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Detection of *Haemophilus influenzae* Type b in Pneumonic Adult Patients in Iraq Represents a Demographic Change in Bacterial Pathogenicity

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ABSTRACT

Background: Among *Haemophilus influenzae* species, *H. influenzae* type b commonly causes pneumonia and meningitis in infants, whereas nontypeable *H. influenzae* commonly causes community-acquired pneumonia in adults.

Methods: We investigated the role of *H. influenzae* type b as a causative agent of pneumonia among adults; hundred blood and serum samples were collected from adult patients with respiratory tract infection and radiologically confirmed pneumonia but with negative results for routinely suspected organisms causing pneumonia. Complete etiological detection was achieved using enzyme-linked immunosorbent assay for both anti-*H. influenzae* type b immunoglobulin M and G antibodies and confirmed by polymerase chain reaction.

Results: Seropositivity to anti-*H. influenzae* type b immunoglobulin G antibodies was observed in 26% pneumonic adult patients, 53.8% of whom also had anti-*H. influenzae* type b immunoglobulin M antibodies. Confirmatory polymerase chain reaction revealed that all 26 seropositive patients were positive, whereas only 2 of the patients seronegative for both immunoglobulin M and immunoglobulin G antibodies were positive to *H. influenzae* type b.

Conclusion: These findings indicated that H. influenzae type b is recently being considered as an important etiological agent of community-acquired idiopathic pneumonia in adults in Iraq; this change in the target population represents a demographic change in *H. influenzae* type b pathogenicity. Therefore, elderly patients must be included in the immunization schedule and therapeutic prophylaxis against *H. influenzae* type b infection.

Keywords: Hib target group; Eastern Mediterranean region; Iraq

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INTRODUCTION

Haemophilus influenzae is a small, nonmotile, Gram-negative coccobacillus that has been classified according to the presence of capsule into the noncapsulated strain, also known as nontypeable *H. influenzae*, and the encapsulated strain, which are further classified according to their antiphagocytic capsule into six serotypes (a to f). *H. influenzae* type "b" (Hib) accounts for 95% of all strains that cause invasive diseases in infants, which is why this is the type against which the Hib vaccine is produced [1].

H. influenzae species is considered as a part of the nasopharynx normal flora in 75% of healthy children and adults. Only a minority, approximately 3%–7%, of healthy individuals intermittently harbor the encapsulated strain in their upper respiratory tract (URT) [2].

The unencapsulated strain causes URT infections in adults such as sinusitis and otitis media, which are generally noninvasive. In contrast, Hib represents a serious health concern in communities where large-scale immunization schedules have not been established, and Hib primarily causes pneumonia and meningitis in infants and young children in such communities, with the highest burden being among children aged 4–18 months [2, 3].

According to World Health Organization (WHO) reports, approximately 20 million cases of childhood pneumonia are reported each year in the Eastern Mediterranean region (EMR; Iraq is one of the countries in this region), among which 10% require hospitalization [4]. Furthermore, a study conducted by EMR Lower Respiratory Infections Collaborators (GBD) in 2015 reported that *H. influenzae* represents the third pathogen of concern responsible for causing lower respiratory tract infections in all the 22 countries of the EMR, preceded only by *Streptococcus pneumoniae* and respiratory syncytial virus, respectively [5].

A previous study conducted in the United Kingdom indicated that Hib was the first cause of bacteremic pneumonia in children before the era of the introduction of Hib vaccination in England in 1992, a step taken for implementing Hib vaccine as a part of the UK infants immunization schedule, which appeared to cause a change in the incidence of Hib disease accompanied with a change in clinical presentation in the UK. Over a 4-year period, it was found that 73% of invasive Hib infections occurred in adults, with 56% of them manifesting as bacteremic pneumonia [6].

According to the WHO and United Nations Children's Fund estimates, the Hib3
vaccine, a conjugate vaccine (a polysaccharide chemically conjugated to a protein carrier), was first implemented in the Iraqi national immunization schedule in 2012 as tetra, penta, and hexa combinations for infants aged from 2 to 18 months; the vaccine coverage in Iraq subsequently increased from 36% in 2012 to 84% in 2018 [7, 8] (Table 1).

Table 1: Iraqi national immunization schedule for Hib vaccine

Age	Vaccine
2 months	HEXA-1 (DPT+Hep-B+Hib+ injectable POL)
4 months	HEXA-2 (DPT+Hep-B+Hib+injectable POL)

6 months	HEXA-3 (DPT+Hep-B+Hib+ injectable POL)
18 months	PENTA (DPT+Hep-B+Hib)
4-6 years	TETRA (DPT+Hep-B+Hib)

METHODS

Study design

In this prospective cross-sectional study, a total of 100 blood and serum samples were collected over a period of 6 months (October to March, 2017-2018) from adult patients with respiratory tract infection and radiologically confirmed pneumonia. Although this study was conducted during the postlicensed Hib vaccination era in Iraq, the Iraqi national immunization schedule includes only infants, and hence all included samples were not vaccinated as they were adults.

Adult patients with suspected pneumonia depending on the clinical symptoms

examined by the physician (cough, fever, severe sore throat, breathing difficulty, difficulty and pain during swallowing and abnormal sounds during breathing) were sent for laboratory diagnosis to identify the suspected pneumonia agents (*S. pneumoniae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus* group A, and *Pseudomonas aeruginosa*) and for X-ray examination. Hib was not included in the abovementioned laboratory diagnosis as it is not recommended for adults. Radiographs examined by the radiologist (interpretation depending on the standard WHO criteria) along with the bacterial diagnosis and clinical symptoms formed the basis for including the samples in this study.

Complete etiological detection of Hib infection was achieved by serological

techniques using anti-Hib immunoglobulin (Ig) M and IgG antibodies by indirect enzyme- linked immunosorbent assay (ELISA) using ELISA kits (ATZ, life technologies, PVT., Ltd. India. This kit enables quantitative detection of free polyribosyl ribitol phosphate (PRP)- moiety but not the conjugated PRP such as that found in anti-diphtheria and anti-tetanus toxoids. Patient result was considered as negative if it was equal or less than negative control, but in case of positive results, the precise concentrations were determined using the standard curve.

For further verification of all serum samples, polymerase chain reaction (PCR) was conducted as a confirmatory test. Genomic bacterial DNA was extracted from the plasma according to the manufacturer's instruction (Presto Mini gDNA bacteria kit, Geneaid, Biotech, Taiwan). The quality of the extracted DNA was then verified by electrophoresis; 5µl of DNA and 2µl of loading dye were loaded and resolved using 1% agarose for 1hr. The PCR run was performed by adding 2µl DNA, 1µl of each forward and revers primers, 5µl Master mix from (Promega Corporation) and 11µl nuclease-free distilled water. Recommended PCR conditions were 95°C/300 s, 94°C/30 s, 60°C/30 s, 72°C/30 s, and 72°C/7 min (40 repeats). The PCR product was further verified by loading 5µl DNA product and resolving it on 1.5% agarose for 2hrs.

The following primers designed for the detection of *H. influenzae* capsular types (b) were targeted within variable region II of the encapsulation cap locus (Bioneer, South Korea): Forward primer TGTTCGCCATAACTTCATCTTAGC, TM(C) 60 Concentration (nM) = 900, nucleotide position = 5579–5602. Reverse primer GGTCTGCGGTGTCCTGTGTT, TM(C) 60 Concentration (nM) = 900, nucleotide position = 2382–2363. Amplicon length Hib 147, GenBank accession number X78559 [9].

Data analysis

Data were presented as frequency and percentage, SPSS version, 23. P-value was detected via Fishers exact test.

RESULTS

The 100 radiologically confirmed pneumonic adult patients were aged 18–88 years and consisted of 49 females and 51 males. The serum samples of all patients were subjected to both ELISA and PCR techniques. Results of indirect ELISA revealed that 26/100 (26%) of the study patients had anti-Hib IgG antibodies, and 14/26 also had IgM antibodies, indicating that 53.8% of the pneumonic adult patients had both anti-Hib IgG and IgM antibodies. However, none of them had anti-Hib IgM antibodies alone.

Results of the confirmatory PCR test revealed that 28% (28/100) of the pneumonic adult patients were positive for the variable region II of the encapsulation cap locus to which the primer was designed. Of these 28 patients, 26 were seropositive to anti-Hib IgG antibodies as indicated by the ELISA test. The remaining two patients were seronegative anti- Hib antibodies.

Considering the results of both of ELISA and PCR techniques, it was found that the majority of Hib infections (57.14%) occurred in the age group of 51–70 years, whereas 42.8% of the infections were observed in the other age groups, referring to a significant difference P-value = 0.021 (Table 2). Regarding sex distribution, 42.85% (12/49) of all Hib infections were detected among females comparing to 57.14% (16/51) in males. The case fatality ratio was zero.

Table 2: Age groups distribution for patients infected with Hib

Age groups (years)	Total number	Number of infections	Infection percentage in each age group	Infection percentage according to all Hib infections (28)
<30	15	2	13.3%	7.14
31-40	18	3	16.6%	10.71
41-50	18	4	22.2%	14.28

51-60	15	8	53.3%	28.57*
61-70	23	8	34.7%	28.57*
>70	11	3	27.2%	10.71
Total	100	28	28	99.98

*By comparing number of infections of the two age groups (collectively) with other age groups, a significant difference was detected, P-value= 0.021.

DISCUSSION

This study was conducted to identify the role of Hib as an etiological agent in idiopathic pneumonic adult patients in Iraq.

Iraq is one of the developing countries located in the EMR occupying an area of 438.317 km2. With Baghdad being its capital, the total population of Iraq is 36 * 106 [10].

Community-acquired pneumonia is a major cause of morbidity and mortality in both children aged <5 years in developing countries and elderly individuals, especially those with chronic pulmonary disease [1]. *H. influenzae* is considered as the third most important etiological agent for pneumonia in the EMR [5].

Considering the limited data regarding Hib burden in Iraq along with the availability of other studies outside Iraq indicating the possible role of Hib in pneumonic adult patients, this study was conducted to determine the burden of this bacterium as an etiological agent for pneumonia in adults. The study subjects included adult patients of both sexes with clinical symptoms of lower respiratory tract infection with radiologically confirmed pneumonia diagnosed in a tertiary care hospital.

In this study, only ELISA and PCR techniques were used for complete serotype detection, and not the conventional tests for *H. influenzae* detection, which include capsular serotyping by slide agglutination using type-specific antisera and cultivation. Conventional methods are typically associated with misinterpretation and problems such as autoagglutination, cross-reaction, and observer variation in the agglutination test [11]. Moreover, detection of *H. influenzae* by differential blood culture rarely gives a positive result, primarily because of methodological problems, and also requires precise concentrations of certain parameters such as V and X factors [12].

In this study, Hib was detected using the capsular genotyping method through a PCR- based technique, which is considered as a definitive method of typing Hib. Molecular typing using appropriate primers could allow an accurate differentiation between typeable, nontypeable, and other strains of *Haemophilus* species, such as those that possess a partial capsule locus and are unable to export their polysaccharide capsule to the cell surface, thereby misinterpreting it as NTHi through conventional methods [11]. For instance, 11% of isolates detected by slide agglutination were encapsulated and had nonfunctional capsular genes as evaluated by the PCR technique [13].

Our results showed that Hib infected 28% of the pneumonic adult patients, which mismatches the common demographic feature of *H. influenzae*, indicating the relationship between Hib and meningitis and pneumonia in young children but not in adults. Nevertheless, NTHi is often responsible for pneumonia in adults [1].

Only a few studies have been conducted in the world regarding *H. influenzae* infection in adults, thus indicating the limitation of data in this age group [14]. The percentage of Hib infections detected in the present study appears to be much higher than that recorded in a previous retrospective study conducted in the USA [15]. In that study, the authors observed that the majority of (5/105) of *H. influenzae* infections (pneumonia and meningitis) in elderly subjects aged >65 years were caused by NTHi compared to Hib infections, which were responsible for only a minor number (0.08/105); moreover, they recorded 19.4 CFR in their study compared to 0 among Iraqi patients in the present study.

Heath T et al (1997) [16] recorded two fatal cases of Hib infection in a nursing home; both were females and roommates, and one of them was diagnosed with pneumonia, and the other one with extensive epiglottitis, laryngitis, and tracheitis, with no evidence of pneumonia.

Moreover, severe infections caused by Hib such as pneumonia and epiglottitis are increasingly being diagnosed since the 90s among the elderly and immunosuppressed adults; however, such infections occur in the form of sporadic cases, although clusters have been rarely reported [17, 18]. Other studies have revealed that recently, in the post vaccination era, invasive Hib disease more frequently occurs in adults, elderly, and immunocompromized children. A study conducted in USA revealed that Hib is responsible for 9% of invasive diseases in adults ranging from 18 to ≥65 years of age [19, 20].

In agreement with our results that the majority of Hib adult infections were detected among the age group of 51–70 years, a previous study conducted in the United Kingdom also reported that the majority of community-acquired pneumonia cases caused by Hib were detected in people aged 45–65 years and not in the very elderly people [1]. In addition, data recorded by the Academic Medical Center in the Netherlands regarding Hib infections in adults had indicated Hib invasive infections represented by meningitis in 38% of meningitis cases among adults aged >50 years [21]. Our findings are consistent with those of other studies that have described changes in the bacterial epidemiology following the implementation of Hib vaccination [6].

It is speculated that this epidemiological change is a multifactorial phenomenon.

Natural genetic competence and exchange of DNA fragments between different *H. influenzae* strains at a high frequency may result in the acquisition of new virulence genes that would ultimately result in increased Hib virulence and acquisition of new traits, especially NTHi that already exists as normal flora in the URT of 75% of healthy children and adults, whereas Hib is found in only a minority of approximately 3%–7% of healthy individuals [22].

Moreover, it is believed that the change in the Hib infection pattern caused by the dissemination of bacteria beyond the

respiratory tract mucosa is due to the inherent capability Hib to overcome host barriers and the age-related decline of both cellular and humoral immune responses, thereby rendering elderly subjects more susceptible to infectious diseases [23]. Mucosal IgA is the primary antibody class found in the respiratory mucosa, which activates phagocytosis and the membrane attack complex that efficiently kill NTHi, but Hib can also produce IgA protease. Moreover, the polysaccharide polyribosylribitol capsule prevents phagocytosis and reduces the attachment of C3 to the bacterial surface, thus inhibiting both classical and alternative pathways, the primary mechanisms for killing Hib [24]. Such capabilities of Hib support its survival and resistance to mucosal barriers to pass through and reach other tissues.

Improvements in bacterial detection strategies and serotyping using molecular techniques are one of the substantial factors related to increased accuracy in detecting bacterial species and serotypes.

Although bacterial culture is the gold standard for Hib detection, the major disadvantage is that it requires several days for complete detection and confirmation of serotyping, in addition to other methodological problems such as operator experience and the need for special growth factors. In contrast, slide agglutination is the gold standard for serotype identification; however, this technique is prone to misinterpretation due to autoagglutination, nonspecific agglutination, and cross-reaction [25].

Therefore, there is a transition toward the application of molecular techniques. PCR technique has also been developed to detect the capsular locus genes represented by *bexA* and *bexB*; this technique has substantially improved the accuracy of *H. influenzae* serotyping. Moreover, real-time PCR assays have been developed to detect protein D gene (*hpd*), which is a surface exposed lipoprotein that shows a high molecular conservation among typeable and NTHi strains. On the other hand, real-time PCR for identification of *ompP2* and *bex A* genes is to identify typeable strains only. Real-time PCR is more sensitive than conventional methods and has shorten the time need for detection and does not involve the application of special ingredients like X and V factors required in conventional methods for differential bacterial growth [26 , 27].

A limitation of the present study is that neither the role of non-b encapsulated *H. infleunzae* nor the predisposing factors for idiopathic pneumonia were verified.

Indeterminacy of the causative pathogen(s) makes disease control a dilemma. According to our findings, it is recommended that Hib should be tested in adults diagnosed with idiopathic pneumonia, and the elderly should be involved in the national immunization schedule and chemoprophylaxis to substantially control Hib disease.

In conclusion, this study has confirmed the role of Hib as an etiological agent for community- acquired pneumonia in idiopathic cases in adults. It appears that the role of Hib in pneumonia has been underestimated or has recently changed after the Hib vaccination era. Hib appears to exhibit a demographic alteration by changing the target age group from infants and young children to adults. Therefore, it would currently be valuable to include idiopathic pneumonic adult patients for the detection of this pathogen in the laboratory diagnosis. Elderly people should also be included in the national immunization schedule against Hib infection.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

ETHICAL APPROVAL

This project was reviewed and approved by the Institute Review Board, / College of Medicine/ Al- Nahrain University (IRB)-314-No: 20191133, Baghdad, Iraq.

KEY POINTS

- Demographic change in Hib epidemiology is being recorded in different areas of the world, and according to the present work, Iraq is one of them.
- Hib should be considered as an expected causative agent for idiopathic pneumoia in adults
- Elderly should be included in Hib vaccination schedule and should be subjected to therapeutic prophylaxis.

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