

Genomics

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

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

Typing of K. kingae Isolates

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

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

Pulsed-Field Gel Electrophoresis

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

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

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

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

Multilocus Sequence Typing

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

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

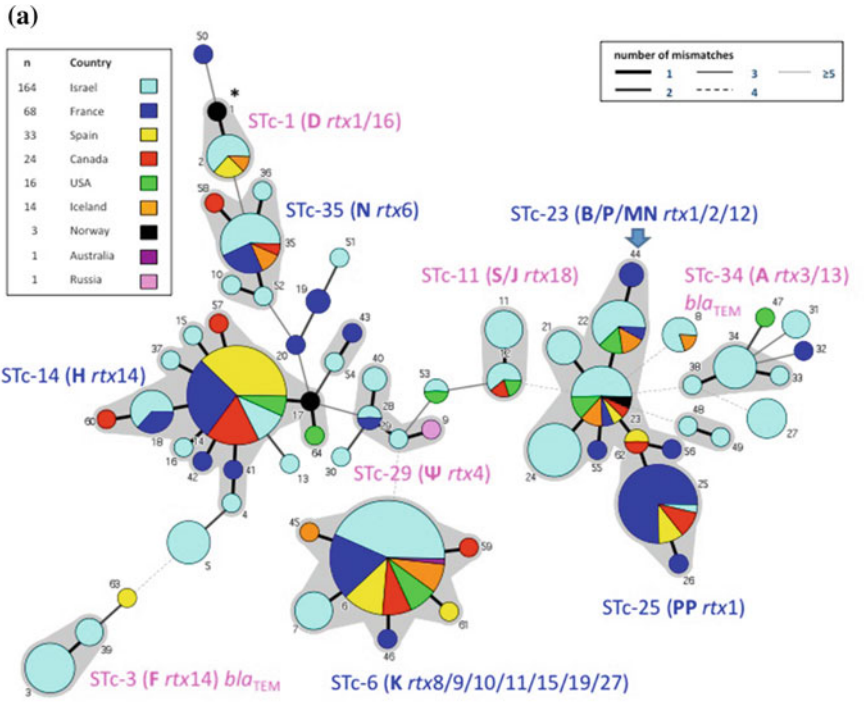
In an important study, a collection of 324 *K. kingae* isolates from Israel, Europe, North America, and Australia was investigated by the novel MLST method. Most European and American isolates were associated with invasive infections, while Israeli isolates came from both invasive infections and asymptomatic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

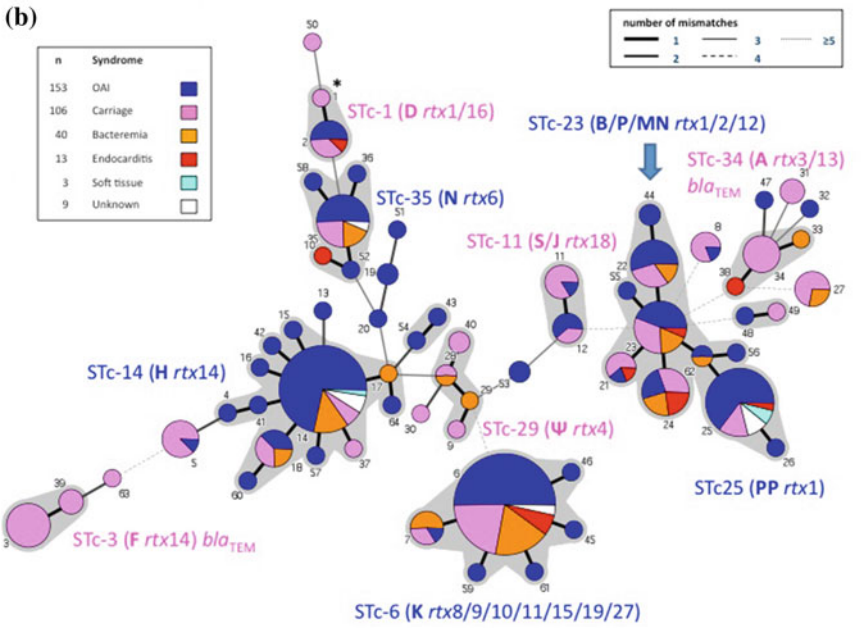
Population Structure

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

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

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


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


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

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

Geographic Distribution

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

Clonal Evolution

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

Population Structure and Virulence

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

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

Association with the RtxA Gene Alleles

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

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

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Epidemiology and Clinical Manifestations of *Kingella kingae* Disease

Kevin J. Downes

Abbreviation

OAI Osteoarticular infection

Epidemiology

A precise description of the epidemiology of *Kingella kingae* disease was limited historically by the inability of standard culture techniques to reliably isolate the organism from tissue, body fluids, and blood. *K. kingae* is a facultative anaerobic β-hemolytic gram-negative bacterium that grows poorly on standard solid media [1, 2]. Until recently, most data regarding *K. kingae* disease stemmed from case reports and small case series. However, over the past 20 years, with the more routine use of selective culture media and molecular diagnostics by microbiology laboratories, the clinical relevance of this emerging pathogen has become more fully appreciated.

Age

Age is the most important factor influencing *K. kingae* oropharyngeal carriage [3, 4] and hence *K. kingae* invasive disease. *K. kingae* is carried on the tonsillar surfaces of young children, generally without producing symptoms [2]. Carriage is rare in the first few months of life [4–6] and begins to appear around 6 months of age, achieving rates of approximately 3–12 % in children 6–48 months old [3–7]. In a random sample of patients undergoing throat culture in Southern Israel from 1998 to 2001, Yagupsky and colleagues found carriage rates of 3.2 % in children

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0–3 years of age, 1.5 % in children 4–17 years of age, and 0.8 % in individuals 18 years and older [3]. More recent studies have reported higher rates of pharyngeal carriage among young children [4, 7]. For example, Amit and colleagues found that carriage ranged between 5 and 12 % in children 6–30 months of age in Southern Israel [4]. Similarly, Anderson de la Llana et al. found that the mean prevalence of carriage was 8.7 % (range 7.6–10.4 %) in a cohort of healthy, asymptomatic Swiss children 7–48 months of age, with no differences across subgroups of age [7].

Oropharyngeal carriage is believed to be the first step in the pathogenesis of invasive disease, a conclusion supported by studies that have detected universal carriage in patients with invasive disease [8]. Yagupsky et al. observed that nearly all cases (98.6 %) of *K. kingae* invasive disease occurred in children younger than 48 months of age, with an incidence that was more than four times higher among children aged 7–12 and 13–24 months of age than among children 25–36 months of age [3]. In a nationwide study of *K. kingae* invasive disease in Israel, almost all children (96 %) were between 6 and 29 months of age, as highlighted in Fig. 1, which displays the age distribution among the 290 children [9]. The higher incidence of invasive disease among children 6–24 months old may coincide with decreased humoral immunity against the pathogen [10].

Osteoarticular infections (OAIs) are the most common form of *K. kingae* invasive disease [9]. In a recent review of 566 cases of *K. kingae* OAI reported in the English literature from 2000 to 2014, 80 % occurred in children between the ages of 4 months and 4 years [11]. In a cohort of children in France with OAIs, the median age of patients with *K. kingae* infection was 14.6 months (range 6.8 months to 6.8 years), with 95 % of these *K. kingae* infections occurring in children younger than 3 years [12]. Similarly, in a prospective cohort study of children 7–48 months of age in Geneva, Switzerland with confirmed OAIs due to *K. kingae,* children 7–24 months of age had an increased odds of OAIs [7].

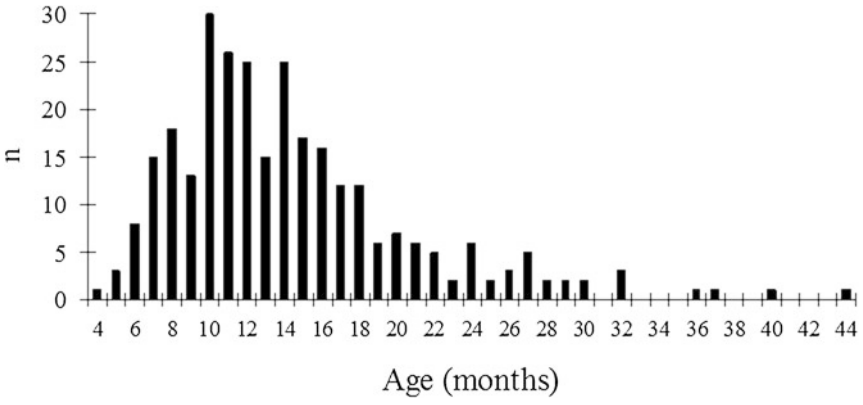

Fig. 1 Age distribution of invasive *K. kingae* infection in otherwise healthy children. Reproduced with permission from Dubnov-Raz et al. [9]. Copyright © Wolters Kluwer Health, Inc.

Few studies have reported the population incidence of *K. kingae* disease [9, 13, 14]. Among children 6–48 months of age in Geneva, the annual incidence of OAIs due to *K. kingae* was estimated to be less than 1 % (0.0664 %) [13]. Based on the population-based studies in Southern Israel, the annual incidence of invasive *K. kingae* disease was 9.4 per 100,000 in children age 0–4 years [9] and roughly 20 per 100,000 in children less than 2 years of age [14].

Gender

The influence of gender on the risk of oropharyngeal carriage and invasive disease with *K. kingae* varies among studies. In a study of asymptomatic Swiss children, Anderson de la Llana and colleagues reported that males were more likely than females to be carriers (10.9 % vs. 6.4 %, $p < 0.05$) [7]. However, other studies have found similar rates of carriage among male and female children [3]. Some studies have reported that invasive disease develops more often in males [3, 15], as highlighted in a cohort of 74 children with invasive disease in Southern Israel that included 50 males ($p < 0.045$) [3]. In contrast, the series of 566 *K. kingae* OAIs reported between 2000 and 2014 identified an equal gender distribution of cases: 1.14 male per 1 female [11].

Seasonality

There may be a seasonal pattern to the development of invasive disease due to *K. kingae*. In the large study by Anderson de la Llana et al., the rate of asymptomatic pharyngeal carriage did not differ by time of year, but nearly two-thirds of OAIs occurred between July and November [7]. Similarly, in a series of studies in Israel, more cases of invasive disease occurred between July and December [3, 9, 14]. In a single-center study from France, the peak incidence of *K. kingae* septic arthritis was in October [16]. The frequent presence of upper respiratory tract symptoms in children with *K. kingae* invasive disease suggests that viral coinfections may predispose children to the development of invasive disease [14, 17–19]. Thus, the seasonal distribution of invasive *K. kingae* disease may mirror that of viral upper respiratory pathogens.

Geographic Location

Investigators in Israel, Switzerland, and France have contributed disproportionately to the current understanding of the epidemiology of *K. kingae* carriage and disease. It can be argued that the populations studied from these regions are diverse,

allowing findings to be extrapolated to other pediatric populations. The similar rates of pharyngeal carriage [3, 4, 7] and invasive disease [9, 12, 13] across these countries support the conclusion that *K. kingae* is a universal pathogen in young children. Yet, there has been a relative paucity of systematic investigation from other countries. Undoubtedly, young children are at highest risk for invasive *K. kingae* disease globally, but the prevalence of disease and the influence of specific risk factors (e.g., gender and season) on carriage and disease may differ among geographic regions.

Recent data indicate that β-lactamase production varies among *K. kingae* isolates from different countries [20, 21], highlighting the possibility that there are important genetic differences in the circulating strains that are associated with carriage and that cause disease in different geographic locations [22]. Additional data indicate that specific clinical syndromes [23], transmissibility [24], and colonization [25] vary based on the *K. kingae* clone involved. Thus, until systematic epidemiologic investigations are conducted in more parts of the world, the global impact of this pathogen cannot be fully understood.

Socioeconomic Factors and Childcare Attendance

The socialization of young children likely contributes to *K. kingae* carriage and disease. As with other bacteria that reside in the upper respiratory tract, child-to-child transmission is believed to be the primary mechanism of spread [1]. During an 11-month longitudinal study of 48 daycare attendees, nearly three quarters of the children studied (n = 35, 73 %) carried *K. kingae* at least once [6]. In a more recent study of carriage among children followed in well-newborn clinics in Israel, only 40 % (283 or 716) of children 2–30 months of age carried the organism one or more times [4]; half of the children in this study attended day care. The difference in carriage rates between these two studies raises the possibility that daycare attendance increases the risk of *K. kingae* acquisition. This conclusion is supported by other studies that found daycare attendance to be a strong independent risk factor for *K. kingae* carriage [5]. Reports of outbreaks of invasive *K. kingae* disease in daycare settings suggest that child-to-child transmission of virulent strains also occurs [26–28].

Amit et al. compared the epidemiology of *K. kingae* invasive disease between Jewish children and Bedouin children less than 4 years of age during a 23-year period in Southern Israel [15]. Despite the fact that both populations lived in the same part of the country and received medical care from the same tertiary care center through the same insurance, Jewish children had a significantly higher incidence of invasive disease (12.21 vs. 5.53/100,000, $p < 0.05$). According to the authors, children of these two ethnicities have similar carriage rates, implying that other factors must play a role in the discrepant rates of invasive disease. There were important differences in age (Jewish children were younger) and socioeconomic status (Bedouin children more often live in poverty and crowded conditions) that

could explain the difference in rates between the two populations. However, Jewish children also had a clustering of *K. kingae* strains, with a single clone accounting for more than 40 % of infections. Other studies from these investigators have demonstrated that daycare attendance is a risk factor for carriage among Jewish children but not among Bedouins [5], suggesting the possibility that social factors and the amount of close contact with other young children play a role in the transmission of strains causing invasive disease.

Clinical Manifestations

Kingella kingae causes a number of different types of invasive disease. The most common forms of *K. kingae* disease are OAIs and bacteremia. Endocarditis is rare but can be severe [9, 29]. As with asymptomatic carriage, invasive disease occurs almost exclusively in previously healthy young children [9, 30]. Children with underlying chronic health conditions who develop invasive *Kingella* infections tend to be older: mean age 51.6 ± 51.9 months versus 14.3 ± 6.4 months in otherwise healthy children [9]. Table 1 displays presenting clinical and laboratory data from the largest case series to date of children with invasive *K. kingae* disease (n = 322) [9].

The pathogenesis of invasive disease likely involves bacterial translocation across the oropharyngeal mucosal barrier and entry into the bloodstream [2]. Children with *K. kingae* invasive disease frequently have concurrent or preceding symptoms of other acute infections, such as upper respiratory tract infections, aphthous stomatitis, or acute gastroenteritis [9, 17, 19, 31]. Compromise of the normally protective respiratory epithelium can lead to dissemination of the bacterium to various sites in the body, including joints, bones, and endocardium. The capacity of *K. kingae* to cause invasive disease may vary based on the specific clone involved, resulting in different clinical syndromes [23].

Osteoarticular Infections

Osteoarticular infections (OAIs) are the most prevalent form of invasive disease caused by *K. kingae* [9]. Septic arthritis, osteomyelitis, and spondylodiscitis make up the majority of *K. kingae*-associated OAIs. In a systematic review of *K. kingae* OAI cases reported between 2000 and 2014, septic arthritis accounted for 73.1 % (n = 404/553) of cases, osteomyelitis for 15.7 % of cases, and spondylodiscitis for 5.4 % of cases [11]. Less common forms of osteoarticular disease include cartilage matrix infections [32] and tenosynovitis [33].

With incorporation of specialized culture techniques and molecular diagnostic methods into routine clinical microbiology laboratory practices, several studies have reported that *K. kingae* is the leading cause of OAIs in children less than four

Table 1 Clinical and laboratory data of children with invasive *K. kingae* infections

	Occult bacteremia (n = 145)	Bacterial endocarditis (n = 8)	Skeletal infections (n = 169)
Age (mo)	13.2 ± 6.3 (4–176)	25.4 ± 21.7* (10–66)	15.5 ± 7.2* (5–40)
Maximal temperature (°C)	38.8 ± 0. 8 (36.7–40.0)	39.6 ± 0.7* (38.5–40.5)	38.3 ± 1.0* (36.0–40.0)
Symptom duration before diagnosis (d)	3.7 ± 2.2 (1–14)	7.8 ± 6.0 (1–14)	4.0 ± 4.6 (1–31)
Symptom duration after diagnosis (d)	3.0 ± 2.4 (1–14)	13.5 ± 11.8* (5–30)	6.7 ± 5.2* (1–30)
Blood WBC count (cells/mm^3)[a]	14,350 ± 6170 (1200–41,300)	18,600 ± 13,200 (5660–47,040)	14,797 ± 4383 (5860–28,450)
CRP (mg/dL)[a]	2.3 ± 1.9 (0.4–6.6)	11.0 ± 8.7* (6.0–24.7)	3.7 ± 3.8 (0.15–17.0)
ESR (mm/h)[a]	32 ± 20 (4–115)	90 ± 25* (60–120)	44 ± 25* (5–140)
Synovial WBC count (cells/mm^3)[a]	–	–	105,368 ± 72,296 (800–325,000)

Reproduced from Dubnov-Raz et al. [9], with permission from Wolters Kluwer Health, Inc.
Values are expressed as mean ± SD. Values in parenthesis mean range
*Values that differ significantly ($p < 0.05$) from those found in the occult bacteremia group
[a]Upon diagnosis

years of age, responsible for 40–70 % of confirmed cases [8, 12, 16, 34, 35]. Ferroni and colleagues found that *K. kingae* was the cause of 53 % (n = 44/83) of proven OAIs in children <15 years of age [34]; *K. kingae* was the most common pathogen isolated and was found exclusively in children less than 4 years of age. Similarly, in a study examining OAIs in patients at DeBrousse Hospital in Lyon, France, Chometon and coworkers discovered that *K. kingae* was the predominant pathogen in children less than 4 years of age, with *Staphylococcus aureus* assuming a dominant role in older children (see Fig. 2) [12]. In another case series of children <16 years of age hospitalized for infectious and non-infectious arthritis in France, *K. kingae* was the most common cause of septic arthritis (69 %) and the most common cause of arthritis of any form, infectious or non-infectious [16]. In a recent report from the University of Texas Southwestern Medical Center, *K. kingae* was the second most common cause of septic arthritis among children less than 18 years of age over a 2-year period [36].

Symptoms of *K. kingae* OAI can be insidious, particularly in patients with osteomyelitis or spondylodiscitis. In a cohort study of consecutive cases of OAIs, the mean duration from onset of symptoms to hospitalization among the 23 cases of *K. kingae* infection was 9.3 days [35]. In the large case series by Dubnov-Raz et al., the mean time to presentation was 3.2 ± 3.0 days for patients with septic arthritis and 9.2 ± 9.4 days ($p < 0.001$) for patients with osteomyelitis [9]. A study by Gene et al. reported an average time to diagnosis of *K. kingae* osteomyelitis of more than two weeks [30]. Thus, diagnosis of *K. kingae* OAI may be delayed due to the subacute nature of infection with this pathogen, especially in cases of *K. kingae* osteomyelitis.

K. kingae may elicit a less intense inflammatory response compared with other pathogens. In a retrospective study comparing 30 children with *K. kingae* OAIs to

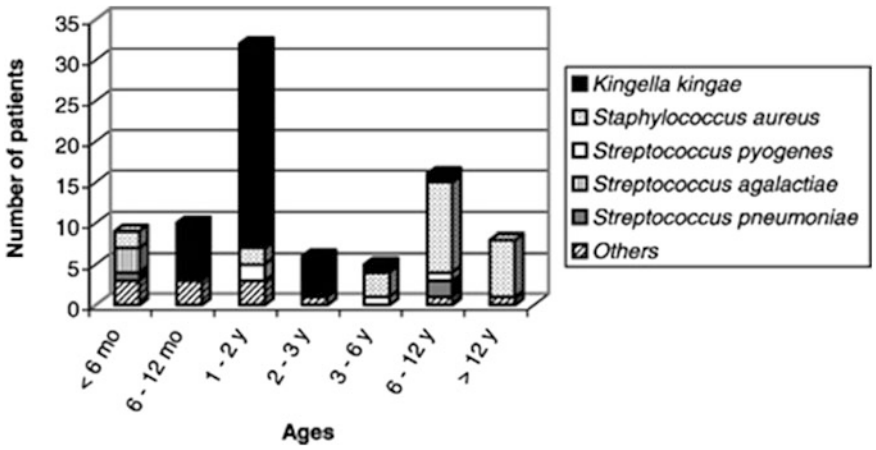

Fig. 2 Distribution of pathogens of 86 bacteriologically documented osteoarticular infections according to the age of patients. Reproduced with permission from Chometon et al. [12]. Copyright © Wolters Kluwer Health, Inc.

30 children with OAIs due to other pathogens [37], only 10 % of children with *K. kingae* infection had a fever (>38 °C) at admission compared to 96.7 % of children with other pathogens. The area under the receiver operator curve for temperature at admission was 0.981 to distinguish OAIs due to *K. kingae* from OAIs due to other pathogens [37]. The laboratory evidence of infection in *K. kingella* OAIs is also more often absent. Dubnov-Raz et al. found that fewer than 50 % of patients with *K. kingella* OAIs had a peripheral white blood cell count above 15,000 cells/mm^3 [9]. Of 23 OAIs caused by *K. kingae* reported by Ceroni et al. [35], only 2 had an elevated peripheral white blood cell count. In a prospective cohort of children with varying OAIs of all causes, CRP was lower in children with documented *K. kingae* infections compared with infections due to other pathogens: mean 4.2 mg/dL versus 8.2 mg/dL, $p < 0.005$ [34]. Similarly, Chometon reported only a modest increase in CRP (mean 3.2 mg/dL) at admission among 33 patients with *K. kingae* OAIs [12].

The outcomes of children with *K. kingae* OAIs are generally favorable [11, 34, 38]. In a study comparing children with *K. kingae* septic arthritis and children with *S. aureus* septic arthritis [38], patients in both groups were similar with regard to numerous factors at initial presentation: symptom duration, fever, CRP, WBC count. However, patients with *K. kingae* had more rapid resolution of fever and normalization of CRP and fibrinogen than did patients with *S. aureus* septic arthritis, suggesting a more rapid response to therapy. Additionally, patients with *K. kingae* had significantly fewer complications (<2 % vs. 23 %), although the types of complications were not defined in this study [38]. Severe cases of *K. kingae* OAI appear to be uncommon [39].

Septic Arthritis

Septic arthritis is the most common type of OAI due to *K. kingae* [9, 11, 12]. In a large case series of invasive *K. kingae* infections from 8 centers in Israel, septic arthritis accounted for 83 % (140/169) of all OAIs [9]. Lower extremities tend to be affected most often [12, 35], with the knee the most commonly infected joint [9, 11, 40]. In a case series from France, *K. kingae* more often caused septic arthritis of the knee, while other organisms more commonly infected the hip and ankle ($p < 0.01$) [16]. Figure 3 displays the cumulative site distribution of *K. kingae* septic arthritis cases as reported by Al-Qwbani et al. in their systematic review [11].

Similar to patients with other etiologies of septic arthritis, children with *K. kingae* septic arthritis present with localized pain, joint swelling, erythema, immobility, and evidence of joint effusion [30]. Fever may be a less pronounced presenting symptom than in *S. aureus* septic arthritis [36]. When present, fever resolves more quickly after initiation of appropriate treatment in patients with *K. kingae* than *S. aureus* septic arthritis. In a single center's experience, the mean duration of fever was 0.2 days (range: 0–3) in children with *K. kingae* septic arthritis versus 3.5 days (range: 0–27) in children with *S. aureus* septic arthritis ($p < 0.0001$) [38]. Synovial fluid aspiration generally reveals inflammation

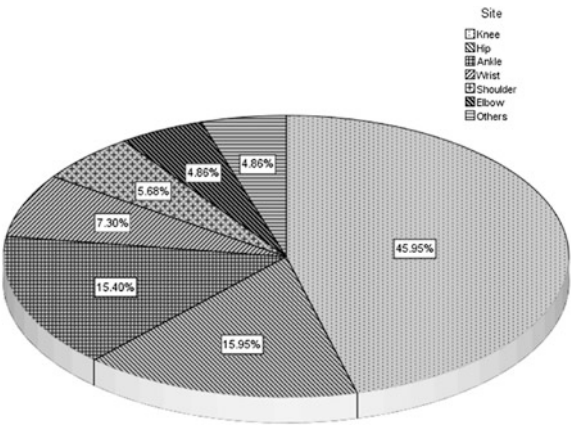

Fig. 3 Site distribution of septic arthritis caused by *K. kingae*. Reproduced from Al-Qwbani et al. [12]. Copyright © SAGE Publications

consistent with pyogenic arthritis [11]. However, among 78 children with septic arthritis who had synovial fluid sampling in the large nationwide study in Israel by Dubnov-Raz et al. [9], 18 (23 %) had synovial fluid WBC with <50,000 cells/mm^3 (mean 105,368 ± 72,296).

Osteomyelitis

Osteomyelitis is less common than septic arthritis as a manifestation of *K. kingae* infection [9, 11, 39]. Infections may develop in any bone, but there are numerous reports describing atypical locations such as the calcaneus [9, 41], sternum [42], and clavicle [43]. Figure 4 displays the site distribution of *K. kingae* osteomyelitis cases as reported by Al-Qwbani et al. [11]. Most cases present subacutely [9], sometimes resulting in formation of a Brodie abscess by the time the diagnosis is made [9, 18, 44]. Although uncommon, severe cases of osteomyelitis with metaphyso-epiphyseal or epiphyseal abscess formation can occur [39]. *K. kingae* has also been described as the most common cause of primary epiphyseal or apophyseal subacute osteomyelitis in children less than 4 years of age [45]. This form of subacute osteomyelitis affects the epiphysis and generally presents as an indolent process with few laboratory signs of infection. The femur (70 %) and tibia are involved most often [45].

Spondylodiscitis

Spondylodiscitis is an infection of the intervertebral disk space and adjacent vertebral bodies. Diagnosis can be challenging in young children whose symptoms can include refusal to sit or walk, abnormal gait, or back stiffness [46]. *K. kingae* is among the most common causes of spondylodiscitis in children, accounting for at

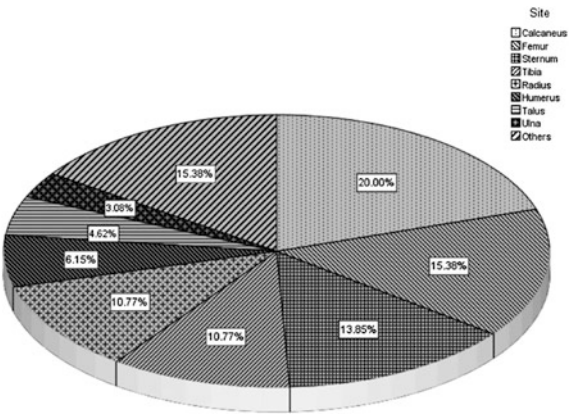

Fig. 4 Site distribution of osteomyelitis caused by *K. kingae*. Reproduced from Al-Qwbani et al. [12]. Copyright © SAGE Publications

least 25 % of confirmed cases [46]. Similar to other OAIs due to *K. kingae*, spondylodiscitis occurs predominantly in young children and presents in an indolent manner [46–48]. The lumbar spine is affected most often, and asymptomatic narrowing of the disk space is the expected long-term outcome, as with all causes of spondylodiscitis [47].

Bacteremia

Bacteremia can occur concurrently with other invasive infections [9, 49] or in isolation as occult bacteremia [9, 31]. Positive blood cultures are obtained in about one in four patients with OAIs in general and are a prerequisite for the diagnosis of bacterial endocarditis [9]. Because of the inferiority of culture methods compared to DNA-based techniques to detect *K. kingae*, accurate estimates of the population incidence of bacteremia are not known. Over an eleven-year period (1996–2006) at Schneider Children's Medical Center of Israel, a tertiary care pediatric hospital in central Israel, there were 42 bloodstream infections with *K. kingae*, including 4 that occurred during cases of endocarditis and none associated with osteomyelitis [31]. In a study of 53,503 blood cultures obtained over a 35-month period in the pediatric emergency department at Soroka University Medical Center in Southern Israel [50], 16 were positive for *K. kingae.* At the Children's Hospital of Philadelphia, 301,716 blood cultures were obtained from 53,544 children between 2004 and 2014, including 5 that were positive for *K. kingae* (unpublished data). Thus, there may be regional differences in rates of *K. kingae* bacteremia, although this issue has not been formally investigated.

Occult bacteremia is defined as bacteremia without a focal infection and is the second most common manifestation of invasive *K. kingae* disease [9]. Children with bacteremia may have osteoarticular complaints without overt evidence of bone or joint infection [31]. In a large case series of 322 invasive infections from 8

institutions in Israel, 140 (43.6 %) children had occult bacteremia [9]. Inflammatory markers in this series were generally low: mean CRP 2.3 ± 1.9 mg/dL and ESR 32 ± 20 mm/h. Many children with bacteremia have a concomitant acute illness such as aphthous stomatitis, upper respiratory tract infection, or acute gastroenteritis [9, 31]. One of these conditions was present in 79 % of children with *K. kingae* bacteremia based on the retrospective review by Dubnov-Raz et al. [31].

Endocarditis

Kingella kingae is one of the HACEK organisms (*Haemophilus* species; *Aggregatibacter* species; and *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* species), a group of fastidious gram-negative bacilli that are responsible for approximately 5–10 % of native valve endocarditis cases in adults [51] and children [52]. These organisms are also believed to be responsible for a portion of culture-negative endocarditis cases, due to challenges in isolating these organisms using conventional culture techniques. Some case series have detected *K. kingae* in approximately 7 % of cases of pediatric infective endocarditis [53, 54]. Poor dental health and dental procedures are reported risk factors for endocarditis due to HACEK pathogens, including *Kingella* [51, 55]. Prosthetic valve endocarditis due to *K. kingae* is rare and is generally treatable with antibiotics alone, although complications such as paravalvular abscess have been described [56].

Kingella kingae endocarditis in children is uncommon, accounting for 2–7 % of invasive *K. kingae* infections [9, 31]. Whereas *K. kingae* OAIs occur almost exclusively in young children, *K. kingae* endocarditis may affect older children as well [9]. In a recent PubMed review of 42 reported cases of pediatric *K. kingae* endocarditis, 20 % occurred in children >4 years of age [57]. Underlying congenital heart disease may be a risk factor for *K. kingae* endocarditis. In a review by Foster et al., congenital heart disease was described in a third of reviewed cases, although a native valve was infected in 95 % of the cases [57]. *K. kingae* endocarditis in children is often severe, resulting in valve damage [9, 58, 59] or cerebral complications such as meningitis or stroke [57, 60–63].

Other Clinical Manifestations

Case reports have described *K. kingae* as the cause a number of other invasive infections, including soft tissue abscesses [18], meningitis [64], peritonitis [49], urinary tract infection [65], and pericarditis [66]. Ocular infections such as endophthalmitis [67] and keratitis [68] have also been reported. The routine use of molecular diagnostic techniques on various clinical specimens will likely lead to additional reports in the future and a better understanding of the role of *K. kingae* as an invasive pathogen.

Conclusions

Kingella kingae is an emerging pathogen worldwide. With the increasing incorporation of molecular diagnostic tests into routine microbiological practice, knowledge of the impact and clinical relevance of this organism is growing. Although reports of infections in adults exist, oropharyngeal carriage and invasive disease predominantly affect young children. OAIs such as septic arthritis, osteomyelitis, and spondylodiscitis as well as occult bacteremia disproportionately affect children less than 4 years of age. *K. kingae* is a prominent pathogen causing OAIs in young children, and septic arthritis is the most common form of *K. kingae* disease. The time to medical care is often delayed because of the relatively indolent nature of *K. kingae* disease, but outcomes are generally favorable. *K. kingae* endocarditis is a rare but significant infection in patients of all ages and tends to be associated with poor outcomes and significant complications in children.

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Pathogenesis of *Kingella kingae* Disease

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Colonization

The pathogenesis of *Kingella kingae* disease is believed to begin with colonization of the oropharynx. In order to colonize this site, the organism must adhere to respiratory epithelial cells. Early studies demonstrated that *K. kingae* expresses hair-like surface fibers and suggested that these fibers may be type IV pili, in particular given that they react with antibodies raised against *Kingella denitrificans* type IV pili. Kehl-fie et al. [1] confirmed that the *K. kingae* fibers are type IV pili and established that *K. kingae* type IV pili mediate adherence to respiratory epithelial cells [2]. The density of piliation correlates with three different colony morphologies when *K. kingae* is grown on solid medium: a spreading/corroding colony type associated with expression of abundant phase variable pili, a non-spreading/noncorroding colony type associated with sparse pili, and a domed colony type associated with no pili [3, 4].

Type IV pili are found in a wide variety of gram-negative species, with examples including enteropathogenic *Escherichia coli*, *Salmonella enterica serovar typhi*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Vibrio cholerae*. These polymeric surface fibers can be up to several μm in length and are composed primarily of a major pilus subunit. Type IV pili major pilus subunits share a number of characteristics, including a short hydrophobic signal peptide that is cleaved during pilus biogenesis, a methylated N-terminal residue, and two cysteine residues near the C-terminus [5]. Structurally, type IV pili major pilus subunits are predicted to have an N-terminal alpha-helical domain and a C-terminal globular domain [6–8].

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Type IV pili are unique in their ability to be retracted by the organism [9]. Retraction is responsible for a form of surface motility called twitching motility, allowing the organism to pull itself across a surface as a consequence of sequential rounds of extension, anchoring, and then retraction of pilus fibers [10]. Type IV pilus retraction is also involved in DNA uptake, an essential step in *K. kingae* natural competence.

Functional expression of type IV can require over 20 gene products, depending on the particular bacterial species. The main steps in type IV pilus biogenesis are as follows: The major pilus subunit (PilA1 in *K. kingae*) is translated as a pre-protein, which undergoes cleavage of the signal sequence by a dedicated inner membrane peptidase called PilD [11]. After processing, the mature subunit can be incorporated into the growing type IV pilus fiber by the pilus assembly complex, which contains the cytoplasmic protein PilM, the periplasmic proteins PilN, PilO, and PilP, the inner membrane spanning protein PilG, and the oligomeric outer membrane secretin PilQ [12–15]. Pilus extension is powered by ATP hydrolysis by the PilF (PilB in the *Pseudomonas* type IV pilus nomenclature) cytoplasmic assembly ATPase [16, 17]. Retraction of the pilus through the PilQ secretion is mediated by the PilT retraction ATPase [18, 19].

Gene deletion studies have revealed that *pilA1* and *pilF* are essential for type IV pilus expression in *K. kingae* [2]. The *pilT* gene is essential for pilus retraction, and deletion of *pilT* results in accumulation of dense nonretractile fibers [20]. Representative transmission electron micrographs of negatively stained wild type, *pilF* mutant, and *pilT* mutant derivatives of *K. kingae* strain 269–492 are shown in Fig. 1. Abundant pili are observed emanating from the surface of the wild-type strain (Fig. 1a) and the retraction-deficient *pilT* mutant (Fig. 1c) but are absent in the *pilF* assembly mutant (Fig. 1b). *K. kingae* contains minor pilins called PilA2 and FimB, which are likely incorporated into the pilus fiber but have unclear functions, as elimination of these proteins via deletion of the relevant gene has no effect on type IV pilus phenotypes [2].

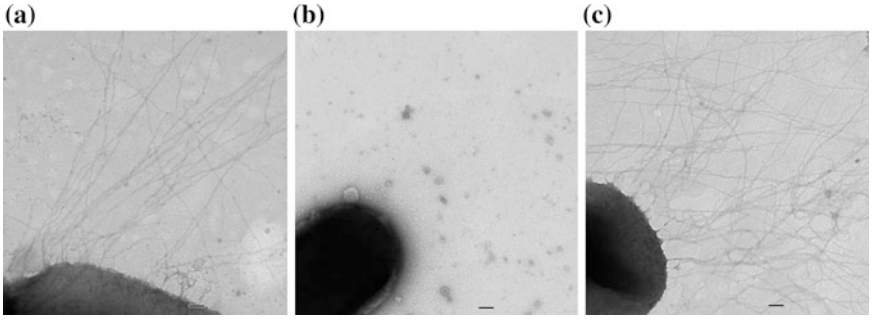

Fig. 1 Transmission electron micrographs of *K. kingae* strain 269–492 type IV pili surface fibers. **a** Abundant pili are seen emanating from the surface of wild-type strain 269–492. **b** Pili are absent from the surface of an isogenic *pilF* assembly ATPase mutant. **c** Mutation of the *pilT* retraction ATPase results in a hyper-piliated phenotype. Bar, 100 nm

The adhesive components of the *K. kingae* type IV pilus are likely the PilC1 and PilC2 pilus-associated proteins, which also play a role in pilus biogenesis [2, 21]. Both of these proteins have similarity to the well-characterized PilC1 and PilC2 pilus adhesins of the pathogenic *Neisseria* sp. and the PilY1 adhesin in *P. aeruginosa* type IV pili. However, unlike the PilC proteins in the pathogenic *Neisseria* sp., the *K. kingae* PilC proteins share limited amino acid sequence homology, with overall identity of 7 % and similarity of 16 % [2]. Elimination of either PilC1 or PilC2 results in a decrease in *K. kingae* adherence to cultured human epithelial cells, and elimination of both PilC1 and PilC2 results in a non-piliated phenotype [2, 21]. While there is not yet any direct evidence showing that the *K. kingae* PilC1 and/or PilC2 proteins mediate direct interaction with host cells, recent studies demonstrating that a *K. kingae* mutant that is able to express pilus fibers lacking PilC1 and PilC2 is non-adherent suggest that these proteins are the adhesive components of the fiber (Porsch and St. Geme, unpublished data). The host cell receptor for *K. kingae* type IV pili is currently unknown.

Similar to *P. aeruginosa* PilY1 and the pathogenic *Neisseria* sp. PilC proteins, both PilC1 and PilC2 in *K. kingae* contain calcium-biding sites [21–23]. Structural analysis of the PilY1 C-terminal domain showed a β-propeller fold containing a bound calcium ion, and mutational studies of the 9 amino acid calcium-binding pocket revealed that calcium binding is essential for the ability of PilY1 to potentiate PilT-mediated pilus retraction and twitching motility [23]. In *K. kingae*, both PilC1 and PilC2 contain calcium-binding pockets, with PilC1 containing a 9 amino acid PilY1-like calcium-binding site and PilC2 containing a 12 amino acid calmodulin-like calcium-binding site [21]. Calcium binding is necessary for PilC1 to facilitate twitching motility and adherence but has little to no effect on PilC2 function [21]. Although both PilC1 and PilC2 facilitate twitching motility and adherence, they appear to do so by different mechanisms.

The current model for *K. kingae* adherence to epithelial cells involves initial adherence by type IV pili, pilus retraction, and subsequent tight adherence mediated by the non-pilus trimeric autotransporter adhesin Knh (*Kingella* NhhA homolog). Trimeric autotransporters are members of the Type V Secretion family and contain an N-terminal signal sequence, an internal passenger domain, and a C-terminal β-barrel outer membrane anchoring domain [24]. The N-terminal signal sequence directs the protein through the Sec secretion system into the periplasm. The protein then integrates into the outer membrane as a trimer, with the C-terminal outer membrane domains trimerizing to form a pore. The internal passenger domain is translocated through this pore and is displayed on the bacterial surface. Trimerization of the passenger domain is facilitated by a coiled-coil domain adjacent to the outer membrane pore [25].

Knh is a 1783 amino acid protein with a predicted molecular mass of approximately 179 kDa. The N-terminal signal sequence consists of amino acids 1–54, and the C-terminal membrane domain consists of amino acids 1695–1783 [20]. Amino acids 55–1694 constitute the internal passenger domain, which is constructed in a modular manner and contains multiple YadA-like head domains, Trp domains, KG domains, Neck domains, and a single iSneck2 domain, as

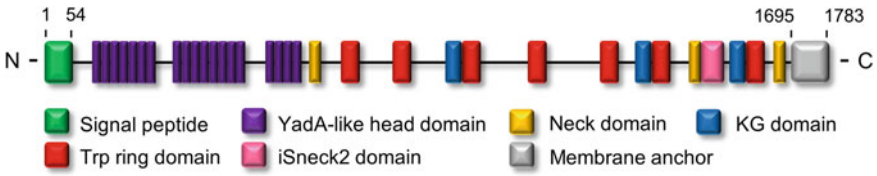

Fig. 2 Domain annotation of the trimeric autotransporter adhesin Knh expressed by *K. kingae* strain 269–492. The structural domain annotation was carried out using the Domain Annotation of Trimeric Autotransporter Adhesin (daTAA) program (toolkit.tuebingen.mpg.de/dataa) [26]

diagrammed in Fig. 2 using the Domain Annotation of Trimeric Autotransporter Adhesin program (daTAA) [26]. Mutation of *knh* results in an approximately 50 % reduction in adherence to epithelial cells, and full Knh-mediated adhesive activity is dependent on Knh glycosylation by the cytoplasmic N-linking glycosyltransferase called $HMW1C_{Kk}$ (the name $HMW1C_{Kk}$ reflects the homology to the well-characterized HMW1C glycosyltransferase in nontypable *Haemophilus influenzae*) [27]. $HMW1C_{Kk}$ modifies a least 32 asparagines throughout the Knh passenger domain [27].

Invasion

The pathogenic model of invasive *K. kingae* disease postulates that the source of invading bacteria is the colonizing population in the upper respiratory tract. According to this model, it should be possible to isolate genotypically identical bacteria from the oropharynx and the site of invasive disease (i.e., blood, joint fluid, and bone) in patients with invasive *K. kingae* disease. In support of this model, multiple studies have shown invading organisms to be genetically identical to colonizing isolates in individual patients [28, 29]. For the bacterium to transition from colonizer to invasive pathogen, it must breach the respiratory epithelial barrier in the oropharynx and enter the bloodstream.

Multiple epidemiological studies of patients with invasive *K. kingae* disease have revealed an association with antecedent or coincident viral infection. Yagupsky et al. [30] found that approximately 50 % of children with invasive *K. kingae* disease displayed symptoms of upper respiratory tract infection, 15 to 20 % had stomatitis, and 15 to 20 % had diarrhea. In agreement with these findings, a study in France found that 19/21 patients with proven *K. kingae* osteoarticular infection and only 3/8 patients with non-*K. kingae* osteoarticular infection had at least one respiratory virus present in the oropharynx, including human rhinovirus (12/21), coronavirus OC43 (4/21), parainfluenza virus 1, 2, 3, or 4 (3/21), enterovirus (2/21), or adenovirus (2/21) [31]. Of note, human rhinovirus infection is known to enhance bacterial adherence to respiratory cells and is associated with invasive bacterial infection [32, 33]. Recently, Houmami et al. [34] described a

series of five epidemic and four sporadic *K. kingae* osteoarticular infections between April and October of 2013 in a French hospital. Of the seven patients in this report who were younger than 24 months of age, five had antecedent hand, foot, and mouth disease and two had antecedent stomatitis. Both patients older than 2 years of age did not have evidence of antecedent or concurrent upper respiratory tract viral infection. Further analysis of an 11-month-old with septic arthritis and antecedent hand, foot, and mouth disease revealed the presence of *K. kingae* DNA in stored joint fluid and coxsackievirus-A6 in a stored stool specimen [34]. These studies strongly support the hypothesis that upper respiratory tract viral infection contributes to the development of *K. kingae* invasive disease, possibly related to virus-induced damage to the respiratory epithelium.

In addition to virus-induced damage to the respiratory epithelial barrier, *K. kingae* may breach the epithelium by means of a potent RTX cytotoxin. The *K. kingae* RTX toxin is a member of the repeats-in-toxin family and has been shown to be cytotoxic to a wide range of cell types in vitro, including respiratory epithelial cells, synovial cells, macrophage-like cells, and red blood cells [35]. The *K. kingae* RTX toxin was discovered following the observation that introduction of *K. kingae* onto a cell monolayer resulted in cell rounding and lysis. A transposon library screen identified a locus with high homology to the Mbx locus in *Moraxella bovis* that is absent in the less pathogenic *Kingella* species *K. oralis* and *K. denitrificans* [35]. This locus also has high homology to RTX toxins in *H. influenzae* and *N. meningitidis*, suggesting that *K. kingae* acquired the *rtx* genes through horizontal gene transfer [35]. Testing on a wide range of cultured human cells showed that synovial cells and macrophages are more sensitive to RTX toxin effects than respiratory epithelial cells, although respiratory epithelial cells are still lysed [35]. Additional investigation revealed that the RTX toxin is abundant in *K. kingae* outer membrane vesicles, which induce cytotoxicity when directly delivered to host cells [36].

The *K. kingae* RTX toxin contains glycine-rich nonapeptide calcium-binding repeats and is secreted by the type I secretion system [37]. It is a pore forming toxin and forms 0.94 nm radius pores in the host cell membrane that display voltage-dependent gating, with a channel conductance similar to general diffusion [37]. Comparison of a wild type and an isogenic RTX mutant strain in an infant rat model of invasive *K. kingae* disease revealed decreased virulence by the RTX mutant [38]. The *rtx* genes are present in all *K. kingae* strains examined to date, and the *rtxA* and *rtxB* genes have proven to be reliable targets for PCR-based detection of *K. kingae* in clinical samples [39–41].

Immune Evasion

Once *K. kingae* breaches the respiratory epithelial layer and enters the bloodstream, the organism must be able to evade host immune mechanisms in order to persist and disseminate to sites of invasive disease. While there are only a few studies

addressing potential *K. kingae* immune evasion strategies, current evidence suggests that expression of a polysaccharide capsule and antigenic variation of selected surface-displayed proteins play a role in survival of the bacterium during invasive disease.

Examination of *K. kingae* strains after 48 h of growth on chocolate agar reveals a striking mucoid colony morphology, consistent with production of a polysaccharide capsule [20]. A number of pediatric pathogens (e.g. *N. meningitidis*, *H. influenzae*, and *Streptococcus pneumoniae*) express a polysaccharide capsule, a layer of surface-associated polysaccharide chains that surround and 'encapsulate' the bacterium. Polysaccharide capsules are considered classical virulence factors and have been shown to play many roles in host–pathogen interactions, including masking of antigenic surface epitopes, prevention of complement factor deposition, prevention of phagocytosis, and host mimicry [42]. Disruption of a predicted *ctrABCD* capsule export operon in *K. kingae* eliminates the mucoid colony morphology and ablates the polysaccharide capsule, as highlighted in Fig. 3 [20]. Structural analysis of the polysaccharide capsule purified from *K. kingae* strain 269–492 revealed a repeating unit of N-acetylgalactosamine (GalNAc) and 3-deoxy-D-manno-octulosonic acid (Kdo) with the structure of $[\rightarrow 3)$-β-Gal*p*NAc-$(1 \rightarrow 5)$-β-Kdo*p*-$(2 \rightarrow]_n$ [43]. A mutant lacking surface capsule was significantly less virulent in the juvenile rat infection model, demonstrating the likely importance of capsule as a *K. kingae* virulence factor.

Further study by Starr et al. [44] established that the genetic requirements for encapsulation in *K. kingae* include the predicted *ctrABCD* export operon, *lipA*, *lipB*,

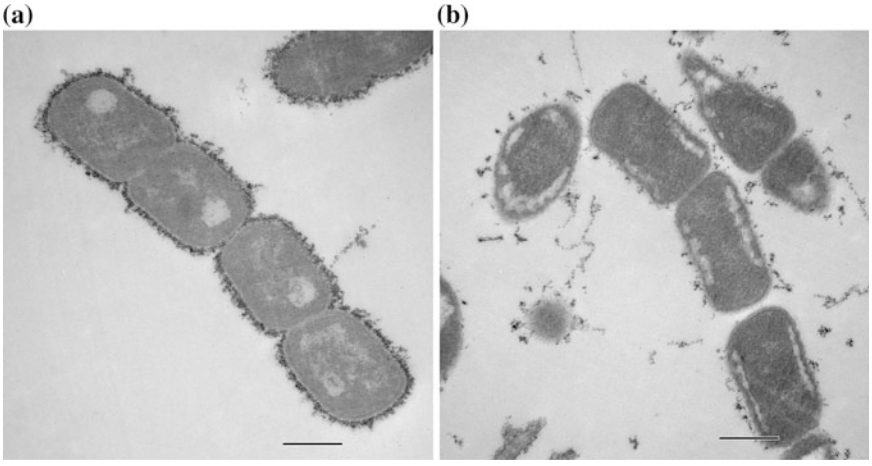

Fig. 3 Transmission electron micrographs of thin-sectioned cationic ferritin-stained wild-type *K. kingae* strain 269–492 (**a**) and an isogenic *ctrA* capsule export mutant (**b**). A layer of electron density due the cationic ferritin interactions with the surface capsular polysaccharide is visible on the surface of the wild-type strain (**a**) but is absent from the nonencapsulated ctrA mutant (**b**). Bar, 500 nm

and *csaA* (capsule synthesis region a gene A). Based on homology to the *N. meningitidis lipA* and *lipB* gene products and the *Escherichia coli kpsC* and *kpsS* gene products, the *K. kingae lipA* and *lipB* gene products likely function as retaining βKdo transferases that create the poly-βKdo linker that connects the polysaccharide to the phosphatidylglycerol membrane anchor [44–46]. Mutational studies revealed that CsaA is a bifunctional glycosyltransferase responsible for synthesizing the GalNAc-Kdo capsule polymer [44].

Examination of a large collection of Israeli healthy carrier and invasive disease isolates revealed a total of four *K. kingae* capsule types, including the GalNAc-Kdo-containing capsule (designated type a), an N-acetylglucosamine-Kdo-containing capsule with a [→6)-α-GlcpNAc (1→5)-β-Kdop-(2→] repeat unit (designated type b), a ribose-Kdo-containing capsule with a [→3)-β-Ribf-(1→2)-β-Ribf-(1→2)-β-Ribf-(1→4)-β-Kdop-(2→] repeat unit (designated type c), and a GlcNAc-galactose-containing capsule with a [→3)(β-Gal-(1→4)]-β-GlcNAc-(1→3)-α-GlcNAc-1-P-(O→] repeat unit (designated type d) [47] (Starr, Porsch, and St. Geme, submitted). In the Israeli collection of isolates, approximately 96 % of invasive disease isolates expressed either the type a or type b capsule. In contrast, only 70 % of carrier isolates expressed the type a or type b capsule, suggesting that the type a and type b capsule structures may provide a selective advantage for the organism in the bloodstream and contribute to the development of *K. kingae* invasive disease (Starr, Porsch, and St. Geme, submitted). Given that these initial studies focused on Israeli clinical isolates, further research is necessary to determine the global distribution of *K. kingae* capsule types.

Porsch et al. [20] demonstrated that the polysaccharide capsule is able to mask the adhesive activity of Knh. When functional pili are present, retraction of these appendages following initial adherence to host cells is thought to displace the polysaccharide capsule, allowing Knh to interact with the host cell and mediate high affinity adherence. A mutant lacking type IV pili but still expressing Knh is non-adherent, presumably because Knh is masked by the polysaccharide capsule. Adherence by this mutant can be restored by eliminating capsule expression, uncovering Knh, and allowing Knh access to host cell receptors [20]. While not demonstrated experimentally, it is likely that the polysaccharide capsule is also able to mask other surface antigens, many of which are smaller than Knh.

In addition to the outer membrane-associated polysaccharide capsule, *K. kingae* has also been found to secrete a galactofuranose exopolysaccharide homopolymer called PAM galactan. Bendaoud et al. [47] reported a PAM galactan structure of [→3)-β-Galf-(1→6)-β-Galf-(1→]$_n$ in *K. kingae* strain PYKK181, and Starr et al. reported a PAM galactan structure of [→5)-β-Galf-(1→]$_n$ in strain 269–492 [43]. The PAM galactan in strain PYKK181 was reported to have broad spectrum biofilm inhibitory activity, which may play a role in dispersal of the organism from a biofilm community or may prevent biofilm formation by competing bacteria in the upper respiratory tract [47]. The potential role of the PAM galactan as a virulence factor has not been investigated, but exopolysaccharides from other gram-negative bacteria have been shown to have protective roles during pathogenesis [48, 49].

K. kingae can cause uncomplicated bacteremia or can disseminate from the bloodstream to joints, bones, or the endocardium [50, 51]. Analysis of a collection of *K. kingae* systemic isolates showed that strains recovered from the blood of patients with uncomplicated bacteremia were generally piliated but typically expressed relatively few pili [52]. In contrast, strains recovered from joint fluid samples, bone aspirates, or the blood of patients with endocarditis were generally non-piliated [52]. It is possible that low-density piliation facilitates a tropism for joints, bones, and the endocardium and potentiates an inflammatory response, which in turn selects against piliated organisms. Consistent with this possibility, pili promote efficient adherence to cultured synovial cells. Similar to type IV pili expressed by other pathogens such as *P. aeruginosa*, *K. kingae* pili are regulated by a transcription factor called σ^{54} and by the PilS/PilR two-component sensor/regulator system [53]. Mutations in the PilS sensor result in a reduced density of pili, similar to the relative reduction in density of pili observed in isolates recovered from the bloodstream compared to isolates from the posterior pharynx. Mutations in the PilR response regulator completely eliminate piliation, similar to the absence of pili observed in isolates from joints and bones [53]. It is unclear which environmental factors influence the activity of σ^{54}, PilS, and PilR and thereby control the density of piliation in *K. kingae*. The spreading/corroding colony morphology associated with dense piliation, the non-spreading/non-corroding colony morphology associated with sparse piliation, and the domed colony morphology associated with no pili may reflect the relative activity of PilS and PilR [52].

Immunity

To investigate the role of the host immune system in development of *K. kingae* disease in healthy individuals, Slonim et al. [54] examined serum IgA and IgG levels against outer membrane proteins in 19 children with invasive disease. As expected, there were significant increases in serum IgA and IgG levels in children convalescing from invasive disease. Further study revealed that the age incidence of disease is inversely correlated with serum IgA and IgG levels in healthy individuals. Infants younger than 6 months of age have undetectable levels of IgA but high levels of serum IgG, suggesting that protection from invasive disease in this age group is due to maternally derived IgG [54]. Children 6 to 18 months of age have the highest incidence of disease and the lowest serum IgG levels [55]. Serum IgG and IgA levels in children 2 years of age and older progressively increase while the incidence of disease progressively decreases, suggesting that *K. kingae* carriage or exposure in the first 2 years of life may be an immunizing event. However, *K. kingae* outer membrane protein epitopes have been shown to be polymorphic among diverse strains, raising the possibility of strain specific immune responses that may not prevent recolonization by an antigenically distinct strain [56]. In agreement with these studies, many of the *K. kingae* surface factors examined to date show strain-to-strain variation, suggesting that immune pressure potentially

drives antigenic diversity in this species. The PilA1 subunit of type IV pili is expressed by all piliated strains, yet only 52 % of PilA1 amino acid residues are identical across a collection of clinical isolates [52]. Recent evidence also suggests that there is strain-to-strain sequence diversity in Knh and the PilC proteins (Porsch and St. Geme, unpublished data).

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Carriage and Transmission of *Kingella kingae*

Eric A. Porsch and Kevin J. Downes

Carriage

Kingella kingae is commonly carried on the tonsillar surfaces of young children [1]. Using selective media to facilitate recovery of *K. kingae,* Yagupsky et al. [2] collected cultures from the nasopharynx and tonsils of 48 healthy infants and toddlers every other week for 11 months. They found that 17.5 % of 624 tonsillar cultures grew *K. kingae,* while no nasopharyngeal specimens were positive. Similarly, Amit and coworkers performed serial cultures of the oropharynx and nasopharynx of 716 children between the ages of 2 and 30 months of age [3] and observed that 8.7 % of 4472 oropharyngeal cultures and only 1 of 4472 nasopharyngeal cultures were positive for *K. kingae* [3]. Figure 1 displays the prevalence of *K. kingae* oropharyngeal carriage among 624 children sampled longitudinally in this study [3].

Age is the most important factor influencing oropharyngeal carriage. In a random sample of all patients undergoing throat culture at a single institution in southern Israel from 1998 to 2001, the highest rate of oropharyngeal carriage was among young children less than 4 years of age [4]. Carriage rates were 3.2 % in children 0–3 years old, 1.5 % in children 4–17 years of age, and 0.8 % in individuals 18 years and older ($P < 0.001$ for trend). Isolation of the organism in children younger than 6 months of age is very rare [2, 3, 5], perhaps reflecting protection by transplacental antibodies. The decreasing rates of colonization beyond

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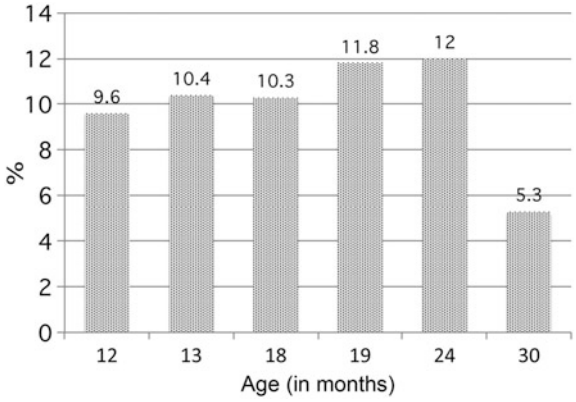

Fig. 1 Carriage of *K. kingae* between 12 and 30 months of age. Reproduced from Amit U et al. [3], with permission from Oxford University Press

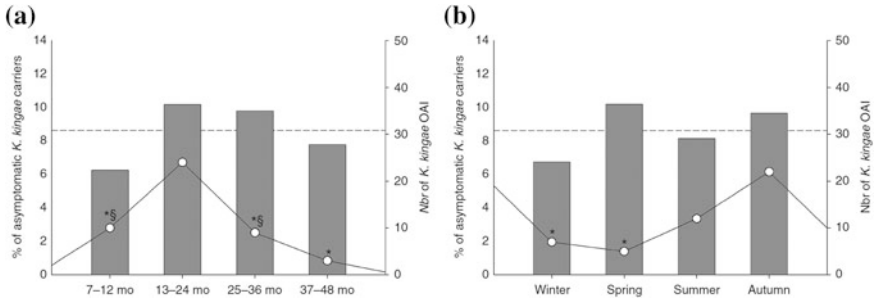

Fig. 2 *K. kingae* asymptomatic carriage rate and osteoarticular infection (OAI) cases by age group (**a**) and season (**b**). Bars represent carriage rates, white circles correspond to the number of OAI cases diagnosed between January 2009 and December 2012, and the dashed line marks the mean carriage rate. For carriage rate, data total $n = 744$ and for OAI cases, total $n = 46$. **a** $^*P < 0.001$ versus 13–24-mo OAI cases, §$P < 0.05$ versus 37–48-mo OAI cases; **b** $^*P < 0.001$ versus autumn OAI cases, Mann–Whitney U-test. Reprinted by permission from Macmillan Publishers Ltd: Anderson de Llana R, et al. *Pediatric Research,* volume 78(5), ©2015

4 years of age suggest that children may develop protective antibodies over time that prevent colonization at a later age [6].

In studies investigating respiratory tract colonization among children 6–48 months of age, the prevalence of asymptomatic oropharyngeal carriage ranges from 3 to 12 % [2–5, 7]. Andersen de la Llana and colleagues reported a mean prevalence of asymptomatic carriage of 8.7 % among their prospective cohort study of 744 healthy Swiss children aged 7–48 months [7]. Although slight variations based on age were found, there were no significant differences in carriage rate by age group. Figure 2 displays the asymptomatic carriage rate by age and season in this study [7]. There was no seasonal variation in *K. kingae* carriage among this Swiss cohort, differing from previous reports from Israel that described seasonal peaks [2, 4].

Children may carry *K. kingae* multiple times during early childhood, but there appears to be turnover of the specific strains that are carried. In the longitudinal study of daycare attendees by Yagupsky et al. [2] that sampled attendees every 2 weeks over an 11-month period, children carried *K. kingae* at varying frequencies and for different durations. While some children had short, intermittent carriage, others had repeatedly positive cultures over weeks or months. Nearly 75 % of children in this cohort carried *K. kingae* at least once, and half of all children carried the organism two or more times. Slonim et al. [8] assessed the genetic relatedness of 50 *K. kingae* strains isolated from this same population and found that individual children often carried specific strains continuously or intermittently for weeks or months until the strains were replaced by new ones. In the longitudinal study by Amit et al. [3], nearly 40 % (n = 283/716) of children were colonized at least once, and 11.1 % of children were colonized at least twice. Among the children with *K. kingae* isolated on multiple visits, 90 % of strains isolated within 2 months of one another were genetically similar, whereas only 22 % of isolates identified greater than 5 months apart were genetically similar. These data demonstrate that carriage of specific strains is relatively transient in healthy children, lasting weeks to months. Strains that colonize the oropharynx may be replaced, and long-term carriage by a specific strain is rare.

Because *K. kingae* is not known to colonize other body sites, oropharyngeal carriage is believed to be a prerequisite for the development of invasive infection. In a study by Ceroni et al. [9], all children 6–48 months of age presenting with osteoarticular infections over a 4-year period in Geneva, Switzerland had oropharyngeal swabs collected for *K. kingae*-specific PCR. *K. kingae* was detected in the oropharynx in 100 % (n = 30/30) of children with *K. kingae* osteoarticular infections and in only 7 % (n = 8/84) of children with non-*Kingella* osteoarticular infections. These same investigators assessed oropharyngeal carriage rates by PCR and tracked osteoarticular infections among healthy 6- to 48-month-old children in Geneva, Switzerland [10]. They found a carriage rate of 9 % and identified osteoarticular infections in just 0.8 % of carriers, demonstrating that the risk of invasive disease among carriers is low [10].

Bacterial density in the oropharynx does not differ between patients with osteoarticular disease and asymptomatic carriers [11]. Similarly, oropharyngeal colonization density does not vary by age [12]. Thus, while oropharyngeal colonization is a necessary step in the development of invasive disease, factors other than bacterial burden must influence development of colonization versus invasive disease.

Important genetic differences may exist between clones that are carried asymptomatically in the oropharynx and clones that cause invasive disease [13]. Yagupsky et al. studied the prevalence of beta-lactamase production and the genetic clonality of 190 invasive isolates and 429 randomly selected carriage isolates from Israeli patients and found a significant difference, with a prevalence of 1.1 % in invasive isolates and a prevalence of 15.4 % in carrier isolates ($P < 0.001$) [14]. The authors hypothesize that beta-lactamase production confers increased colonization fitness and may impede the invasive potential of these isolates.

Transmission

Relative to the general population, close contacts of children with *K. kingae* are at higher risk of acquiring the organism. In a two-year cohort study in Israel, daycare attendance was significantly associated with *K. kingae* carriage among Jewish children (odds ratio 8.86, 95 % CI: 2.74–28.64), suggesting that daycare facility attendance is a risk factor for colonization [5]. Other studies have also reported high rates of *K. kingae* carriage among daycare attendees [2, 8, 15–17]. The first longitudinal study to evaluate the person-to-person transmission of *K. kingae* strains among daycare facility attendees was conducted in Israel in the mid-1990s [8]. Fifty isolates were collected over an 11-month period by sampling two cohorts of daycare facility attendees every 2 weeks. These isolates were compared to a collection of 60 epidemiologically diverse respiratory tract and invasive isolates. Based on a variety of typing techniques, children were found to routinely carry the same strain continuously or intermittently for weeks or months. Different strains were able to periodically replace the originally detected strain. Interestingly, a single strain represented 28 % of the isolates and was most prevalent over the first 6 months of the study, and a different strain represented 46 % of the isolates over the remaining 5 months [8]. Epidemiologically unrelated strains showed much greater variability in relatedness compared to isolates from the daycare facility cohorts.

In addition to daycare facility classmates, siblings of *K. kingae* carriers are at higher risk for *K. kingae* acquisition. In a small cohort study examining *K. kingae* carriers and their 6- to 48-month-old siblings, 40 % of siblings of carriers (n = 10/25) were positive by PCR [18]. In addition, 53.8 % of siblings of patients with *K. kingae* osteoarticular infection were asymptomatic carriers. Conditional probability analysis indicated that there is an approximately 50 % chance that a sibling of an oropharyngeal carrier in the 6–48 month age range will carry the organism as well [18]. The high rate of asymptomatic carriage among children in close contact with one another implicates direct child-to-child transmission of colonizing strains. This observation has been corroborated in outbreak investigations, demonstrating indistinguishable *K. kingae* strains among daycare classmates [17].

The presence of Israeli Jewish and Bedouin populations in separate cities and towns in southern Israel enabled Yagupsky et al. [19] to study asymptomatic carriage and the molecular epidemiology of circulating *K. kingae* strains in these two populations. In the Bedouin population, which is traditionally nomadic but has recently settled in towns and has maintained ancestral tribal divisions, significant spatial clustering of *K. kingae* clones was detected in towns and households, suggesting interfamilial and close contact transmission [19]. Children in the Bedouin population typically do not attend daycare facilities, and most of their social interactions take place in family units or clans [19]. Conversely, no spatial clustering of *K. kingae* clones based on residency was observed in the Jewish population. It is speculated that there is obscured spatial distribution of *K. kingae* strains in Jewish children because they live in westernized conditions, attend

daycare facilities from an early age, and are connected by more and complex social interactions [19]. In agreement with the observation that there is very little personal and social interaction between the two groups, the strains isolated from Bedouin children tended to differ from those isolated from Jewish children [19].

The mechanism of person-to-person spread of *K. kingae* has not been directly investigated. Considering that *K. kingae* colonizes the oropharynx, it is likely that the organism is able to spread via droplets produced during coughing or sneezing. In addition, the propensity of young children to put objects in their mouths and the frequent sharing of toys in daycare facilities suggests that saliva may be a potential route of transmission.

Daycare Facility Outbreaks

To date, there have been a total of 10 reports of daycare facility outbreaks of invasive *K. kingae* disease, including two in the United States, two in France, and six in Israel [15–17, 20–25]. The first reported outbreak occurred in Minnesota in 2003 and involved three diagnosed cases of *K. kingae* osteomyelitis/septic arthritis in children 16–24 months of age from the same classroom [17]. Oropharyngeal sampling of other attendees of the same facility revealed an overall *K. kingae* carriage rate of 13 % (15/122) and a peak colonization rate of 45 % (9/20) in the classroom of 16- to 24-month olds. All three children had evidence of upper respiratory tract illness before or during the episodes of invasive *K. kingae* disease. In North Carolina in 2007, a case of confirmed *K. kingae* endocarditis and potential meningitis and a case of suspected intervertebral discitis and osteomyelitis were identified in children who attended the same daycare facility within a two-week period [20].

One case of confirmed *K. kingae* septic arthritis and four cases of presumed osteomyelitis occurred in France in 2011 during a one-month period in two adjoining classrooms of children aged 10–16 months [16]. The overall attack rate was 20.4 % (5/24), and 85 % (11/13) of the classmates who were present for oropharyngeal sampling and were not on antibiotics at the time were positive for *K. kingae* carriage [16]. During a two-week period in 2013, five children aged 11–17 months who attended the same classroom presented to local hospitals with osteoarticular infections (2 septic arthritis, 3 tenosynovitis) [15]. The attack rate was 23.8 % (5/21), and the carriage rate among classmates was 93.3 % (14/15). The investigation revealed that there was an outbreak of herpangina and hand, foot, and mouth disease with an attack rate of 38.3 % (8/21) two weeks prior to the first osteoarticular infection diagnosis. In addition, fever and upper respiratory tract infections were prevalent in the index classroom at the same time [15].

In Israel in 2005, one confirmed and two suspected cases of *K. kingae* skeletal infections were diagnosed in children from the same daycare facility [21]. Evaluation of the other children at the same facility established that 4 out of 11 were colonized. Genotyping analysis showed that invasive and oropharyngeal isolates

from the source daycare facility were genotypically identical and that oropharyngeal isolates from a control facility were genotypically distinct [21]. In 2012, one confirmed case of bacteremia and one presumed case of septic arthritis were identified [24]. The overall attack rate in the index classroom was 5.6 % (2/36), and the carriage rate among classmates was 11.8 % (4/34). In 2013, two cases of confirmed septic arthritis were identified in a classroom with an attack rate of 15.4 % (2/13); oropharyngeal sampling among classmates revealed a carriage rate of 45.4 % (5/11) [24]. In 2014, three additional outbreaks in Israel were identified [22, 25]. These outbreaks had a classroom attack rate of 14.3–16.7 % and a carriage rate among classmates of 33.3–58.3 % [22, 25]. Interestingly, four of the documented Israeli outbreaks occurred in so-called closed communities, including three on military bases and one in a "kibbutz" commune, suggesting that circulation of *K. kingae* among young children is facilitated by the social commonalities in these environments [25].

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Advances in Diagnosis of *Kingella kingae* Disease

Pablo Yagupsky

Culture of *Kingella kingae* from Skeletal System Specimens

Although *Kingella kingae* was first identified and characterized in the 1960s, publications on *K. kingae* disease were limited to a few case reports and small case series over the ensuing few decades, leading to the impression that this organism is an unusual cause of human disease [1]. The role of *K. kingae* as an important pediatric pathogen began to emerge in the 1990s, catalyzed by a pioneering study in which synovial fluid aspirates obtained from young children with arthritis were inoculated into blood culture vials (BCVs) in an attempt to improve the bacteriologic diagnosis of joint infections [2]. In this study, 11 of 100 aerobic pediatric BCVs of the BACTEC 460 radiometric system (Becton Dickinson, Cockeysville, MD) inoculated with synovial exudates grew the organism compared to only one of the conventional cultures performed in parallel on routine solid media [2]. Following this initial observation, other studies convincingly demonstrated that inoculation of synovial fluid specimens into aerobic BCVs from a variety of commercial blood culture systems such as the BACTEC (Becton Dickinson, Cockeysville, MD) [2, 3] and BacT/Alert (Organon Teknika Corporation, Durham, N.C.) [4–7] series of instruments, Hémoline DUO (bioMérieux, Paris, France) [8, 9], and Isolator 1.5 Microbial Tube vials (Wampole Laboratories, Cranbury, N.J.) [10], and an in-house-made liquid medium [11] significantly improves the detection of *K. kingae* relative to traditional culture methods [2, 12–14]. The question of which automated blood culture system is best for recovering *K. kingae* from skeletal system infections was addressed by Høst et al. using an in vitro model in which different *K. kingae* strains and pooled synovial fluid from adult patients with noninfectious conditions were inoculated into BCVs [7]. Of the 24 tested strains,

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all 24 were detected in BacT/Alert Aerobic and BacT/Alert Pedi-bacT bottles, 21 (87.5 %) were detected in BacT/Alert FAN aerobic bottles, and 15 (62.5 %) were detected in BACTEC Peds Plus vials. The time to detection in the three BacT/Alert vials was significantly shorter than in the comparator, suggesting that this blood culture system is superior to the BACTEC and may reduce the number of cases of culture-negative septic arthritis [7]. However, in a study by Wilms et al., when BacT/Alert 3D vials were spiked with different quantities of *K. kingae*, the organism was not detected by the automated instrument despite prolonged incubation [15]. Thus, the question is far from settled, with no in vivo validation to date employing clinical specimens from actual pediatric patients [3, 7].

When BCVs detected as positive by the automated blood culture instruments are subcultured onto routine media such as blood agar and chocolate agar plates, *K. kingae* grows readily, indicating that solid media adequately support the nutritional requirements of the organism. This observation suggests that synovial fluid possesses potent antibacterial properties [16], hindering the recovery of *K. kingae* and possibly other fastidious organisms. Consistent with this conclusion, dilution of the small volumes of synovial fluid aspirated from a small pediatric joint in a large volume of liquid medium improves the recovery of *K. kingae* [2], presumably related to decreased concentration of inhibitory factors. In studies conducted in Israel and France in which synovial fluid aspirates obtained from children less than 3 years of age with arthritis were inoculated into BCVs, *K. kingae* was isolated in 48 % of the patients with culture-proven disease [17, 18]. The Isolator 1.5 Microbial Tube enables quantitation of the original number of bacteria in a biological fluid and recovery of already phagocytosed but still viable organisms [10]. Use of this blood culture system for processing joint aspirates has established that the concentration of *K. kingae* in synovial fluid is low and that many organisms have been engulfed by neutrophils, adding to the difficulties of recovering the organism [10].

Despite the impressive improvement in detection of *K. kingae* by BCVs compared to traditional bacteriologic methods, recovery of the organism from skeletal system exudates remains unsatisfactory. In a study by Gené Giralt et al. [19] comparing pediatric and adult BacT/Alert BCVs and examining factors that influence the isolation of *K. kingae*, the pediatric vial was clearly inferior to the adult vial in terms of sensitivity and time to detection of growth and was improved by supplementation of the culture broth with human blood. Additional work by the same investigator has demonstrated wide differences among *K. kingae* clinical isolates in terms of their ability to grow in BacT/Alert BCVs, with improved growth by all strains when BCVs are supplemented with human blood (Gené Giralt personal communication). Supplementation of BCVs with human blood may reproduce the features of many synovial fluid exudates that become contaminated with blood during aspiration of joint fluid or arthrotomy, thus mimicking real life conditions. Together these studies suggest that *K. kingae* has important unidentified

growth requirements and that supplementation of BCVs with human blood may be a practical means for improving detection of this elusive pathogen.

Detection of *K. kingae* in Blood Cultures

Epidemiologic evidence suggests that the pathogenesis of invasive *K. kingae* disease begins with colonization of the oropharyngeal mucosa by a virulent strain and then involves breaching of the respiratory epithelium, perhaps facilitated by the *K. kingae* RTX toxin, by a concomitant viral infection, or by the interaction of the RTX toxin and viral infection [12, 20–24]. Subsequently, the organism enters the bloodstream and in some cases then seeds a joint, a bone, or the endocardium, sites for which *K. kingae* exhibits a particular tropism.

Naturally, blood cultures play a critical role in the diagnosis of *K. kingae* bacteremia without focal infection ("occult bacteremia"), which is the second most common presentation of invasive pediatric infections caused by the organism [12], and in establishing the etiology of *K. kingae* endocarditis [25]. Modern blood culture systems such as the BACTEC or BacT/Alert enable detection of the organism within 4 days, and prolonged incubation of BCVs does not improve recovery [26]. Because the pathogenesis of skeletal system infections in children usually involves a bacteremic phase, blood cultures have also been advocated for determining the etiology of suppurative arthritis and osteomyelitis. Blood samples are easy to obtain compared to joint and bone exudates and tissue specimens and are positive in approximately 40 % of pediatric patients with osteomyelitis [27] and approximately 50 % of pediatric patients with septic arthritis [28, 29]. The sensitivity of blood cultures for diagnosing *K. kingae* osteoarticular infections is much lower. In particular, among 81 children with culture-proven *K. kingae* skeletal system infections, the organism was recovered from joint or bone exudates in 74 (91.4 %), from the blood in 6 (7.4 %), and from both synovial fluid and blood in a single patient (1.2 %) (Yagupsky, unpublished data), suggesting that the bacteremic phase of the disease is transient and of short duration.

Culture Detection of *K. kingae* in Respiratory Cultures

K. kingae has been clearly established as a member of the resident oropharyngeal microbiota. The colonized mucosal surface of the oropharynx is the portal of entry to the bloodstream for invasive strains [30] and the source of person-to-person transmission of the organism [31, 32]. Because of the high density of the resident flora in oropharyngeal specimens, *K. kingae* is difficult to detect by culture of upper respiratory tract specimens and can be entirely missed. To facilitate the recovery of

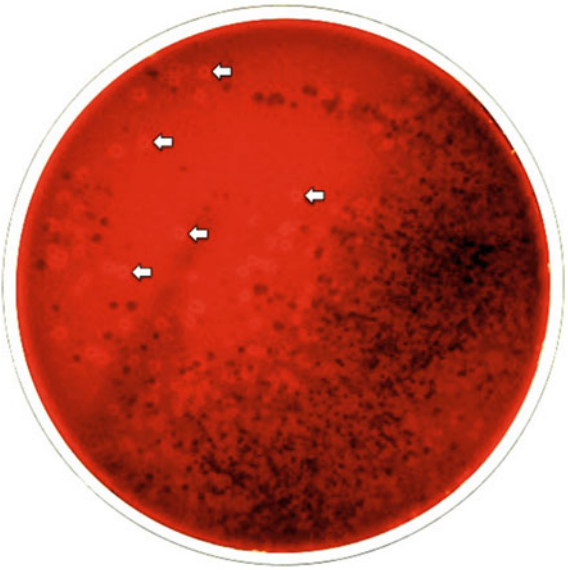

Fig. 1 Oropharyngeal specimen cultured on BAV medium. Note the conspicuous β-hemolytic colonies of *K. kingae* (*arrows*)

K. kingae, a selective medium that consists of blood agar with added vancomycin at a concentration of 2 mcg/mL (BAV) has been developed. Inoculated BAV plates are incubated in aerobic conditions at 35 °C in a 5 % CO_2-enriched atmosphere and examined over two days. The rationale for BAV medium and the associated culture conditions is to accentuate the β-hemolysis surrounding *K. kingae* colonies with blood agar, to suppress gram-positive bacteria with vancomycin, and to inhibit growth of anaerobic members of the buccal and oropharyngeal flora with aerobic conditions (Fig. 1). In a prospective blinded evaluation, 34 positive pharyngeal cultures were detected by the BAV medium, 9 positive cultures were detected by both the BAV medium and routine blood agar plates, and only one positive culture was detected exclusively by routine blood agar plates ($P < 0.001$) [33]. The original BAV medium [33] and a similar medium based on Columbia agar [34] have been successfully employed in epidemiological studies investigating respiratory carriage of the organism and the dissemination of the invasive strains among attendees of day care facilities where clusters of *K. kingae* disease have occurred [35–39].

Confirmation of *K. kingae* as the etiology of pediatric septic arthritis, osteomyelitis, and spondylodiscitis is hampered by the low yield of blood, synovial fluid, and bone exudate cultures and by the fact that joint and intervertebral disk aspirates and bone biopsies are obtained infrequently in young children [40, 41]. To overcome these difficulties, demonstration of *K. kingae* in the oropharynx has been proposed as a means to establish the bacteriological diagnosis of bone or joint infections and spondylodiscitis when cultures are not obtained or are negative [34, 41, 42]. This approach has the obvious advantage of being noninvasive and easy to perform. However, the predictive value of a positive pharyngeal culture is limited, because *K. kingae* is normally carried in the respiratory tract by 10–12 % of

children in the 6 months to 4 years age-group [43] and an even higher percentage of children attending day care facilities [42, 44].

Oropharyngeal cultures on BAV medium have been adopted as part of the routine evaluation of children younger than 4 years presenting to the emergency department at Soroka University Medical Center in Israel with monoarthralgia or monoarthritis, suspected osteomyelitis, or spondylodiscitis. In a substantial fraction of children lacking a positive blood or skeletal system culture or an alternate diagnosis, *K. kingae* is isolated from the oropharynx, and typing reveals that the recovered strain belongs to one of the invasive clones (as determined by pulsed-field gel electrophoresis) that are usually recovered from patients with positive cultures of blood, joint fluid aspirate, or bone exudate in the country (unpublished data).

Although it seems reasonable to conclude that failure to isolate *K. kingae* from an oropharyngeal culture probably excludes the organism as the cause of an osteoarticular infection, the sensitivity of an oropharyngeal culture as the sole diagnostic test for invasive *K. kingae* disease has not been established, precluding the routine use of this approach at this point in time.

Detection of *K. kingae* by Nucleic Acid Amplification Assays

Because of the risk for long-term orthopedic sequelae in pediatric patients with bone and joint infections, prompt bacteriological confirmation of the diagnosis and early initiation of appropriate antibiotic therapy are critical to prevent disability. However, even when specimens are inoculated into BCVs, a substantial fraction of infections remains bacteriologically unconfirmed. The diagnosis of *K. kingae* septic arthritis and osteomyelitis is further complicated by the fact that the microscopic examination of the exudate rarely reveals the presence of *K. kingae* [12, 45, 46], probably because of the low bacterial concentration (range 8–300 colony forming units (CFU)/mL, median 15 CFU/mL) [10] and the difficulty in visualizing gram-negative bacilli against the pink-stained fibrin background [47–54].

In recent years, the development and gradual adoption of nucleic acid amplification assays (NAAAs) have revolutionized the field of infectious diseases. These culture-independent methods exhibit improved sensitivity compared to cultures, enabling detection of fastidious and uncultivable bacteria, making possible the determination of the etiology of infections in patients already being treated with antibiotics, and shortening the time to detection and identification of the causative organism from days to a few hours [55, 56]. Therefore, NAAAs are an attractive alternative to culture detection of *K. kingae* infections and are being employed increasingly to diagnose the presence of this organism.

The technology of NAAAs has evolved over the years from the initial conventional PCR to real-time PCR that is less prone to contamination and from the

use of universal primers to primers that amplify species-specific targets and show improved sensitivity. The use of universal primers involves extraction of DNA from clinical samples, incubation of the DNA with broad-range oligonucleotide primers that anneal to constant regions of the 16S rRNA gene, and amplification of the intervening sequence, which varies according to the bacterial species [48, 57]. The resulting amplicons are then sequenced and compared with sequences deposited in the Genbank database for species assignment, making it possible to diagnose a wide array of different bacterial pathogens. Alternatively, the amplicons may be hybridized with species-specific probes belonging to the most plausible etiologies chosen on the bases of clinical and/or epidemiological considerations. In a seminal study by Stahelin et al. [57], DNA was extracted from the synovial fluid aspirate from a young child with culture-negative arthritis. Amplification of the 16S rRNA gene followed by sequencing of the amplicon revealed the presence of *K. kingae* [57]. Use of the same strategy allowed Moumile and coworkers to detect *K. kingae* in 17 children with septic arthritis and one child with osteomyelitis; in this study, blood culture was positive in only one (5.9 %) child, the conventional culture of joint or bone exudate was positive in 2 of 15 (13.3 %) children, and BCVs inoculated with joint or bone exudate were positive in 11 of 17 (64.7 %) children [48]. In a comparative study, Rosey et al. [58] detected *K. kingae* by the combined use of cultures on solid media and in BacT/Alert BCVs in 6 of 94 pediatric patients with suspected septic arthritis, whereas a real-time NAAA targeting the 16S rRNA gene detected *K. kingae* in 15 additional patients with culture-negative samples. In a study by Verdier et al. [59] in which 171 children with suspected osteoarticular infections were enrolled, cultures (both conventional and BCVs) confirmed the disease in 64 (37.4 %) children and demonstrated *K. kingae* in 9 children. The 107 culture-negative specimens were examined by 16S rRNA PCR, and 15 samples were positive, all yielding *K. kingae* DNA sequences [59]. The performance of NAAAs that employ universal primers for detecting *K. kingae* is summarized in Table 1.

More recently, PCR assays that amplify *K. kingae*-specific targets have been developed and have been associated with higher sensitivity and reliability [60–68]. The RTX toxin is produced by all *K. kingae* strains, and thus the RTX locus genes

Table 1 Performance of cultures and nucleic acid amplification assays targeting the 16S rRNA gene for detecting *Kingella kingae* in children with joint and bone infections

Year	Reference	Culture method	+*K. kingae* culture/total	PCR method	C-PCR target gene	RT-PCR target gene	+PCR/total
1998	[57]	C	0/1	C	16S rRNA	NA	1/1
2003	[48]	C + BCV	13/18	C	16S rRNA	NA	18/18
2005	[59]	C + BCV	9/24	C	16S rRNA	NA	24/24
2007	[58]	C + BCV	6/21	C + RT	16S rRNA	16S rRNA	20/21
Total (%)			28/64 (43.8)				63/64 (98.4)

C conventional; *BCV* blood culture vial; *RT* real-time; *NA* not applicable

Table 2 Performance of culture methods and nucleic acid amplification assays targeting species-specific DNA sequences in children with *Kingella kingae* infections of the skeletal system

Year	Reference	Culture method	+*K. kingae* culture	PCR method	C-PCR target gene	RT-PCR target gene	+PCR/Total
2007	[61]	C + BCV	17/39	C + RT	16S rRNA	*cpn*60	39/39
2008	[70]	BCV	2/4	C + RT	16S rRNA	*cpn*60	4/4
2008	[40]	C	0/1	C	16S rRNA	NA	1/1
2009	[60]	C + BCV	7/31	RT	ND	*cpn*60	31/31
2009	[62]	C	0/2	C + RT	16S rRNA	*rtx*A + *rtx*B	2/2
2009	[73]	C + BCV	5/18	RT	ND	?	18/18
2010	[63]	C	0/23	RT	ND	*rtx*A + *rtx*B	23/23
2011	[64]	ND	2/20	RT	ND	*rtx*A	20/20
2013	[66]	C + BCV	5/44	RT	ND	*cpn*60	43/44
2013	[69]	C	?	C + RT	16S rRNA	*cpn*60	11/11
2014	[67]	C + BCV	2/24	C + RT	ND	*rtx*A	27/27
2014	[71]	C	0/3	RT	ND	*cpn*60	3/3
Total			40/209 (19.1 %)				324/325 (99.7 %)

C conventional; *BCV* blood culture vial; *RT* real-time; *ND* not done; *NA* not applicable

appear to be pertinent targets for molecular detection of the bacterium in normally sterile body fluids and oropharyngeal specimens [62–64, 67, 68]. The *cpn*60 gene that encodes the *K. kingae* chaperonin 60 protein responsible for assisting misfolded or unfolded proteins to reach their native state has also been employed successfully for this purpose [60, 61, 66, 69–71].

The NAAAs developed to amplify conserved segments of the *rtx*A and/or *rtx*B genes that encode the RTX toxin have a one order of magnitude higher sensitivity than PCR tests that target the 16S rRNA gene [61, 66, 72] or the *cpn*60 gene [64], are highly specific, can be applied to a wide variety of clinical specimens, and allow detection of *K. kingae* strains exhibiting RTX locus polymorphisms, including non-synonymous ones [72]. The published data on the performance of species-specific NAAAs for the detection of *K. kingae* in skeletal system specimens are summarized in Table 2.

Chometon and coworkers sequentially analyzed bone and joint samples using blood culture vials, conventional PCR with universal primers, and real-time PCR with primers derived from the *K. kingae cpn*60 gene and showed that the detection rate of the organism increased from 29 to 41 % and 45 %, respectively, of all microbiologically proven cases [61]. These results made *K. kingae* the most common cause of suppurative arthritis and osteomyelitis in their series, accounting for ~80 % of cases among children younger than 3 years [61]. In a study by Ilharreborde et al. [60] that included 89 children with suspected septic arthritis, joint fluid was cultured using BCVs and was examined by a real-time PCR assay employing highly specific primers that amplify a 169-bp fragment of the *K. kingae cpn*60 gene (Fig. 2). The diagnosis of septic arthritis was confirmed by culture in 36

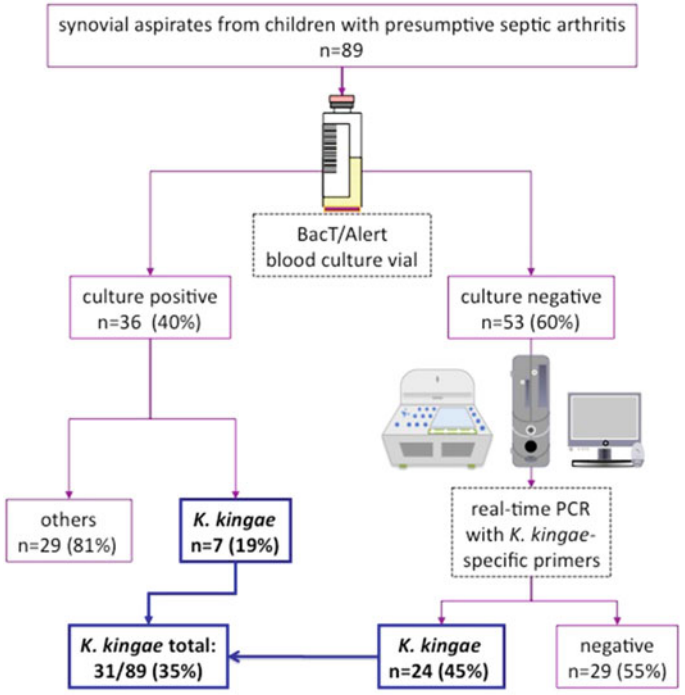

Fig. 2 Detection of *K. kingae* from synovial fluid aspirates from children with arthritis by the combined use of BCVs and PCR with species-specific probes (adapted from [60])

(40 %) specimens, including 7 that grew *K. kingae*. Real-time PCR identified 24 additional cases of *K. kingae* infection among the 53 culture-negative patients. Of note, the NAAA detected *K. kingae* in all 7 samples that grew the bacterium and was negative in all specimens in which other microorganisms were identified, indicating that the test is highly sensitive and highly specific. Altogether, *K. kingae* was present in 31 (51.7 %) of the 60 bacteriologically documented cases [60].

In a large study conducted in a French pediatric orthopedic unit, bone and joint exudates from children with suspected skeletal system infections were inoculated into BacT/Alert BCVs and subjected to amplification of the *K. kingae cpn*60 gene. *K. kingae* was detected by BCVs and/or NAAAs in 35 of 46 (76.1 %) children under 4 years of age with septic arthritis and in 9 of 17 (52.9 %) children younger than 4 years with osteomyelitis or concurrent septic arthritis and osteomyelitis [66]. The standard culture on solid media failed to recover the organism in all 44 patients with NAAA-confirmed *K. kingae* bone and/or joint infection. The BCVs were positive in only five specimens (including four exudates and one blood culture), and a positive NAAA was the sole source of diagnosis of *K. kingae* in 39 of the 44 (88.6 %) patients [66]. Similar results were obtained by Juretschko et al. [73], who compared the yield of BacT/Alert FAN or PEDs-F blood culture bottles with the

yield of real-time PCR in children with osteoarticular infections. Overall, the BCVs were positive in only 5 of 21 (23.8 %) specimens positive for *K. kingae* by the NAAA.

A sequential approach was employed by Levy et al. in adult and pediatric patients with culture-negative osteoarticular infections. Initially, the skeletal system exudates were subjected to amplification of the 16S rRNA gene and the amplicons resulting from positive tests were sequenced [69]. *K. kingae* accounted for 8 % of the organisms identified and was found exclusively in children. Positive results for *K. kingae* obtained with the broad-range PCR primers were confirmed in all cases by amplification of specific *cpn*60 gene sequences [69].

The relative merits of universal and *K. kingae*-specific primers have been compared in vitro by Cherkaoui et al, who found that the PCR assay targeting the *rtx*A gene exhibited a sensitivity of 30 CFUs, an order of magnitude higher than the semi-nested broad-range NAAA that amplified the 16S rRNA gene [62]. This large difference in the sensitivity of the molecular tests has important diagnostic implications, because the concentration of *K. kingae* organisms in the synovial fluid is usually below 300 CFU/mL, explaining the relatively poor performance of the NAAAs that targets the universal 16SrRNA gene. These experimental results were recently confirmed in an in vivo study by Filleron et al, who found that the PCR-specific assay detected *K. kingae* in 54 patients and the PCR assay using universal primers was positive in only two patients [68]. Similarly, Ferroni et al. found that amplification with species-specific *cpn*60 primers succeeded in 9 of 44 (20.5 %) cases of skeletal *K. kingae* infections in which the universal 16S rRNA primers failed [66]. Because *K. kingae* is far and away the most common etiology of septic arthritis and osteomyelitis below the age of 4 years, the researchers advised the use of NAAAs that target *K. kingae*-specific sequences as the initial diagnostic test for joint and bone specimens from children in this age-group, reserving the tests that amplify the 16S rRNA gene for exudates in which *K. kingae*-specific assays yield negative results [66].

Of note, Grivea and colleagues reported that the NAAA targeting the *rtx*A gene was still positive on joint fluid samples from a child with *K. kingae* arthritis 5 days after initiation of antibiotic treatment [74]. Filleron et al. reported similar findings using NAAAs that amplify the 16S rRNA universal target or the *K. kingae*-specific RTX toxin-encoding genes in joint and bone specimens [68]. Together, these reports indicate that the molecular diagnosis of *K. kingae* infections may still be possible in patients with partially treated disease.

In summary, the accumulated clinical experience clearly demonstrates that culture recovery of *K. kingae* from skeletal system exudates remains unsatisfactory, even when specimens are inoculated into BCVs. NAAAs that amplify the 16S rRNA gene sequence double the detection rate of *K. kingae* in skeletal system exudates, and NAAAs that amplify *K. kingae*-specific DNA sequences result in a 5-fold increase in yield, even in antibiotic treated patients (Tables 1 and 2). Use of molecular tests reduces the fraction of culture-negative infections, shortens the time to detection, and confirms that *K. kingae* is the leading bacterial etiology of suppurative arthritis and osteomyelitis in young children. However, while the

sensitivity of *K. kingae* cultures is suboptimal compared to NAAAs, isolation of the organism has the obvious advantages of enabling antibiotic susceptibility testing and genotyping of isolates, two important considerations for the clinical management of patients and for the study of the epidemiology and the pathogenesis of disease due to *K. kingae*.

Use of NAAAs to Detect *K. kingae* in Oropharyngeal Specimens

Because of the technical difficulties posed by the aspiration of pediatric joints and bones and the relative inaccessibility of intervertebral disk samples, these normally sterile body fluids and exudates are not always available for culture or NAAA testing. Given that colonization of the pharyngeal mucosa precedes invasion of the bloodstream and skeletal system [32], search for the presence of the organism and/or its specific DNA sequences in oropharyngeal specimens has been advocated as a surrogate detection strategy [34, 41, 42].

The sensitivity of cultures and NAAAs for detecting *K. kingae* colonization has been compared in a single study in which the yield of both methods was evaluated in the investigation of a large cluster of invasive infections in a French day-care center facility [75]. Pharyngeal specimens that were obtained from attendees and members of the staff of the facility to assess the spread of the organism among contacts of the index cases were cultured by the BAV method and analyzed by a PCR assay that targeted the *cpn*60 gene [75]. Twelve of 18 (66.7 %) individuals were positive by real-time PCR, and 6 of 18 (33.3 %) individuals were positive by culture on modified BAV medium ($P < 0.01$), indicating that NAAAs are more sensitive than cultures to determine the true carriage state. 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 for eradicating *K. kingae* from colonized children, cultures have the advantage of detecting living bacteria, whereas the viability of *K. kingae* organisms in NAAA-positive/culture-negative specimens is unclear [75].

Basmaci et al. [34] grew *K. kingae* from pharyngeal cultures of 8 of 12 NAAA-positive/culture-negative synovial fluid specimens of young children with arthritis. These investigators 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 and 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 [34].

Ceroni et al. [75] employed a real-time PCR assay that amplifies the *rtx*A and *rtxB* genes and detected *K. kingae*-specific sequences in the oropharynx of 8.1 % of

431 young asymptomatic children and in all 27 patients with culture positive and/or NAAA-proven invasive infections. More recently, the same investigators detected *rtx*A gene sequences in the oropharynx of all 10 children aged 6–48 months with a radiographically confirmed diagnosis of spondylodiscitis, suggesting that *K. kingae* was the causative agent [41]. In a study in which 123 patients aged 6–48 months with skeletal system complaints were enrolled, all 30 children in whom the joint exudate was positive for *K. kingae* by culture and/or NAAA had a positive molecular test on an oropharyngeal specimen [42]. However, in the same study, 8 pharyngeal samples obtained from 84 children (9.5 %) with microbiologically unconfirmed arthritis or osteomyelitis or in whom other bacteria were detected were also positive for *K. kingae*-specific DNA sequences [42]. These observations suggest that the sensitive NAAAs have a high negative predictive value and that failure to detect *K. kingae*-specific genomic sequences in an oropharyngeal specimen practically excludes *K. kingae* as the etiology of a skeletal system infection. On the other hand, because of the high background respiratory carriage of *K. kingae* in children 6–48 months of age, and especially among attendees to out-of-home care facilities, the predictive value of a positive pharyngeal NAAA, as well as isolation of the organism from the upper respiratory tract, is limited.

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Antibiotic Susceptibility of *Kingella kingae*

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Antibiotic Susceptibility

Because the true importance of *K. kingae* as an invasive human pathogen has only been recently recognized, species-specific microbiological, clinical, and pharmacokinetic/pharmacodynamic break points have not yet been defined. Information on the antibiotic susceptibility patterns of *K. kingae* remains limited primarily to reports on individual patients or small series of patients. In these reports, the methodology for determining susceptibility (including culture media, incubation conditions, and incubation duration) has been inconsistent and the interpretive criteria for susceptibility have varied.

Information conveyed by these anecdotal data suggests that *K. kingae* isolates are generally susceptible to aminoglycosides, macrolides [1], co-trimoxazole (trimethoprim-sulfamethoxazole) [2], fluoroquinolones, tetracycline, and chloramphenicol, with rare exceptions [3–8]. *K. kingae* is resistant to trimethoprim and has relatively high MIC values to isoxazolylpenicillins such as oxacillin, cloxacillin, and dicloxacillin (MIC_{50}: 3 μg/mL; MIC_{90}: 6 μg/mL). The organism is often resistant to clindamycin and is always resistant to glycopeptide antibiotics such as vancomycin, a serious concern in areas where joint and bone infections caused by community-associated methicillin-resistant *Staphylococcus aureus* are prevalent, and clindamycin or vancomycin is initially administered to children with osteoarticular infections, pending culture, and antibiotic susceptibility results [9–11].

In the absence of species-specific criteria for *K. kingae*, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has recommended use of

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general pharmacokinetic/pharmacodynamic-based break points [12]. The US Clinical and Laboratory Standards Institute (CLSI) has formulated general guidelines for the antibiotic susceptibility testing of the HACEK group of microorganisms, which includes *K. kingae*. Although it is widely accepted that susceptibility break points need to be specifically tailored to different bacterial species or closely related organisms, these uniform recommendations gather together heterogeneous and taxonomically unrelated bacteria chosen on the common grounds of gram-negative staining, fastidious growth, commensal nature, a shared oropharyngeal niche, and association with bacterial endocarditis, disregarding their profound biologic differences. Equally odd, the interpretive criteria for chloramphenicol for the HACEK group have been adapted from the criteria formulated for *Streptococcus* species and for the other antimicrobial drugs have been extrapolated from the criteria for *Haemophilus influenzae*.

The CLSI guidelines recommend the use of cation-adjusted Mueller–Hinton broth with added 2.5–5 % lysed horse blood, a 0.5 % McFarland standard concentration inoculum, and incubation for 24–48 h at 35 °C in aerobic conditions in a CO_2-enriched atmosphere [13]. However, thus far no large-scale results on the antibiotic susceptibility of *K. kingae* based on these principles have been published.

Under the auspices of the EUCAST, a recent study investigated the antibiotic susceptibility of *K. kingae* in a systematic fashion, employing a uniform protocol and well-standardized culture conditions. The MIC values for 13 clinically relevant antimicrobial agents were determined in a collection of 159 *K. kingae* strains recovered from asymptomatic carriers and patients with invasive infections from diverse geographic origins, employing the broth microdilution method and following the guidelines of the ISO standard 20776-1 with EUCAST media for fastidious organisms [14, 15]. In addition, antibiotic susceptibility testing was performed in parallel by the disk diffusion method according to the EUCAST recommendations for fastidious organisms and was correlated to the corresponding MIC values (examples in Fig. 1). The frequency of wild-type isolates (i.e., those that lack acquired antibiotic resistance genes) for each of the antimicrobial agents is shown in Table 1. With few exceptions (benzylpenicillin, ampicillin, tetracycline, and co-trimoxazole), more than 99 % of the isolates lacked acquired resistance.

Susceptibility to β-Lactam Antibiotics and β-Lactamase Production

K. kingae is usually highly susceptible to the β-lactam antibiotics such as ampicillin and second- and third-generation cephalosporins that are empirically administered to young children with suspected invasive bacterial diseases [16, 17], correlating with the excellent therapeutic response observed in most infections caused by the organism, with the notorious exception of endocarditis. However, production of β-lactamase has been detected in some *K. kingae* isolates [18, 19], with a

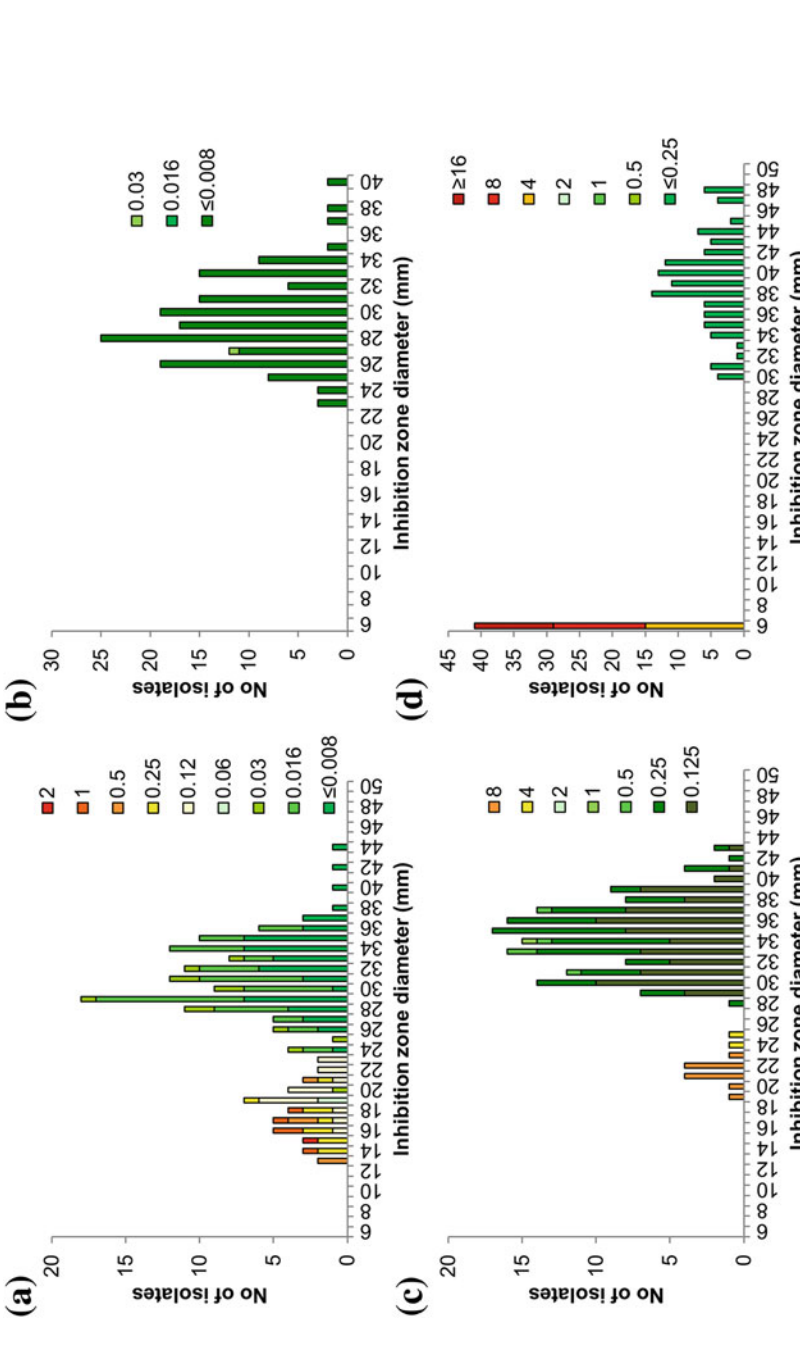

Fig. 1 Inhibition zone diameter distribution for 159 *K. kingae* strains versus selected antimicrobial agents with corresponding MIC values as *colored bars*. All testing was performed according to EUCAST recommendations for fastidious organisms. **a** benzylpenicillin (1 unit); **b** amoxicillin (2 μg)–clavulanic acid (1 μg); **c** tetracycline (30 μg); **d** trimethoprim (1.25 μg)/sulfamethoxazole (23.75 μg)

Table 1 Antimicrobial susceptibility testing results for 159 *K. kingae* strains using EUCAST media for fastidious organisms

Antimicrobial agent	Disk content (μg)	Wild-type		Non-wild-type	
		zone diameter[a] (mm)	BMD MIC[b] (mg/L)	zone diameter[a] (mm)	BMD MIC[b] (mg/L)
Benzylpenicillin	1 unit	24–44	≤0.008–0.03	13–24	0.016–2
Ampicillin	2	24–43	≤0.008–0.06	16–26	0.03–2
Amoxicillin–clavulanic acid	2–1	23–40	≤0.008–0.03		
Cefotaxime	5	28–48	≤0.016–0.06		
Ceftriaxone	30	31–48	≤0.016–0.125		
Cefuroxime	30	30–48	≤0.03–0.25		
Meropenem	10	30–49	≤0.008–0.06		
Ciprofloxacin	5	28–46	≤0.25		
Levofloxacin	5	29–47	≤0.25		
Erythromycin	15	20–37	≤0.06–1		
Tetracycline	30	28–43	0.125–1	19–25	4–8
Rifampicin	5	20–40	≤0.016–0.5		
Co-trimoxazole	1.25–23.75	30–48	≤0.25	6	4–>8

[a]MH-F agar
[b]MH-F broth

Table 2 Global prevalence of β-lactamase production among invasive *K. kingae* isolates

Location	β-lactamase positive	β-lactamase negative	Total # of isolates	% positive
Minneapolis, MN, USA	3	12	15	20.0
Montreal, Quebec, Canada	0	24	24	0
Reykjavik, Iceland	4	10	14	28.6
France	0	56	56	0
Barcelona, Spain	0	32	32	0
Israel	2	188	190	1.0

prevalence that shows wide variability between countries and among clinical conditions (i.e., invasive infection or asymptomatic carriage). Because the vast majority of reports on *K. kingae* infections have originated in the developed world, global data on the prevalence of β-lactamase production are scarce and fragmentary [3] (Table 2). The lack of information from resource-poor countries is probably not the result of lack of *K. kingae* morbidity but is instead related to the shortage of costly automated blood culture systems and advanced detection technology. Available data suggest that β-lactamase production is rare among invasive strains in continental Europe, including countries with high antibiotic consumption such as Spain and France [20, 21]. In a large study in which β-lactamase production was

determined in *K. kingae* isolates from different geographic origins, β-lactamase was detected in four of 14 (28.6 %) invasive and 3 of 15 (20 %) respiratory strains from Reykjavik (Iceland) and in 5 of 17 (29.4 %) invasive isolates from Minneapolis (Minnesota, USA) but was absent in 56 French, 32 Spanish, and 24 Canadian isolates associated with clinical infections (Table 2) [20].

In an Israeli study, β-lactamase production was detected in 43 of 406 (10.6 %) randomly chosen oropharyngeal isolates but in only 2 of 190 (1.1 %) strains recovered from patients with bacteremia, osteoarticular infection, or endocarditis ($P < 0.001$) [22]. Production of β-lactamase in Israeli isolates was found to be strictly clonal and limited to three distinct subpopulations as defined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) (PFGE clone A/MLST sequence-type complex (STc)-34, PFGE F/STc-3, and the less common PFGE ψ/STc-29). Of note, all isolates belonging to these clones contain the β-lactamase [22]. The fact that β-lactamase production was found to be widespread among carrier isolates suggests that the enzyme may confer a selective advantage to *K. kingae* organisms colonizing the oropharynx of young children, coinciding with the highest rates of oropharyngeal carriage of the organism and enhanced exposure to β-lactam antibiotics [23–26]. On the other hand, because β-lactamase production is rare among invasive organisms, it is possible that Israeli *K. kingae* strains expressing β-lactamase are less capable of entering the bloodstream and seeding deep tissues.

The local prevalence of β-lactamase-producing isolates among oropharyngeal colonizers and invasive isolates probably reflects the selective pressure exerted by antibiotic use patterns and the colonization fitness and intrinsic virulence of the strains harboring the $bla_{\text{TEM-1}}$ gene. The Minneapolis and Reykjavik β-lactamase-producing isolates belong to PFGE K/STc-6, are genomically identical by PFGE and MLST, and contain the same *rtxA* and *por* gene alleles [20]. This full genomic match between *K. kingae* strains derived from two relatively isolated locations situated an ocean apart is remarkable, suggesting transatlantic crossing and transmission. Of note, clone K/STc-6 is globally distributed, virulent, responsible for 28.2 % of all cases of invasive *K. kingae* disease in Israel [27, 28] and frequently carried by the healthy pediatric population [29], although the Israeli version does not produce β-lactamase [22]. The French β-lactamase producer strain belongs to STc-14, another globally distributed clone [21] that is being frequently isolated from patients with osteoarthritis and bacterial endocarditis worldwide [30], although other STc-14 isolates usually lack the enzyme.

The TEM-1 β-lactamase is encoded by the $bla_{\text{TEM-1}}$ gene [31], which is located on a plasmid in the Minneapolis-Reykjavik K/STc-6 strain and the Israeli F/STc-3 and STc-29 strains [32] and is integrated into the chromosome in the Israeli A/STc-34 strain and the French STc-14 strain [21, 32]. In Israel, the $bla_{\text{TEM-1}}$ gene is present almost exclusively in carrier isolates. In contrast, in Minneapolis and Reykjavik, the $bla_{\text{TEM-1}}$ gene is present in the virulent and transmissible K/STc-6 clone, resulting in widespread dissemination of the organism and high prevalence of the enzyme in invasive isolates in these geographic locations [20].

Mating experiments employing the Minneapolis/Reykjavic strain as the donor cell have demonstrated that the conjugative plasmid containing the *bla* $_{\text{TEM-1}}$ gene can be transferred to other *K. kingae* strains but is lost after ≤4 passages, suggesting that the plasmid is transmissible but cannot be retained unless the recipient strain meets other special and still unidentified requirements [31]. Although this limitation could have contributed to the strict clonality of β-lactamase production in *K. kingae* and prevented dissemination of the *bla* $_{\text{TEM-1}}$ gene to other strains, including more invasive strains, the fact that the β-lactamase-encoding plasmid has already been acquired and maintained by the highly transmissible and virulent Minneapolis-Reykjavik strain is worrisome and could herald further propagation of the resistant trait in the future [32].

The structure of the $bla_{\text{TEM-1}}$ gene has recently been studied in depth by Bidet et al. [33] in *K. kingae* strain KWG1, which was isolated from a French child with septic arthritis and belongs to the STc-14 clone [21]. This work found that the enzyme-encoding gene is located in a large (74 kb) genomic island that is absent in the β-lactamase non-producer ATCC23330-type strain [33]. The island is inserted in the vicinity of Met tRNA, possesses putative phage integrases at its 3′- and 5′-ends, contains genes encoding resistance to sulfonamides, streptomycin, and tetracycline, transposases, phage proteins, elements of type-IV secretion systems, and plasmid transfer and replication proteins [33]. This complex genomic array displaying both plasmid and phage features can be considered an integrative and conjugative element (ICE), similar to those found in approximately 18 % of prokaryotic chromosomes [33]. The sequence similarity of the ICE from strain KWG1 and the contigs of the plasmid-borne β-lactamase-producing A/STc-34 strain (isolate KK247) [34] suggest that a plasmid carrying the $bla_{\text{TEM-1}}$ gene acquired additional antibiotic resistance genes and inserted itself into the chromosome through a phage-like integration process. It is possible that the chromosomal position of the gene confers a biologic advantage in an era of selective pressure exerted by high antibiotic consumption. A stable location of the $bla_{\text{TEM-1}}$ gene in the chromosome ensures that antimicrobial drug resistance is endowed automatically to the daughter cells in each generation, obviating the need for plasmid replication [35].

Production of TEM-1 β-lactamase by *K. kingae* has a modest effect on the MICs of β-lactam antibiotics, even weaker than the TEM-1 hydrolyzing enzyme of *H. influenzae* [35]. The presence of β-lactamase in *K. kingae* increases the ampicillin MIC to 0.25–8 mg/L, with an MIC_{50} of 2 mg/L and an MIC_{90} of 4 mg/L. The enzyme is inhibited by clavulanate in all β-lactamase-producing isolates, suggesting that production of the enzyme is the only mechanism conferring β-lactamase resistance in the species. The MIC values vary slightly between the different β-lactamase-producing clones (e.g., the MIC is higher in PFGE clone F/STc-3 than in other clones), suggesting that production of the enzyme is controlled by the interaction of the *bla* $_{\text{TEM-1}}$ gene with undetermined regulatory mechanisms [33]. The disk containing 1 unit of benzylpenicillin successfully separates β-lactamase-producing isolates from non-producers, whereas use of the ampicillin 2-μg disk results in poorer discrimination [14]. As a measure of caution, β-lactamase production should be routinely determined in all isolates derived from patients with invasive infections using the sensitive nitrocefin (Cefinase) assay.

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Kingella kingae Treatment and Antibiotic Prophylaxis

Pablo Yagupsky and Nataliya Balashova

Treatment and Prognosis

The initial empiric treatment for osteoarticular infections in early childhood usually involves parenteral administration of the following: (1) vancomycin or a penicillinase-stable β-lactam antibiotic, such as oxacillin, and (2) a broad-spectrum second-generation or third-generation cephalosporin [1–3]. *K. kingae* exhibits reduced susceptibility to oxacillin but is generally exquisitely susceptible to cephalosporins, including β-lactamase-producing strains [4]. Following guidelines derived for the treatment of staphylococcal septic arthritis, traditionally patients with *K. kingae* septic arthritis have received antibiotic treatment for a total of 3–4 weeks and patients with *K. kingae* osteomyelitis have received treatment for 4–6 weeks.

In recent years, shorter duration of antibiotic therapy, early switch to oral antibiotics guided by a favorable clinical response and decreasing serum CRP levels below 20 mg/L (without the need for determining serum bactericidal levels), and limited use of surgical procedures are being recommended for the management of childhood septic arthritis and osteomyelitis [3, 5]. A 2–3 week course of sequential parenteral–oral antibiotic agents for uncomplicated septic arthritis and a 3–4 week course of parenteral–oral antibiotics for osteomyelitis caused by gram-positive pathogens (including *S. aureus*) appear to yield results comparable to those obtained with longer treatment courses, provided a well-absorbed oral antibiotic and a four-times-per-day (qid) regimen are used [3, 5]. Although the experience

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with this therapeutic strategy does not include patients with proven *K. kingae* infections, it is highly plausible that this approach is also adequate for treating pathogens such as *K. kingae*, in particular given that the approach is effective in cases of culture-negative osteoarticular infections [6], many of which likely represent undocumented *K. kingae* infections. Once *K. kingae* is reliably identified and β-lactamase production is excluded, the initial broad-spectrum antibiotic coverage can be changed to intravenous ampicillin and can be completed with oral amoxicillin or cefuroxime axetil. The proposed approach to the management and antimicrobial therapy of skeletal system infections caused by *K. kingae* is summarized in Fig. 1. Although the joint space has been aspirated and lavaged in some children with *K. kingae* arthritis [7] and intraosseous abscesses have been surgically drained in some patients with *K. kingae* osteomyelitis [8, 9], most patients can be managed adequately with conservative antibiotic therapy alone [10].

Because the host immune response to suppurative arthritis contributes to cartilage degradation by releasing proteases and collagen-degrading enzymes [11], anti-inflammatory medications have been explored as a strategy to reduce tissue damage and prevent joint destruction and permanent disability. In a rabbit model of pyogenic arthritis, dexamethasone decreased the synovial fluid leukocyte count and the concentration of pro-inflammatory cytokines and stromelysin and reduced the

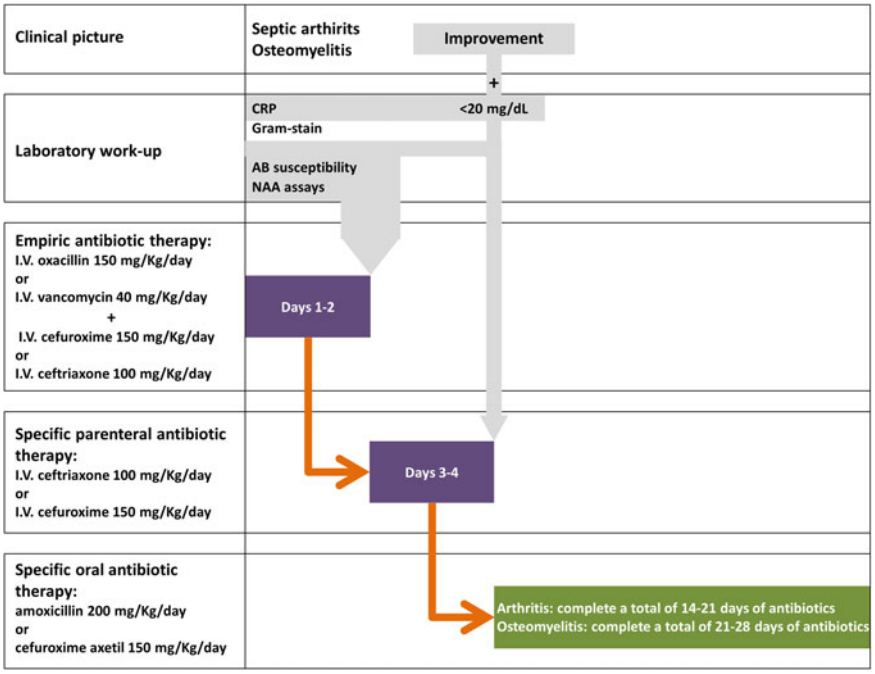

Fig. 1 Therapeutic approach to *K. kingae* septic arthritis and osteomyelitis (adapted from [3, 5], respectively). *CRP* stands for C-reactive protein. *AB* stands for antibiotic. *NAA* stands for nucleic acid amplification

extent of joint damage [12]. Based on these experimental results, two double-blind, randomized, placebo-controlled studies using a 4-day course of dexamethasone have been performed in children with laboratory confirmed or presumptive septic arthritis [13, 14]. In these studies, dexamethasone treatment resulted in a significantly shorter duration of symptoms and reduced residual dysfunction [13, 14]. However, it is important to recognize that there is a delicate balance between a helpful immune response aimed at eradicating the invading organism and over exuberant inflammation that may harm the joint architecture and cause permanent damage. Administration of corticosteroid therapy may reduce the effectiveness of the host anti-infective response and add unnecessary side effects. Because the clinical course of *K. kingae* septic arthritis is usually favorable and is characterized by a prompt response to antibiotic therapy and no immediate orthopedic sequelae, administration of adjuvant dexamethasone therapy for *K. kingae* arthritis is unnecessary and potentially harmful.

Historically, children with *K. kingae* spondylodiscitis have been treated with antibiotics for 3–12 weeks, generally without surgical intervention [10]. Recent studies have demonstrated that antibiotic courses for 3–4 weeks are effective [15]. While some of these patients develop narrowing and reduced height of the intervertebral space, functional neurological disabilities have not been described [8]. With this information in mind, surgery should be restricted to patients in whom there is uncertainty about the diagnosis or evidence of medullary compression, spine instability, or spine malalignment [15–17].

Patients with *K. kingae* bacteremia and no focal infection (occult bacteremia) have generally been treated initially with penicillin, ampicillin, or a cephalosporin by the intravenous route. The initial antibiotic therapy is typically switched to an oral regimen once the clinical condition has stabilized and endocardial involvement has been excluded by a thorough echocardiographic study. The duration of antimicrobial therapy has ranged from 1 to 2 weeks and has generally resulted in prompt improvement and no dissemination of the infection to peripheral sites [10].

Patients with *K. kingae* endocarditis have usually been treated with a high dose of an intravenous β-lactam antibiotic as monotherapy or in combination with an aminoglycoside for 4–7 weeks [10, 18]. Despite the generally favorable clinical course associated with other invasive *K. kingae* diseases and the usual susceptibility of *K. kingae* to antimicrobial agents that are empirically prescribed in patients with suspected bacterial endocarditis, serious complications have been commonly observed in children and adults with *K. kingae* endocarditis [18]. Examples include mycotic aneurysms of the aorta [19], thromboemboli involving the femoral [20–22], brachial [23], and ophthalmic [20] arteries, and embolic septic arthritis [24] and cellulitis [25]. Neurologic complications occur in almost one-third of children with *K. kingae* endocarditis, with examples including cerebrovascular accidents [18, 19, 26–35], meningitis [18, 24, 26, 36, 37], and brain abscesses [18, 22, 26, 36, 38–42]. Severe cardiovascular manifestations also occur in a significant fraction of pediatric patients and include mitral valve perforation [18, 31, 35, 40], mitral valve rupture [36, 43], and paravalvular abscess formation [32, 39, 44], resulting in congestive heart failure [34, 39, 45], cardiogenic shock [21, 46, 47], and pulmonary infarction

[42]. The average mortality rate of *K. kingae* endocarditis approaches 10 % [18, 20, 26, 33, 45, 46]. Emergency surgery has been required for life-threatening cardiovascular complications that do not respond to conservative medical treatment [20, 21, 31, 34, 39, 40, 43, 44, 48–50] and for embolism to large arteries [20–23]. Many patients require late valvuloplasty [35, 42] or valve replacement [18, 38, 44].

Because only a limited number of cases of *K. kingae* meningitis have been reported, no specific guidelines exist for the antibiotic therapy of central nervous system infections caused by *K. kingae*. In the past, patients were treated with a penicillin by itself [51] or in combination with chloramphenicol [52, 53], but currently a third-generation cephalosporin (cefotaxime or ceftriaxone) alone or in combination with an aminoglycoside (especially when the infection is secondary to endocarditis) for a total of 10 days to 4 weeks is preferred [54–56]. The clinical course of *K. kingae* meningitis is severe, with a high mortality rate and with permanent neurological sequelae such as hemiparesis [34], hemiplegia [53], aphasia [34], and ophthalmoplegia [51] among survivors, particularly in patients with concomitant endocarditis.

Antibiotic Prophylaxis

As with other pathogens of respiratory origin, at any given time the population of asymptomatic *K. kingae* carriers is large compared to the number of individuals who develop *K. kingae* disease. In particular, less than 1 % of carriers younger than 4 years develop *K. kingae* invasive disease [57, 58]. With this information in mind, there is no rationale for routine eradication of *K. kingae* from the oropharynx of healthy carriers. However, the risk of serious *K. kingae* disease is greatly increased among children attending daycare facilities with two or more recent cases of invasive *K. kingae* disease. In the past decade, outbreaks of invasive *K. kingae* disease (defined as occurrence of ≥2 cases of confirmed or presumptive infection) have been detected in the USA, France, and Israel, frequently triggered by a viral respiratory infection [26, 59–64]. When the available data from the 10 reported outbreaks of disease occurring in daycare centers are pooled, a total of 29 of 184 (15.8 %) classmates developed *K. kingae* infection within a 1-month period, including fatal endocarditis and meningitis. In addition, 66 of 154 (42.8 %) close contacts of index patients were colonized with *K. kingae*, usually the outbreak strain [26, 59–64]. Typing of the isolates from the daycare attendees who developed disease has revealed that the outbreaks are usually caused by a strain belonging to one of the highly virulent clonal groups responsible for a large fraction of the local burden of disease [63]. The need for administration of antibiotic prophylaxis to close contacts in daycare facilities where clusters of *K. kingae* disease are detected is still disputed, because no prospective studies have been conducted to assess the risk of infection in classmates. In support of withholding antibiotics, the efficacy of antibiotic prophylaxis has not been evaluated in prospective studies, and antibiotic exposure is associated with expense, induction of antibiotic resistance, and alteration of the child's microbiota. However, it is important to recognize that the annual

incidence of invasive *K. kingae* disease in children younger than 5 years of age is only 9.4 per 100,000 [58], and thus, detection of multiple cases of disease clustering in the same daycare center within a few weeks is highly significant. Therefore, antibiotics intended to eradicate colonization and prevent further cases of disease have often been offered to close contacts of the index cases in most daycare center outbreaks [26, 59–62, 65]. Rifampin at a dosage of 20 mg/kg/day in two divided doses for 2 days either alone [59, 61] or in combination with amoxicillin (80 mg/kg/per day) in two divided doses for two [26] or 4 days [60] has been prescribed. In adults, rifampin 600 mg twice a day for two days has been prescribed [65]. Detection of a sporadic case of invasive *K. kingae* disease among attendees of a daycare center is not generally considered an indication for administration of prophylactic antibiotics to close contacts, although the topic remains an open question in the absence of solid data.

Heightened index of suspicion for subsequent disease in the month after the detection of the last case and awareness of parents, facility staff, and primary care physicians of the subtle presentation of invasive infections caused by *K. kingae* are key factors for the identification of additional patients and timely recognition and adequate handling of clusters of disease. General infection control measures such as hand washing, cleaning of toys, and other objects that might become contaminated with oral secretions, and exclusion of sick children should be implemented and reinforced [61].

Upon deciding to administer antibiotic prophylaxis, three different options exist (Fig. 2). One approach would be to prescribe antibiotics for all close contacts as soon as the outbreak is detected, without waiting for surveillance oropharyngeal culture or PCR results or strain typing information (Fig. 2, option 1). In support of this approach, in daycare facilities where clusters of *K. kingae* disease have occurred, the rate of colonization with the organism among attendees is high and prompt administration of antibiotics saves time, is practical, and is justified clinically. As an alternative approach, antibiotics could be prescribed only for contacts who are carrying *K. kingae* as determined by oropharyngeal culture or PCR (Fig. 2, option 2). As a third approach, antibiotics could be prescribed only for contacts who are carrying the outbreak strain based on genotyping of oropharyngeal isolates (Fig. 2, option 3). Options 2 and 3 would limit the use of antibiotics and reduce the possibility of selective pressure but would result in potentially important delay of treatment while testing is being completed.

The rationale for selecting rifampin for prophylactic treatment includes the high level of activity of rifampin against *K. kingae* [59, 66], the secretion of rifampin in saliva and respiratory secretions, and the successful experience with rifampin in the elimination of respiratory colonization by *Haemophilus influenzae* type b and *Neisseria meningitidis* and the resulting prevention of invasive *H. influenzae* and *N. meningitidis* disease in daycare centers [67, 68]. High-dose amoxicillin has been added to the antibiotic regimen in recent outbreaks [26, 60, 64] because only partial success was achieved with rifampin monotherapy in the Minneapolis cluster [61]. On average, eradication of oropharyngeal carriage among colonized daycare attendees who completed the prescribed regimens was 81.1 %, ranging from

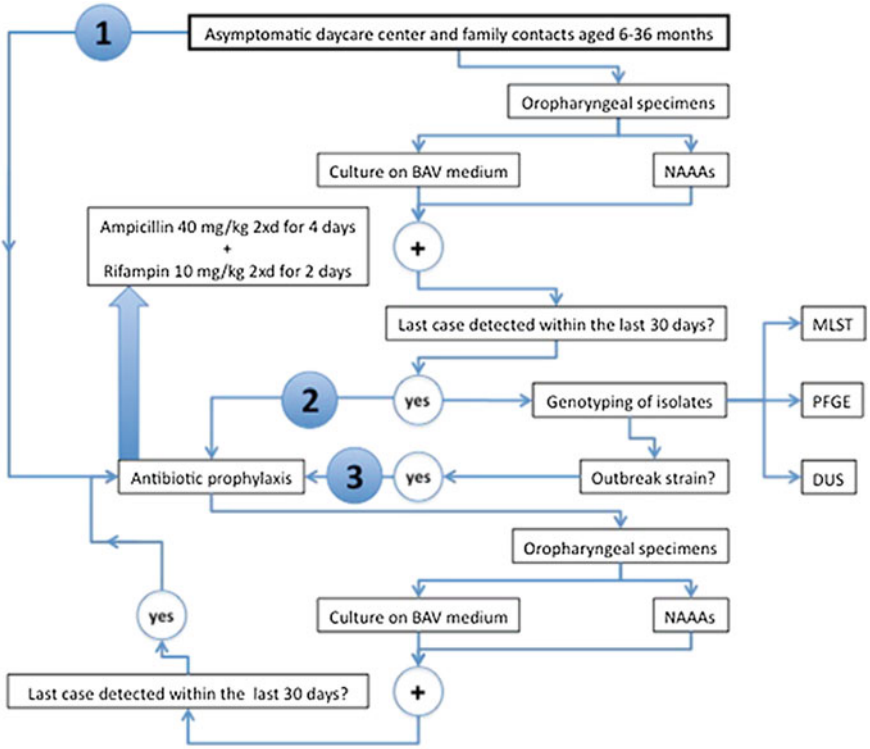

Fig. 2 Algorithm for the management of clusters of invasive *K. kingae* infections in daycare center facilities. *DUS* DNA uptake sequences [70], MLST multilocus sequence typing, PFGE pulsed field gel electrophoresis, NAAA nucleic acid amplification assay

31.2 %, as determined by a sensitive nucleic acid amplification assay [59], to 100 %, as established by culture [62]. This wide discrepancy suggests that the culture approach might have overlooked residual low-level colonization. Recurrent colonization by the original strain has been observed in a few children, with no evidence of primary or acquired resistance to the administered antibiotic regimens by the colonizing strain [59, 61]. Inadequate adherence and/or failure to provide antibiotic prophylaxis to young siblings could have resulted in only partial elimination of the reservoir, potentially leading to renewed spread of the strain among the daycare population [59, 68].

Despite the fact that antibiotic treatment has not been uniformly successful in eradicating *K. kingae* carriage, no new cases of disease have occurred in the affected facilities after administration of prophylaxis, even when elimination of the causative strain was incomplete [26, 59–62, 64]. It is possible that a reduction in density of colonization by antibiotic administration was enough to prevent transmission to additional attendees. Alternatively, it is possible that prolonged mucosal carriage elicited an effective antibody response, decreasing the individual risk of an invasive

infection. It is also possible that circulation of an important co-infecting virus ceased, eliminating the circumstances required for invasive disease [65].

It seems logical that prophylactic antibiotics should be offered primarily to children aged 6 months to 3 years, because over 95 % of cases of *K. kingae* disease occur in this age group [69]. Antibiotic administration to caretakers is probably unnecessary, given that prompt and spontaneous elimination of the organism usually occurs in the adult population, which represents only a temporary and limited reservoir and a "dead end" in the chain of person-to-person transmission [65]. Because patients with *K. kingae* disease cluster within a narrow period of time relative to the index case (3–30 days, median 12 days) [63], it seems reasonable to conclude that prophylaxis is unnecessary if more than one month has elapsed since detection of the last case.

Of note, four of the six clusters of *K. kingae* infections in Israeli daycare centers occurred in closed communities characterized by intensive social interaction [64]. In these closed communities, children spend many hours per day in the daycare facility and also share a connection by family ties and live within a short radius of each other. Thus, each daycare center in this peculiar setting represents a "closed community within a closed community" and *K. kingae* organisms can easily circulate through multiple complex social networks and spread among the susceptible young pediatric population [64]. Accordingly, the current Israeli guidelines for management of cases of *K. kingae* disease in closed communities recommend administration of antimicrobial prophylaxis to the entire population of individuals aged 6–36 months upon detection of a single case of disease, disregarding daycare attendance.

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Experimental Methods for Studying *Kingella kingae*

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Growth Conditions

Kingella kingae is a fastidious facultative anaerobe that grows optimally in a 37 °C humidified atmosphere supplemented with 5–10 % CO_2. The organism can be readily cultured on trypticase soy agar supplemented with 5 % lyophilized hemoglobin or 5 % sheep blood as a hemoglobin source (e.g., sheep blood agar) [1]. The organism also grows well on Columbia base or GC base agar supplemented with hemoglobin (e.g., chocolate agar). *K. kingae* does not grow well in liquid culture conditions using standard media formulations used for other fastidious gram-negative bacteria. However, it has been shown to grow in BD BACTEC™ Standard/10 Aerobic/F Culture Vials (BD, Franklin Lakes, NJ) [1, 2]. Using the same media formulation as the BACTEC vial media, *K. kingae* has been shown to grow in a 5 % CO_2-supplemented atmosphere at 37 °C without shaking, albeit at a slow rate.

Genetic Manipulation

Kingella kingae is naturally competent, resulting in the efficient uptake of extracellular DNA from the environment via natural transformation. If the imported DNA has high sequence homology to a segment of DNA of the recipient strain, the homologous recombination machinery can incorporate the exogenous DNA into the

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genome of the recipient strain, a process known as allelic exchange. This aspect of *K. kingae* biology has been exploited in the laboratory setting to introduce antibiotic resistance cassette-marked gene deletions, mutations, and complements. To transform *K. kingae*, bacteria are cultured overnight on chocolate agar and are then resuspended in brain–heart infusion (BHI) broth to an OD_{600} of ~0.8. Subsequently, the transforming DNA (typically 100–500 ng, but optimization may be necessary) is added to 250 μl of the bacterial suspension in a well of a 24-well plate, and the suspension is allowed to stand at room temperature for 30 min. Next, 250 μl of BHI supplemented with 5 % horse plasma is added to the transformation mixture, and the mixture is incubated at 37 °C in a 5 % CO_2-supplemented atmosphere for 2 h. Diluted and undiluted samples of the transformation reaction are then spread on chocolate agar plates containing the appropriate antibiotic to select for transformants, and plates are incubated overnight. Individual colonies are picked, and genomic DNA from these colonies is analyzed by PCR to confirm the presence and location of the integrated transforming DNA.

Beyond delivery by natural competence, exogenous DNA can be introduced into *K. kingae* by electroporation. Electroporation may be the only option for strains that are recalcitrant to transformation by natural competence, including strains that lack functional type IV pili, which appear to be necessary for transformation by natural competence. Briefly, bacteria are suspended from a chocolate agar plate into 25 ml of BD BACTEC™ Standard/10 Aerobic/F Culture Vial medium to an OD_{600} of ~0.1. After static growth overnight at 37 °C in 5 % CO_2, the bacteria are washed twice with 0.3 M sucrose at room temperature and then resuspended in 0.3 M sucrose. Subsequently, 100 ng of transforming DNA is electroporated into the electrocompetent bacteria at 12.5 kV/cm in a 2-mm cuvette. The electroporated bacteria are recovered in 500 μl of BHI supplemented with 5 % horse plasma and are incubated at 37 °C in a 5 % CO_2-supplemented atmosphere for 2 h prior to plating on selective media.

Plasmids

Attempts to introduce a variety of cloning plasmids with different origins of replication into *K. kingae* have not been successful. Recently, some *K. kingae* clinical isolates have been shown to carry up to two plasmids [3, 4]. More work is necessary to determine whether either of these plasmids will facilitate stable introduction of cloned DNA into *K. kingae*.

Targeted Mutations

The first step to introduce targeted mutations or gene deletions into *K. kingae* involves generating a recombinant targeting plasmid using standard molecular

biology approaches. An example targeting plasmid for deletion of hypothetical gene A (*hypA*) is diagrammed in Fig. 1. Briefly, approximately 1 kb of upstream DNA and 1 kb of downstream DNA flanking the *hypA* gene are cloned into the multiple cloning site (MCS) of a standard cloning plasmid such as pUC19, leaving some restriction sites intact between the two cloned fragments. An antibiotic resistance cassette is then inserted between the upstream and downstream targeting fragments, generating a targeting deletion plasmid. To date, antibiotic resistance cassettes conferring resistance to kanamycin, erythromycin, tetracycline, or chloramphenicol have been used to generate marked *K. kingae* mutants and are detailed in Table 1.

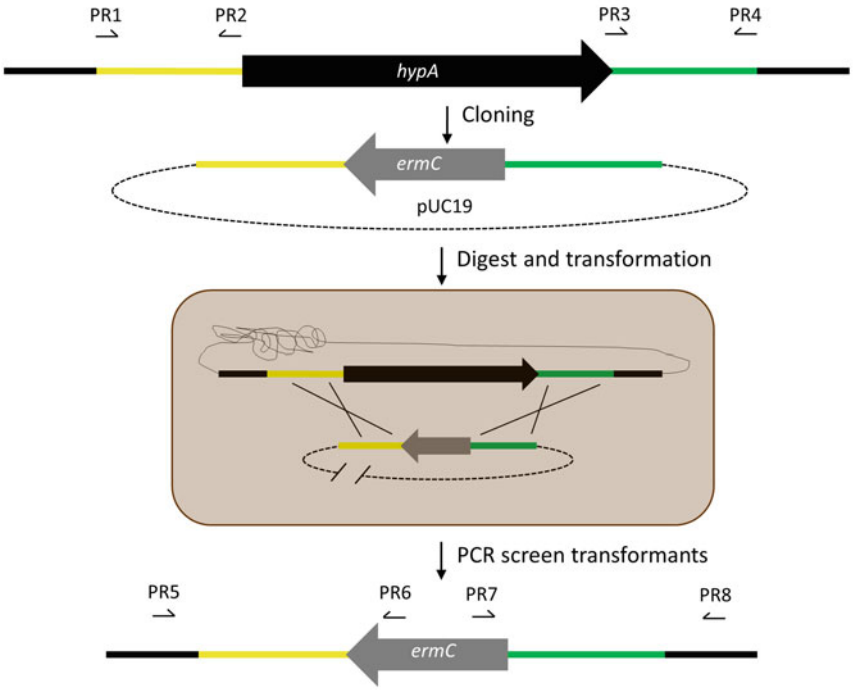

Fig. 1 Strategy for targeted gene deletion in *K. kingae*. Upstream (*yellow*) and downstream (*green*) homologous recombination targeting fragments (~1 kb each) are amplified from genomic DNA of the parental wild-type strain using primer pairs PR1/PR2 and PR3/PR4, respectively. Both fragments are cloned into pUC19, and the *ermC* erythromycin resistance cassette is cloned between the targeting fragments, generating a targeting deletion plasmid. The plasmid is then linearized by restriction digestion with an enzyme that cuts the vector backbone and is transformed into the wild-type parental strain via natural transformation. Individual transformants that grow on selective chocolate agar supplemented with 1 μg/ml erythromycin are single colony purified on the same selective media, and genomic DNA is prepared for use as the template for PCR screening. Two primer sets (PR5/PR6 and PR7/PR8) are used to confirm deletion of the target gene. Each primer set contains one primer that anneals outside of the cloned homologous recombination targeting regions (PR5 and PR8) and another primer that anneals to the *ermC* resistance cassette (PR6 and PR7)

Table 1 Antibiotic resistance cassettes for use in *K. kingae*

Antibiotic	Cassette	Source	Selection (μg/ml)	Reference
Kanamycin	*aphA3*	pFalcon2	50	[7]
Erythromycin	*ermC*	pIDN4	1	[22]
Tetracycline	*tetM*	pHSX*tetM*4	2	[23]
Chloramphenicol	*cat*	pACYC184	1	[24]

To eliminate the possibility of single-crossover homologous recombination and incorporation of the entire targeting plasmid, it is advisable to linearize the targeting plasmid prior to transformation by digesting the plasmid with an enzyme that only cuts in the vector backbone. Using this approach, only double-crossover homologous recombination events in the upstream and downstream targeting regions will result in incorporation of the resistance cassette and thus viable antibiotic resistant transformants.

Similar approaches with various modifications can be used to introduce other types of mutations into the *K. kingae* chromosome, as long as the mutation does not affect viability. Two example mutations other than gene deletions that have been generated in *K. kingae* are shown in Fig. 2. Figure 2a illustrates how a mutated *pilA1* promoter element with a scrambled sequence was generated. A targeting plasmid was constructed containing the entire *pilA1* locus and surrounding sequence in pUC19, with a kanamycin resistance cassette inserted into *recJ*, the gene upstream of *pilA1*. Next, PCR was performed with primers containing overhangs with the desired scrambled sequence and a SpeI restriction site that annealed to the opposite strands of the region surrounding the promoter element of interest. After treatment with DpnI to digest the template plasmid, the resulting PCR product with overhanging SpeI sites was gel-extracted and digested with SpeI. The SpeI-digested PCR product was gel-extracted and then self-ligated, generating a targeting plasmid with a scrambled *pilA1* promoter element with an internal SpeI site. Following sequencing to confirm that the promoter sequence was scrambled as desired, the mutated plasmid was transformed into *K. kingae*. Kanamycin-resistant transformants were picked and subjected to sequencing of the *pilA1* locus. Sequencing was necessary to confirm the presence of the desired mutation in the transformants because selection was solely based on the antibiotic resistance marker, which could potentially be kilobases away from the desired mutation, enabling selection of transformants with a homologous recombination event between the marker and the mutation of interest.

As illustrated in Fig. 2b, in order to introduce a calcium-binding site mutation in the pilus-associated protein PilC1, the entire *pilC1* gene with 1 kb of upstream and 1 kb of downstream targeting sequence was cloned into pUC19, with a kanamycin resistance cassette inserted into the *abcA* gene upstream of *pilC1*. The resulting plasmid was subjected to a standard site-directed mutagenesis procedure using the QuikChange XLII Site-Directed Mutagenesis Kit (Agilent Technologies, Wilmington, DE) to generate the calcium-binding site mutation. After sequencing

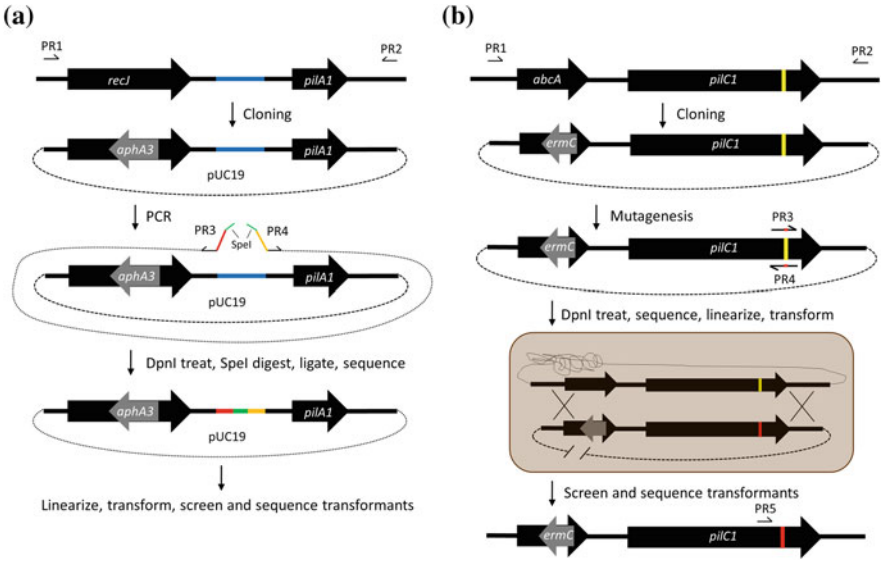

Fig. 2 Mutagenesis strategies in *K. kingae*. The strategy to generate a scrambled *pilA1* promoter element mutation in *K. kingae* is shown in (**a**) [10]. The native promoter element (not to scale) is shown in *blue*. The *pilA1* region is amplified with PR1/PR2 and is cloned into pUC19. The *aphA3* kanamycin resistance cassette is then cloned into the *recJ* ORF (a mutation that was previously shown to have no impact on type IV pilus expression or function). The plasmid is then used as the template for PCR with PR3, which has an overhang with half of the desired promoter scramble sequence (*red*) and a SpeI site (*green*), and PR4, which has an overhang with the other half of the desired promoter scramble sequence (*orange*) and a SpeI site (*green*). After cycling, the reaction is treated with DpnI to digest the template plasmid. The amplicon is then digested with SpeI, ligated, and transformed into laboratory *E. coli* strain DH5α for propagation. After sequencing of the entire *pilA1* locus to confirm the presence of the promoter scramble and absence of unwanted PCR-introduced mutations, the promoter scramble plasmid is linearized and transformed into *K. kingae*. Genomic DNA is isolated from the transformants and is subjected to PCR and sequencing to confirm the presence of the desired promoter scramble. A similar strategy is used to generate coding sequence mutations, including the procedure outlined in (**b**) used to generate a single amino acid substitution in the PilC1 calcium-binding site [12]. The primers PR3 and PR4 are complementary sense and antisense oligos with the necessary nucleotide changes (*red*) to change the native codon (*yellow*). The Agilent QuikChange XLII Site-Directed Mutagenesis kit is used to generate the mutation, and the resulting plasmid is sequenced and transformed as described above. The greater the distance from the selectable marker that the desired mutation is located, the greater the number of transformants will need to be screened by sequencing. This relationship is due to the fact that there is a greater chance of homologous recombination between the marker and the desired mutation as the distance from the selectable marker increases

to determine that only the desired mutation was generated, the plasmid was transformed into *K. kingae*. Introduction of the desired mutation was confirmed as described above.

Genetic Complementation

Given that there are no known plasmids for the introduction of episomal DNA into *K. kingae*, a strategy for chromosomal complementation of mutations was developed [5]. Briefly, a targeting plasmid (complementation plasmid) was created to direct the insertion of individual genes or clusters of genes into the *K. kingae* chromosome. The chromosomal region that the complementation plasmid was designed to target lacks predicted genetic elements (e.g., open reading frames, tRNAs, or regulatory elements) to minimize the chance that the introduction of cloned DNA into this region will affect *K. kingae* biology at the genetic level. The complementation plasmid contains an antibiotic marker and a fragment of the pUC19 MCS, including the restriction sites KpnI, BamHI, XbaI, PstI, and SphI, between the upstream and downstream targeting regions [5]. For complementation analysis, the gene to be complemented (with its native promoter) can be cloned into the MCS, introduced into the mutant strain via natural transformation, and screened for targeted recombination into the complementation locus. A modified version of this complementation plasmid with the *lacI* gene and *trc* promoter has been developed to enable isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced expression of cloned genes without their native promoter [6].

Random Transposon Mutagenesis

Given the natural transformability of *K. kingae*, the organism has been shown to be amenable to random in vitro transposon mutagenesis. Genomic DNA is isolated using the Promega Wizard Genomic DNA Isolation Kit (Promega, Madison, WI) according to the manufacturer's instructions. The isolated DNA is mutagenized using a purified maltose-binding protein (MBP)-*Himar*1 transposase fusion (purified from *E. coli* lysates after expression from the pMAL-*Himar* plasmid) and the plasmid pFalcon2 as the source of a kanamycin-marked mini Solo transposon, as described by Hendrixson et al. [7]. Briefly, 1 μg of genomic DNA is incubated with 500 ng of pFalcon2 and 500 ng of purified Himar1 transposase in a final volume of 80 μl of a solution containing 25 mM HEPES pH 7.9, 250 μg/ml BSA, 1 mM dithiothreitol (DTT), 100 mM NaCl, and 5 mM $MgCl_2$ for 4 h at 30 °C. The mutagenized chromosomal DNA is extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1) and is then ethanol-precipitated. To repair transposon/chromosome junctions, the purified mutagenized DNA is first treated with 1.5 units of T4 DNA polymerase for 20 min at 11 °C. The enzyme is heat-inactivated by incubating the reaction for 15 min at 75 °C. To complete the repair, the DNA is then treated with 600 units of T4 DNA ligase for 1 h at 22 °C. The repaired DNA is then transformed into *K. kingae* using the natural transformation protocol described above. After overnight growth on

chocolate agar containing kanamycin, the recovered colonies can be pooled to generate a transposon mutant library.

To confirm that individual transposon mutants only have one transposon insertion, purified genomic DNA is digested with BspHI and examined by Southern hybridization using a ~500-bp biotinylated fragment of the *aphA3* cassette from pFalcon2 as the probe. Following complete digestion, the presence of one hybridizing band indicates a single insertion. To determine the location of the transposon insertion, arbitrary PCR and sequencing are performed. The first round of a nested PCR is carried out using arbitrary primers ARB1 (5′ GGCCAC GCGTCGACTAGTACNNNNNNNNNNGATAT 3′) or ARB6 (5′ GGCCACGCG TCGACTAGTACNNNNNNNNNNACGCC 3′), where N represents a random nucleotide and specific primer Solo5 Arb#1 (5′ GCCCGGGAATCATTT GAAGGTTG 3′) or Solo3 Arb#1 (5′ CGCGTCGCGACGCGTCAATTC GAGG 3′). Solo5 Arb#1 anneals at the 5′ end of the Solo transposon, and Solo3 Arb#1 anneals at the 3′ end of the Solo transposon. A second round of amplification, using the first PCR product as the template, uses ARB2 (5′ GGCCACGCGTCGA CTAGTAC 3′), which anneals to the 5′ end of ARB 1 and ARB 6 with Solo5 outN (5′ AATATGCATTTAATACTAGCGACGCC 3′) or Solo3 outN (5′ CGCTCTTGAAGGGAACTATGTTG 3′), which are external to Solo5 Arb#1 and Solo3 Arb#1, respectively. The PCR products from the second round of amplification are gel-purified and sequenced using either Solo5 outN or Solo3 outN, as appropriate, to sequence across the chromosome/transposon junction. The utility of random transposon mutagenesis is best exemplified by Kehl-Fie et al., who used this approach to identify the RTX toxin locus responsible for the broad cell-type cytotoxicity of *K. kingae* [8].

Surface Factors

Given the importance of bacterial surface factors in mediating interactions with the host and their potential roles as colonization and virulence factors, much of the *K. kingae* molecular pathogenesis research has been focused in this area. *K. kingae* is generally amenable to a variety of standard techniques previously developed to study surface factors in other organisms. For example, the type IV pili of *K. kingae* are readily visualized by negative-staining transmission electron microscopy (TEM) [8–11], pilus retraction can be assessed using a modified agar plate stab assay [5, 12], outer membrane fractions can be isolated based on sarkosyl insolubility [5], and the presence of the polysaccharide capsule can be visualized by cationic ferritin staining and thin section TEM [5, 6]. The following sections describe experimental methods that have been optimized for the study of *K. kingae* surface factors.

Type IV Pili

Initial studies of *K. kingae* type IV pili examined pilus density and morphology using negative-staining TEM of whole bacteria [8, 9]. A protocol for large-scale purification of pili was developed based on a method for purifying type IV pili from *Eikenella corrodens* [13]. Briefly, this method involves resuspension of bacterial growth from 20 chocolate agar plates in 150 mM ethanolamine pH 10.5, shearing the fibers with a handheld homogenizer, and completion of two rounds of 10 % ammonium sulfate precipitation and dialysis [10, 13], yielding highly pure type IV pilus fibers as assessed by negative-staining TEM and SDS-PAGE separation and Coomassie blue staining of the ~14-kDa major pilus subunit. To complement this method, a small-scale procedure to more rapidly examine the relative piliation levels across multiple *K. kingae* strains or isogenic mutants was developed. The small-scale pilus preparation procedure is delineated in Table 2 and allows quantitative assessment of pilus levels in less than a day.

Type IV pilus-mediated twitching motility can be assessed using a modified agar plate stab assay originally developed for *Pseudomonas aeruginosa* [14]. For *K. kingae*, chocolate agar is poured as a thin layer into tissue culture-treated 100-mm Petri dishes. Following solidification and cooling of the agar, 1 µl of a 0.8 OD_{600} bacterial suspension is stab-inoculated at the plate/agar interface. After incubation under standard *K. kingae* growth conditions for 2–3 days, the agar is carefully peeled away from the plate to expose the twitching zone surrounding the inoculation site. The plate is air-dried, and a 0.1 % crystal violet solution is then applied to stain the twitching zone. The diameter of the zone can be measured to quantitate the level of twitching motility [5, 12].

Table 2 *K. kingae* small-scale type IV pilus purification procedure

Step	Description
1	Grow *K. kingae* strains on chocolate agar for 17–18 h
2	Resuspend bacteria in PBS to an OD_{600} of 1.0
3	Add 1.5 ml of the bacterial suspension to a 1.7-ml microcentrifuge tube
4	Vortex at the maximum setting for 1 min
5	Centrifuge the vortexed suspension for 2 min at 21,000 × *g*
6	Transfer 1.25 ml of the supernatant to a new 1.7-ml microcentrifuge tube
7	Add 250 µl of saturated ammonium sulfate, vortex, and incubate on ice for 2 h
8	Centrifuge for 5 min at 21,000 × *g* to pellet the precipitated, sheared fibers
9	Resuspend the pellet in 40 µl 1 × SDS-PAGE loading buffer
10	Load different volumes of the resuspended pellet onto a 15 % SDS-PAGE gel
11	Stain the gel with Coomassie blue

Outer Membrane Proteins

Outer membrane fractions can be readily isolated from *K. kingae* whole-cell sonicates based on sarkosyl insolubility. Briefly, bacteria are resuspended in 10 mM HEPES, pH 8.0, and sonicated to create whole-cell lysates. Bacterial debris is removed from the lysates by centrifuging at 21,000 × *g* and 4 °C for 2 min. The supernatant is recovered and centrifuged at 100,000 × *g* and 4 °C for 1 h to pellet the total membrane fraction. The pellet is then resuspended in 10 mM HEPES supplemented with 1 % sarkosyl and incubated with gentle agitation for 30 min to solubilize the inner membrane fraction. The sample is centrifuged again at 100,000 × *g* and 4 °C for 1 h to pellet the outer membrane fraction. The outer membrane fraction is washed once with 10 mM HEPES and can be resolved on an SDS-PAGE gel. Prior to SDS-PAGE analysis, the outer membrane fraction can be treated with formic acid to denature extremely stable multimeric proteins, such as trimeric autotransporters [5, 15, 16].

Like other gram-negative bacteria, *K. kingae* produces outer membrane vesicles (OMVs) that can be readily purified for analysis. Maldonado et al. [17] described a purification protocol that involves the scraping of bacterial growth from agar plates and then centrifugation at 150,000 × *g* and 4 °C for 15 min. Following centrifugation, the supernatant is collected, filtered through a 0.45-μm pore membrane, and centrifuged again at 150,000 × *g* and 4 °C for 2 h. The final pellet consists of the OMV fraction, which can be examined by TEM to visualize the OMVs and can be plated on growth media to confirm sterility.

Extracellular Polysaccharides

Kingella kingae expresses a polysaccharide capsule that can be purified by using a method that releases the capsule polymer from the surface of the organism and removes protein and other cell component contaminants [6, 18]. To prepare strains for extraction of capsule polysaccharide, bacterial lawns are grown on 40 chocolate agar plates overnight. Subsequently, the lawns are swabbed from the plates, resuspended in 50 ml of 1 % formaldehyde in PBS, and incubated at room temperature for 30 min. The bacteria are pelleted by centrifugation at 4000 × *g* for 10 min and are then resuspended in 40 ml of 50 mM Tris-acetate pH 5. Following vigorous shaking at room temperature for 1 h, the bacteria are pelleted by centrifugation at 12,000 × *g* for 20 min. The supernatant is filtered using a 0.22-μm filter and is adjusted to pH 7 with 1 M Tris pH 9. To remove contaminating DNA and RNA, a total of 10 units of DNase I and 0.1 mg of RNase A are added to the filtered material, and the mixture is incubated at 37 °C for 4 h. To remove contaminating proteins, 0.18 mg of proteinase K is added, and the sample is incubated at 55 °C overnight. To remove the proteinase K and any remaining protein contaminants, the sample is concentrated using a 100-kDa molecular weight cutoff

filter, extracted with Tris-saturated phenol pH 7.4, and then extracted with chloroform. The extracted material is dialyzed overnight in deionized water, and the purified polysaccharide can then be flash-frozen and lyophilized to allow for extended storage.

Kingella kingae also expresses a secreted exopolysaccharide galactan homopolymer (PAM galactan) that copurifies along with the polysaccharide capsule [18, 19]. To prevent contamination of capsule preparations with the PAM galactan, the *pamABCDE* locus can be deleted in *K. kingae* strains prior to capsule extraction [18]. To specifically purify the PAM galactan, the bacterial growth from a capsule synthesis-deficient or capsule export-deficient strain is directly resuspended in PBS, the suspension is agitated for 1 h, the bacteria are pelleted, and the supernatant is subjected to the same purification protocol as described for the capsule polysaccharide [18].

Interactions with Eukaryotic Cells

The *K. kingae* RTX toxin is present in all *K. kingae* strains examined to date and has broad-range cytotoxicity, including activity against human epithelial cells, synovial cells, macrophage-like cells, and red blood cells [8, 17]. This cytotoxicity precludes studies of in vitro interactions between wild-type *K. kingae* and cultured human cell lines. Early studies aiming to define *K. kingae* adherence factors found that fixation of cell monolayers with 2 % glutaraldehyde protected the monolayers against cytotoxicity and still allowed high-level in vitro adherence [9]. Many of the subsequent studies exploited this fixation protocol to identify and characterize *K. kingae* surface factors that influence adherence to epithelial and synovial cells, including type IV pili, the Knh trimeric autotransporter, and the capsular polysaccharide [5, 10–12, 18]. The protocol for assessing quantitative adherence levels of *K. kingae* to fixed cultured cell monolayers is detailed in Table 3. As an alternative to fixation to allow assessment of *K. kingae* interactions with host cells, the *rtx* locus can be deleted, as described thus far in two *K. kingae* strain backgrounds [8, 20].

Juvenile Rat Infection Model

Basmaci et al. reported the first animal model for examining virulence differences among *K. kingae* clinical isolates [21]. These investigators inoculated 5-day-old Sprague–Dawley rats via the intraperitoneal (i.p.) route with $\sim 10^7$ cfu of three *K. kingae* strains resuspended in PBS from overnight growth on chocolate agar. The animals were examined for 5 days for signs of infection, including skin discoloration, signs of peritonitis, and survival. Signs of infection included necrotic lesion formation around the injection site, abscess formation, lethargy, and death.

Table 3 *K. kingae* in vitro adherence assay procedure with Chang epithelial cells

	Description
Day 1	
1	Start with a T75 or T150 adherent tissue culture flask of Chang epithelial cells at ~80 % confluency cultured in Minimum Essential Medium (MEM) supplemented with 10 % fetal calf serum and 1 × MEM non-essential amino acid mix (complete media)
2	Release the cells with 0.05 % trypsin–EDTA and dilute to 1.8×10^5 cells/ml in complete media
3	Add 1 ml of the cell suspension to each well of a 24-well tissue culture plate (3 wells per *K. kingae* strain) and incubate at 37 °C in a humidified atmosphere supplemented with 5 % CO_2 for 17–18 h
4	Streak out each *K. kingae* strain on chocolate agar and incubate for 17–18 h
Day 2	
5	Remove the complete media from the 24-well tissue culture plate, add 300 µl of 2 % glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4 to each well, and gently rock at 4 °C for 2 h
6	Remove the glutaraldehyde solution, wash the wells 3 times with 500 µl of Tris-buffered saline (TBS), and add 300 µl of fresh complete media to each well
7	Suspend the bacteria in ~3 ml of BHI broth to an OD_{600} of 0.8 (~6.5×10^8 cfu/ml) and add 10 µl of each strain suspension to 3 wells
8	Gently swirl the plate and centrifuge for 5 min at 165 × *g*
9	Transfer the plate to a 37 °C incubator for 25 min
10	During the incubation, prepare a 1:100 dilution series in BHI in duplicate for each inoculating strain, plate on chocolate agar, and incubate overnight (inoculum plates)
11	Remove the media from the wells and wash 4 times with 500 µl of PBS
12	Add 100 µl of 0.05 % trypsin–EDTA to each well and incubate at 37 °C for 20 min
13	Add 900 µl BHI to each well, scrape the entire well surface with a pipette tip, and pipette up and down repeatedly to mix
14	Prepare a 1:100 dilution series in BHI for each well, plate on chocolate agar, and incubate overnight (adherence plates)
Day 4	
15	Determine the total cfu of the inoculum and adherence plates
16	Divide the total number of cfu on the adherence plates by the number of cfu on the inoculum plates to determine the adherence level expressed as % of inoculum

Significant differences in survival were observed among the strains. Chang et al. expanded on the model and tested the role of the *K. kingae* RTX toxin in virulence using both 7-day-old and 21-day-old Sprague–Dawley rats [20]. Significant differences in survival were observed in the 7-day-old pups injected i.p. with ~1.2×10^7 cfu of strain PYKK081 (a wild-type septic arthritis clinical isolate) versus an isogenic RTX-deficient mutant. In contrast, no differences in survival were observed in the 21-day-old animals. This report also found evidence of bacteremia and significant histopathology in the thymus, spleen, and bone marrow [20]. Starr et al. used 5-day-old Sprague–Dawley rats to investigate the role of the *K. kingae* polysaccharide capsule on virulence. Using an i.p. inoculum of 10^8 cfu of

strain KK01 (a stable derivative of septic arthritis isolate 269–492) and isogenic capsule-deficient mutants, the capsule-deficient mutants were significantly less virulent as assessed by survival through the 5-day experiment [6]. The development of the juvenile rat model was a significant first step for examining *K. kingae* virulence, but an animal model that more closely replicates *K. kingae* disease in humans, such as a septic arthritis model, will be critical for future studies. In addition, an animal upper respiratory tract colonization model will be necessary to test the in vivo relevance of potential colonization factors.

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