Abstract

Background: *Kingella kingae* and *Neisseria meningitidis* are Gram-negative bacteria, causing several life-threatening diseases and are considered to be opportunistic pathogens in the upper respiratory tract of healthy carriers. The detection of these both bacterial species is difficult in routine culture methods.

Objective: This study aimed to find the occurrence rate of *K. kingae* and *N. meningitidis* colonizing upper respiratory tract of young Jordanian children, and to determine the antimicrobial susceptibility profile of the isolates.

Methods: A total of 300 samples of throat and nasal swabs were collected from out-patient Jordanian children aged between 6 months and 5 years, who were admitted to the pediatric clinical departments at the Jordan University Hospital and Al-Bashir Hospital over the period October 2018 through January 2019. Samples were cultured for detection of *K. kingae* and *Neisseria* species, including *N. meningitidis*. Their suspected growth was identified and tested using microbiology culture methods and polymerase chain reaction (PCR) method. Additionally, DNA was extracted directly from one 100 samples and was investigated only for *K. kingae* using real-time PCR assay.

Results: This study showed the absence of *K. kingae* in all cultured samples. *Neisseria* species was detected in 21 (7%) including one *N. meningitidis* isolate (0.3%). The results of antibiotic susceptibility testing indicated the presence of a low percentage of *Neisseria* species isolates resistant to clindamycin, oxacillin and vancomycin, whereas all were susceptible (100%) to levofloxacin and gentamycin, and fewer to ampicillin (90.6%) and erythromycin (85.7%), respectively.

Enigma of Respiratory Carriage of *Kingella kingae* and *Neisseria meningitidis* in Young Jordanian Children

Malak A. Khanfar¹, Eman F. Badran², Basma Marrar³, Ekatherina Charvalos⁴, Asem A. Shehabi¹

¹ Department of Pathology-Microbiology and Forensic Medicine, The Jordan University, School of Medicine, Amman, Jordan. Malak A. Khanfar.
² Department of Pediatrics, School of Medicine, Jordan University Hospital, Amman, Jordan. Prof. Eman F. Badran.
³ Al-Bashir Hospital, Ministry of Health, Amman, Jordan. Dr. Basma Marrar.
⁴ IASO Hospital, Kifissias 37-39, Athens Greece 151 23. Prof. Ekatherina Charvalos.

Contact information:
Prof. Dr. Asem A. Shehab; Prof. Dr. Eman F. Badran.

asshehabi2@gmail.com
shehabi@ju.edu.jo
e.badran@ju.edu.jo
Introduction

Kingella kingae is a member of the HACEK (Hae-mophilus species, Aggregatibacter actinomyce-temcomitans, Cardiobacterium hominis, Eikenella corrodens, and K. kingae). HACEK organisms are typically oropharyngeal commensals and have long been recognized as a cause of infective endocarditis in children [1-2].

K. kingae is considered to be a rare human pathogen, occasionally isolated from oropharynx of young children as well as from cardiac valves, synovial fluid and exudates [3-4]. Asymptomatic colonization of the upper respiratory tract by K. kingae is mostly observed in children aged up 6 months [4-5]. The most frequent clinical manifestation of invasive K. kingae infections are osteoarticular infections (OAI) that are frequently diagnosed in children aged between six months and four years [3-4, 6]. The organism invades the blood stream and spreads to other organs after oropharyngeal colonization [4].

Children infected with K. kingae, other than endocarditis, frequently have mild clinical symptoms, and they can have high fever or a mild fever and are in good general condition. Their blood cultures are often negative, suggesting that the bacteremic stage of the disease is transient [4, 7].

Commensal Neisseria species including N. meningitidis are similar to K. kingae, in many biological features and found frequently in the human nasopharynx [8-9]. N. meningitidis colonizes only the human upper respiratory tract, and it has been found asymptotically in the pharynx of 8–25% of the human population in certain regions [8]. The commensal N. lactamica, is genetically and bacteriology closely related to N. meningitides [10]. Carriage rate of commensal N. lactamica is high in the nasopharynx of infants and young children, but declines with age in contrast to N. meningitidis where carriage rate is increased in young adults [11]. There is indication that N. lactamica is a contributing factor in the development of immunity against meningococcal diseases [8].

Both K. kingae and N. meningitidis are difficult to recover during routine culture of pharyngeal samples. Therefore, most current studies use Real-time PCR tests to detect both organisms in oropharyngeal cultures or clinical specimens [12]. Therefore, the detection rates of K. kingae and N. meningitidis in the upper respiratory tract of young Jordanian children will offer important epidemiological data on these both organisms.

Material and methods

Study design

This prospective study was carried out at the Pediatric Clinics of The Jordan University Hospital (JHU) and Al-Bashir Hospital, the main government-run hospital and referral center in Amman. The study was approved by the School of Medicine and Faculty of Graduate Studies, The Jordan University, Amman, Jordan. Permission was also obtained from the Institution Review Board (IRB) at The Jordan Univer-
University Hospital (Permission NO.217/2018) and Al-Bashir Hospital (Permission NO.13978). All parents of participant children were received full information about the purpose of the study prior to get their informed consent.

A total of 300 oropharyngeal and nasal samples has been collected from children aged between 6 months and 5 years. Samples were collected over a period of 4-month from October 2018 to February 2019. The biographical data of each patient was obtained and registered on a special information sheet and these included name, age, sex, presence of fever, pharyngitis, otitis media, cough, runny nose and lower respiratory infection (LRT), taking antibiotic at time of sampling and with in 2 weeks before sampling, and type of antibiotic treatments as shown in Table 1.

**Sample collection**

For each patient sample, nose and throat swabs were collected, and placed in one tube. The first 200 samples were collected by cotton swabs; two swabs from each patient were collected and placed in one tube of Brain Heart Infusion broth (BHI, Oxoid, England) for a few hours to promote enrichment. Additionally, 100 samples were collected using foam swabs and a special transport media which used for culture and direct molecular analyses using Real-time PCR.

(i) All 300 nasal and throat samples were directly cultured for detection of *K. kingae* using selective vancomycin blood culture, chocolate agar for *Neisseria* species and all cultures were incubated for 48 hours at 37 C in 5% CO2 using anaerobic jar. [4]

(ii) The first 200 samples of nasal and throat swabs were mixed together in a 3ml Brain-Heart infusion broth (BHI, Oxoid) tube.

(iii) One 100 samples were mixed together in a 3ml liquid transport media tube recommended to be used for Real-time PCR test. All samples were stored at -70°C for further DNA extraction. *K. kingae* (ATCC 23330) and *N. meningitides* (ATCC 13077™) strains were included for quality control in biochemical identification of the isolates, antibiotic susceptibility testing, and as positive controls in genetic identifications. Nuclease-free water and a control strain of *Haemophilus influenzae* were used as negative controls.

**Culture identification and susceptibility tests**

All suspected growth resembling *K. kingae* or *Neisseria* species were examined for oxidase and catalase reactions, the Gram-stain, and those bacterial cells resembling morphologically to *Neisseria* and *Kingella* were examined by biochemical reactions using Remel Rap ID NH kit (Thermo Fisher Scientific, USA). Antimicrobial susceptibility test using disc diffusion method was performed according to the recommendation of Clinical Laboratory and Standards Institute (CLSI, 2016) [13].

**Molecular testing**

DNA extraction directly from 100 samples for direct detection of *K. kingae* was performed using the QIAMP® DNA mini kit (Qiagen, Germany) according to the manufacturer’s instructions. Real Time PCR assay was used for the detection of *rtxA* and *rtxB* genes of *K. kingae*. [12] DNA was extracted from fresh bacterial growth of preliminarily identified *Neisseria* spp. Using QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer’s instructions regarding the isolation of genomic DNA from bacterial plate cultures. All *Neisseria* DNA recovered were confirmed by Uniplex PCR to detect 16S rRNA gene according to the method of Kremastinou et al. [14].

PCR detection of *N. meningitidis* isolates were performed by amplification of the *ctrA* and *crgA* genes using specific primers as reported by Rizek et al. [15]. Furthermore, *Actin Beta* (*ACTB*) primers which are used for detecting *Homo sapiens ACTB* gene, their sequences, and product sizes (NCBI, NG-
were used as controls in qPCR for direct detecting the presence of *K. kingae* in 100 samples.

**Statistics**

Data generated from the study were tabulated as Microsoft Excel sheets and uploaded to Statistical Package for social Sciences (SPSS version 20). Frequency and percentage were calculated for the categorical data and Pearson chi-square test or Fisher’s exact test to determine whether there are any statistical differences in comparison to age groups the level significant was set at p-value of 0.05 to test the hypothesis of no association. All data analyses were performed using SPSS version 20. The $x^2$ -test was used for statistical analysis. $P \leq 0.05$ was considered statistically significant.

**Results**

Table 1 shows the demographic characteristics of the 300 examined children. The nasal and oral samples collected were distributed randomly in each month during the period from October 2018 through January 2019.

Table 1 shows the demographic characteristics of one 100 samples of examined children. These include age, gender, taking antibiotic at time of sampling and within 2 weeks before sampling, type of antibiotic treatment and important clinical data.

All 300 nasal and throat samples were negative for presence of *K. kingae* (0.0%) using selective vancomycin blood culture. Also, the one 100 prepared DNA samples for direct detection of *K. kingae* were negative for *rtxA* and *rtxB* genes, respectively, using Real-Time PCR (Figures 1 & 2). All suspected growth resembling to *Neisseria* spp. (36/300; 12%) based on Gram-stain, positive oxidase and catalase tests, and 21/36 (58.3%) have the IS1106 gene of *Neisseria* spp. Of these only 1/36 (2.8%) isolate was identified as *N. meningitides*. In addition, 20 out 36 (55.6%) of isolates were classified as *Neisseria* species and the rest 15 out 36 (41.6%) isolates were identified as *Moraxella catarrhalis* (Table 2). All

![Figure 1: Real Time PCR reaction of *rtxA* gene for *K. kingae*.](image)
Neisseria species isolates were highly susceptible to levofloxacin and gentamicin, while lower numbers of isolates were resistant to erythromycin and ampicillin as shown in Table 3.

A total of 21 of isolates Neisseria species were assayed for ctrA and crgA genes using PCR technique. The result shown in Table 2, indicate that only N. meningitides/21 (4.8%) have both ctrA and crgA genes, whereas 15/21 (41.6) of Moraxella catarrhalis have only the crgA gene. PCR product and pri-

Table 2. Detection identity of suspected 36 Neisseria isolates using biochemical tests and specific genes for Neisseria spp.1.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Neisseria spp.</th>
<th>IS1106 gene</th>
<th>CtrA gene</th>
<th>CrgA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>% positive</td>
<td>No.</td>
<td>% positive</td>
</tr>
<tr>
<td>Neisseria spp.</td>
<td>21/36</td>
<td>58.3</td>
<td>21/36</td>
<td>58.3</td>
</tr>
<tr>
<td>N. meningitides</td>
<td>1/21</td>
<td>4.8</td>
<td>1/21</td>
<td>4.8%</td>
</tr>
<tr>
<td>N. lactamica*</td>
<td>15/21</td>
<td>41.6</td>
<td>15/21</td>
<td>41.6</td>
</tr>
</tbody>
</table>

1: Sequencing of 16 Neisseria isolates showing no match with N. meningitides, whereas indicated highly genetically relation to N. lactamica. *: Biochemical tests using RemelRapid NH kit identified isolates only as Moraxella catarrhalis

Table 3. Antimicrobial susceptibility of 21 Neisseria isolates and one N. meningitidis.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Neisseria isolate (N=21)</th>
<th>N. meningitidis isolates (N=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible (%)</td>
<td>Resistant (%)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>19</td>
<td>90.6</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>18</td>
<td>85.7</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>0</td>
<td>21</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>

Neisseria species isolates were highly susceptible to levofloxacin and gentamicin, while lower numbers of isolates were resistant to erythromycin and ampicillin as shown in Table 3.

Figure 2: Real Time PCR reaction oftrxB gene for K. kingae.

Figure 2: Show sequencing result for crgA gene of N. meningitidis.
Discussion

This study investigated the colonization rate of K. Kingae and N. meningitides in the upper respiratory tract of young Jordanian children using selective culture methods and classical PCR and Real-Time PCR tests. The present study shows that no single K. kingae isolate was found among 300 investigated children, whom being examined at two major medical centers in Amman over 4-month period.

Numerous studies from various countries have reported that K. kingae is mostly recovered in low percentage (0.5% to 12%) from the upper respiratory tract of young children aged less than 5 years, and rarely isolated from adults [1, 5]. In consistence to the current study; a recent study from Canada has found no presence of asymptomatic pharyngeal carriage of K. kingae in young children in Vancouver [16]. Moreover, most studies detected that K. kingae are most commonly found in children suffering of osteoarticular infections or rarely in association with endocarditis and other invasive diseases [1-2, 4, 7]. The reasons for our negative result can’t be easily explained now, since there is no single study from Arab Middle East countries has reported about this organism either as cause of disease or prevalence in the throat of children. Furthermore, it has been demonstrated that the incidence K. kingae in the throats of children could be reduced by previous recent exposure to antibiotics, since the organism is extremely susceptible to antibiotics commonly used in treatment respiratory bacterial infections [4-5].

Many recent studies have found that Real-time PCR testing is highly sensitive for detection of K. kingae in clinical samples of children in case of a negative vancomycin culture from oropharyngeal swabs or body fluids [2-4, 6].

This study have documented that 48.67% of the examined children had received antibiotic at least within 2 weeks before collection of nasal and throat swabs, therefore, previous antibiotics treatment of children may explain the negative incidence of K. kingae in our study. Additionally, our study has a number of limitations. We used a convenient sampling of children population visiting the two hospitals, which is not truly representative of the local Jordanian community. In this study, we did not collect data from day care attendance that could potentially affect the rates of carriage in the Jordanian children. Finally, all available clinical data from examined children have not recorded any case of osteoarticular infections which can be due to invasive K. kingae.

The pathogenesis of K. kingae is based on production of a potent cytotoxin (RTX) and presence of type IV pili. Both virulence factors are responsible for colonization of the respiratory tract, attachment to the epithelium, and causing damage of bones and joints [17-19]. However, bacteremia and osteoarticular infections in young children can be associated with rapidly progressive and complicated endocarditis [4, 20].

This study also shows that the occurrence rate of Neisseria species in the upper respiratory tract of examined Jordanian children was 7% (21/300) using both culture and PCR methods, including only one N. meningitides isolate 4.8% (1/21). This result accounted for 0.3% (1/300) of the total children. Furthermore, 15/21 of our Neisseria species isolates were biochemically identified as Moraxella catarrhalis, whereas they are later proved to be highly related to N. lactamica according to the results of their DNA sequencing and closely similar to the DNA sequencing of N. meningitidis.

N. meningitidis is an obligate human pathogen colonizing at one time or other 10% to 35% of children and young adults’ nasopharyngeal mucosa without causing detectable symptoms [21-23]. However, nasopharyngeal carriage of N. meningitides is most prevalent in young adults whereas carriage of commensal Neisseria lactamica is mostly found in young children [11].

In Jordan, according to reports issued by the Ministry of Heath, each year a few cases of meningococcal disease are recorded in children and...
young adults. Jordan implements mandatory meningococcal vaccination with the quadrivalent (A, C, Y, W) vaccine for any person intending to travel for pilgrimage in Saudi Arabia. This measurement has reported to have significantly reduced the incidence of meningococcal disease during Hajj period [24].

Meningococcal disease is potentially fatal and should always be considered as a medical emergency. Admission to a hospital is necessary and diagnosis of disease should be done without delay. Prompt antibiotic treatment is lifesaving in case of bacterial meningitis is suspected in order to prevent serious neurologic complications or death. This study shows that all Neisseria species isolates were highly susceptible to levofloxacin and gentamicin, while lower numbers of isolates were resistant to erythromycin and ampicillin. These results are very similar to recent antibiotic susceptibility results observed in N. meningitidis isolates in Brazil [25]. There are few data on antibiotic resistance among other Neisseria species isolates from Arab neighboring countries including on N. lactamica, which is closely genetically related to N. meningitidis [14]. Furthermore, it has been reported that N. lactamica can transfer antibiotic resistance markers into the closely related species N. meningitides [26]. Several studies suggest that N. lactamica contributes to the emergence of N. meningitidis strains with intermediate resistance to penicillin by horizontal genetic exchange of penicillin binding- protein (PBP) genes [26].

Conclusion

Our findings exhibit that nasopharyngeal carriage of K. kingae was not detected among Jordanian children, during the 4-month period of surveillance, while rarely N. meningitides and commonly N. lactamica were found. This study confirms the value of prospective surveillance to control emerging of potential pathogens.

Acknowledgements

Thanks for the administration of The Jordan University and Al-Bashir Governmental Hospitals in Amman, Jordan, for its permission to carried out this study.

Funding

This work was supported by a grant from The Jordan University, Dean of Research (No. 112/2018 ).

Conflict of interest

The authors declare no conflict of interest.

References


