

Original Research Article

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Haemophilus influenzae in Community Acquired Infections

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ABSTRACT

Haemophilus influenzae is strictly a human pathogen, responsible for many diseases like community acquired pneumonia, meningitis, sinusitis, epiglottitis and otitis media. *Haemophilus* type b (Hib) was the most common strain responsible for all the infections. Non type b strains were at an emerging risk. Ampicillin was commonly used but resistance due to TEM-1 gene is being reported. It is aimed to find out whether Hib continues to be isolated from childhood cases of pneumonia and its susceptibility to the drugs of choice and possible mechanisms of resistance. All purulent respiratory samples were processed for isolation of *Haemophilus influenzae*. They were identified based on its X&V factor requirement and a negative Porphyrin synthesis test. Biotyping and Serotyping was done using standard methods. Ampicillin resistance was characterized by phenotypic and genotypic methods. From the results, out of 1540 purulent respiratory samples, *Haemophilus influenzae* was isolated from 103 cases. Among the 103 isolates, 69 were Nontype b and 34 were type b *Haemophilus influenzae*. All the Hib isolates were from those above 20 years of age. Among 15 Ampicillin resistant isolates, 13 were positive for TEM-1 gene. Finally, Nontype b isolates were predominant and Hib was isolated only from adult age group.

Keywords

Hib, Non type b,
Porphyrin synthesis
test,
X & V factors,
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Introduction

Haemophilus influenzae is strictly a human pathogen responsible for many diseases like community acquired pneumonia, meningitis, sinusitis, epiglottitis and otitis media (Mac Neil *et al.*, 2011). *Haemophilus influenzae* is a short pleomorphic Gram negative bacillary rod which is fastidious in nature. It is mainly recovered from the human respiratory tract and very rarely from other sites. The mode of spread of the infection is mainly airborne droplets (Puig *et al.*, 2014). Among the 6 capsulated strains (a, b, c, d, e, f) type b remains the most common cause of invasive diseases (Puig *et al.*, 2014; Jain *et al.*, 2006). Type b H. influenzae colonizes the respiratory tract of children at a rate of 2- 4% and this has

shown substantial decrease after the introduction of the conjugate vaccine. Non typeable strains account for 25-35% of otitis media in children less than 5 years of age and exacerbations of chronic obstructive pulmonary disease in adults (Urwin *et al.*, 1996).

Haemophilus influenzae is confirmed by performing satellitism, in which the colony grows near a streak of *Staphylococcus aureus*. *Haemophilus influenzae* is a fastidious organism which requires two important factors, namely, factor X (hemin factor) and factor V (Nicotinamide adenine nucleotide factor) for its growth. The detection of

porphyrins produced from b-Aminolevulinic acid is evaluated for use in speciation of the *Haemophilus* genus (Lund *et al.*, 1977). Porphyrin synthesis is positive for the non hemin requiring *H. parainfluenzae* and is negative for *Haemophilus influenzae* which requires both hemin and NAD factor for its growth.

Haemophilus influenzae can be classified in to eight biotypes based on three biochemical tests indole, Ornithine, urease and this has been used as an epidemiological marker for carriage in the upper respiratory tract. Of these biotypes, type b falls mostly in biotype I (Campos *et al.*, 2004).

Infections caused by *H. influenzae* were initially being treated with cell wall active agents like Ampicillin and protein synthesis inhibitors like Chloramphenicol. Now the favoured regimen is Ceftriaxone, Tetracycline and Azithromycin (Barbour *et al.*, 1996; Guevara *et al.*, 2008). Some of the *Haemophilus influenzae* shows resistance to ampicillin which is mainly due to beta lactamase production. Beta lactamase may be due to two genes, TEM-1 and ROB-1 genes, but the most commonly isolated gene is the TEM-1 gene. Both phenotypic and genotypic methods were used to detect Ampicillin resistance (CDC, 1998).

The aim of this study is to identify the serotype predominance in childhood and adult cases of pneumonia and to know the whether the vaccination against Hib has had any effect on the serotype predominance in different age groups and whether the isolates are susceptible to the drugs of choice like Ceftriaxone, tetracycline and Azithromycin. The study also characterizes resistance to Ampicillin, which was used as an empirical treatment by the clinicians in earlier days for upper respiratory tract infections.

Materials and Methods

Study population

The study was done during the period from March 2014 to August 2015. A total of 1540 purulent respiratory samples from patients suspected of having community acquired pneumonia were processed in our microbiology laboratory.

Isolation of *Haemophilus influenzae*

All samples as indicated above were inoculated on Chocolate agar, Fildes agar, Chocolate agar with antibiotics (Vancomycin, Bacitracin and Clindamycin), and Blood agar with Staphylococcus streak.

Blood agar (MacNeil *et al.*, 2011)

Blood agar was streaked with the sample along with Staphylococcus aureus and it was incubated at 37⁰ C in the presence of 5-10% CO₂ in a candle extinction jar for 24 hours. They form low, translucent, convex, pinpoint colonies around the staphylococcus which provides the factor V for its growth. The size of the colony decreases as it goes away from the staphylococcus streak. This is the satellite phenomenon shown by *Haemophilus influenzae*.

Chocolate agar (Kilian, 1976)

Heated blood agar or the Chocolate agar which provides both the hemin and NAD factor was streaked with the sample and it was incubated at 37⁰ C in the presence of 5-10% CO₂ in a candle extinction jar for 24 hours. *Haemophilus influenzae* shows a shiny, tiny, smooth, flat colony which smells like wet fur.

Fildes agar (Campos *et al.*, 2004)

Fildes enrichment is an enzymatic digest of blood used to enhance the growth of fastidious organisms. The resultant digest is rich in growth factors including X and V factor required by *Haemophilus influenzae*. To the BHI chocolate agar of 100 ml, 5ml of Fildes enrichment broth was added and allow the media to set. The sample is streaked and incubated at 37⁰C in the presence of 5-10% CO₂ in a candle extinction jar for 24 hours. Colony morphology is very similar to that on chocolate agar.

Chocolate agar with VBC (Harrison *et al.*, 2009)

The CHOC-VBC media was made by preparing chocolate agar and the following antibiotics were added in concentrations of 5 microgram/ml Vancomycin, 300 microgram/ml of Bacitracin, 1 microgram/ml of clindamycin. After that the media is allowed to dry and the samples are directly streaked on to the plates and incubated at 37⁰C in the presence of 5-10% CO₂ in a candle extinction jar for 24 hours. *Haemophilus influenzae* shows a shiny, tiny, smooth, flat, slightly yellow colony which smells like wet fur.

Antibiotic susceptibility testing (Nag *et al.*, 2001)

Antibiotic susceptibility testing was done on chocolate agar for susceptibility to Ceftriaxone, Azithromycin, Aztreonam, ciprofloxacin, ampicillin, tetracycline, Cotrimoxazole and Meropenem based on 2014 CLSI guidelines. Ampicillin resistant strains were then tested for beta lactamase production by acidimetric method. One of the genes responsible for beta lactamase production (TEM-1) was identified by conventional PCR. The following primers are used for detection of TEM-1 gene:-

5'TGGGTGCACGAGTGGGTTAC3' and
5'TTATCCGCTCCATCCAGTC3'

Porphyrin synthesis test (Campos *et al.*, 2004)

This test indicates the absence of requirement of X factor. If α -Amino levulinic acid is provided to the bacterium, it synthesizes it and excretes porphobilinogen and other porphyrins. This test is carried out by using Kovacs's indole reagent (Para dimethyl amino Benzaldehyde) and formation of pinkish red colour in the lower water phase indicates the synthesis of porphyrin and absence of X factor requirement.

X and V requirement (Madore, 1996)

Make a lawn culture in the Muller Hinton agar plate and keep the commercially available discs X and V from HIMEDIA laboratories. The X factor (hemin) and V factor (Coenzyme- Nicotinamide adenine dinucleotide NAD⁺) are impregnated on the sterile filter paper discs of diameter 6 mm. If both X and V factors are

Typing

- i) Biotyping was done using the biochemical tests as follows: - Indole production, Ornithine decarboxylation and urease production (Campos *et al.*, 2004).
- ii) Serotyping was done using –Becton-Dickinson --- Kit.
- iii) BD Difco catalogue no. Type b-222361
- iv) Type a-222501
- v) Type f-227921

Results and Discussion

Among the 1540 cases of suspected pneumonia, the purulent respiratory samples yielded. *Haemophilus influenzae* were noted in 103 cases. Out of 103 isolates, 84 were

from inpatient samples and 19 were from outpatient samples. Among 84 inpatient cases, 33% were associated with COPD, 26.2% with Bronchial asthma and 18.44% were provisionally diagnosed as pyrexia of unknown origin.

Distribution of *Haemophilus influenzae* in various age groups

Among the 103 *Haemophilus influenzae* isolates, 95 (92%) of them were isolated from patients above 20 years of age and 8 (7.7%) from below 20 years. Among the 95 strains of *Haemophilus influenzae* isolated from adults, 63 (61.2%) were in males and 40(38.8%) in female patients. The strains isolated were more during winter months of October to January (Figure 1).

Serotype

All the isolates of *Haemophilus influenzae* were serotyped with type b, type a and type f antiserum. A total of 34(33%) were type b and the remaining 69 were non-type b. Among the 69 non type b strains, 46.6% were other serotypes, 10.7% were type a and 9.7% were type f. (Figure 2). Comparison of *Haemophilus influenzae* serotype b in different age groups showed that all isolates of type b were from those above 20 years, whereas no type b were isolated from below 20 years and this is statistically significant ($p < 0.05$).

Biotypes

Haemophilus influenzae can be divided in to eight biotypes based on three biochemical tests, namely, Indole, Ornithine and Urease production. Out of the 103 *Haemophilus influenzae* isolates, strains belonging to all 8 biotypes were detected and 47 strains belonged to biotype I (Table 1).

Antibiotic sensitivity pattern

Antibiotic sensitivity testing of 103 isolates of *Haemophilus influenzae* showed that 91.2% were sensitive to Ceftriaxone, 96.1% to Azithromycin, 99.02% to Aztreonam, 94.2% to ciprofloxacin, 85.4% to Ampicillin, 96.1% to Meropenem, 97.08% to Tetracycline and 65.04% to Cotrimoxazole.

Detection of Ampicillin resistance

Among the 103 isolates of *Haemophilus influenzae* isolated, 15(14.6%) strains showed Ampicillin resistance. Out of the 15 Ampicillin resistant strains, all 15 produced beta lactamases by acidimetric method and 13 showed presence of TEM-1 (Figure 3).

Haemophilus influenzae causes a variety of community acquired respiratory infections, including sinusitis, acute otitis media, bronchitis and community acquired pneumonia. Earlier, these infections were mainly caused by *Haemophilus influenzae* serotype b (Hib), but after the introduction of vaccines, the incidence of *Haemophilus influenzae* type b has markedly reduced. In our study, Non type b *Haemophilus influenzae* has emerged as an increasing cause of the pneumonia (67%) as compared to type b and this is similar to other studies also where it is isolated in 70-90% cases (Mac Neil *et al.*, 2011; Puig *et al.*, 2014) and Jain *et al.*, (2006).

Earlier studies done during the period from 1993-1997, show a high rate (86%) of isolation of Hib amongst children below 5 years of age (Lund *et al.*, 1977). Studies between 1996 and 2004 have reported lower isolations of 26-28% among children (Urwin *et al.*, 1996; Campos *et al.*, 2004 and Barbour (1996).

Among children in Asia and central European countries a considerable decrease has been demonstrated from 21 cases per 1, 00,000 populations per year in 1994 to 5 cases per 1, 00,000 populations per year by 2013 (MacNeil *et al.*, 2011; Puig *et al.*, 2014). A comparative study in Costa Rica have also reported a significant decline in the incidence of type b *H. influenzae* when the data from 1992 -1997 was compared with that between 1999 and 2004 (Guevara *et al.*, 2008). By June 2011, Hib vaccine was included in the National Immunization program of 170 countries in all regions of the world. After that the incidence of Hib invasive disease and oropharyngeal carriage in young children has drastically decreased to 0.3-0.7 cases per 1, 00,000 populations per year wherever vaccination programs have been implemented (Campos *et al.*, 2004; Barbour, 1996 and Madore, 1996). In our study none of the isolates were from cases below 20 years of age.

The *H. influenzae* isolates in this study were more commonly seen among male patients (61%), which are similar to other studies (Urwin *et al.*, 1996). In contrast, a study reports higher prevalence among female elderly patients, and a lower prevalence in female children (Mac Neil *et al.*, 2011). This infection has always shown a seasonal variation. Isolation of *H. influenzae* has shown higher incidence during winter months across many countries (Kilian, 1976; Zhu *et al.*, 2015). In our study also 61% were isolated during winter months. An increase in isolations during rainy season is also observed in some studies (Guevara *et al.*, 2008; Zhu *et al.*, 2015 and Leibovitz *et al.*, 2004).

In this study, strains belonging to all known biotypes were isolated and 44% were Biotype I whereas in other studies 60-70% belonged to this biotype (Landgraf *et al.*, 1993). COPD was an important risk factor for the development of *Haemophilus influenzae*

pneumonia and their association was 25 to 30% percent in many studies (Jain *et al.*, 2006; Urwin *et al.*, 1996), when compared to 33% in our study. Association of *Haemophilus influenzae* with bronchial asthma cases were 26.2% in our study which correlated well with many studies where 28% association is noted (Jain *et al.*, 2006; Urwin *et al.*, 1996).

Ceftriaxone being the drug of choice for invasive *H. influenzae* infections, 91% of our isolates were susceptible, and this correlated well (95%-97%) with other studies in both developed and developing countries (Zhu *et al.*, 2015; Harrison *et al.*, 2009). Infections caused by *Haemophilus influenzae*, particularly sinusitis and otitis media are now frequently treated with fluoroquinolones and their sensitivity were 94% in our study which is comparable to other studies (Farrell *et al.*, 2005).

A susceptibility of 94.5% in 2012 as compared to 54% in the year 2000 was observed with tetracycline and this remarkable increase in susceptibility is reportedly due to decrease in its usage in the past 20 years (Bennett *et al.*, 2014). In this study also 97% of isolates were sensitive to tetracycline.

Azithromycin is an acid stable orally administered Macrolides antimicrobial drug that appears to be more active against many Gram-positive pathogens and several other Gram negative pathogens, notably *Haemophilus influenzae*. After oral administration, serum concentrations of Azithromycin are lower reflecting the rapid and extensive movements of the drug from the circulation into intracellular compartments resulting in increased tissue concentrations (Puig *et al.*, 2015). In our study 99% were susceptible to Azithromycin, which was similar to other studies (Appelbaum *et al.*, 2003; Jacobs *et al.*, 1999; Doern *et al.*, 1997).

High level resistances to Macrolides in *H. influenzae* (0.5%-2%) are rare as is reported in many studies (Appelbaum *et al.*, 2003; Jacobs *et al.*, 1999; Doern *et al.*, 1997). Macrolides are of particular use in children who are allergic to beta lactams (Jacobs *et al.*,

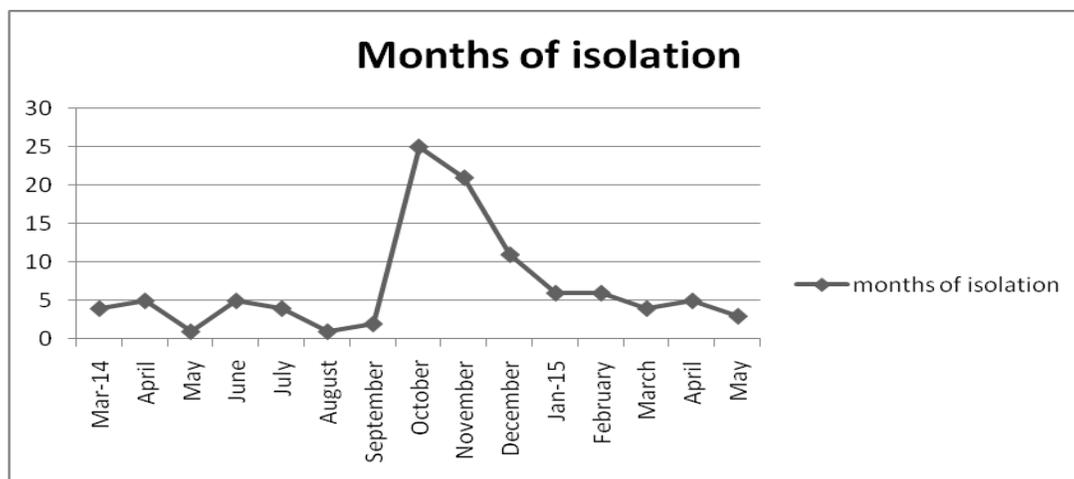
1999). There was no difference in susceptibility between type b and non-type b isolates to Ceftriaxone, tetracycline, Azithromycin and ciprofloxacin (Zhu *et al.*, 2015; Nag *et al.*, 2001).

Table.1 Biotype distribution among different isolates of *Haemophilus influenzae*

Biotypes	Number of isolates showing different biotypes	Percentage
I	47	45%
II	6	6%
III	5	5%
IV	1	1%
V	2	2%
VI	5	5%
VII	4	4%
VIII	33	32%

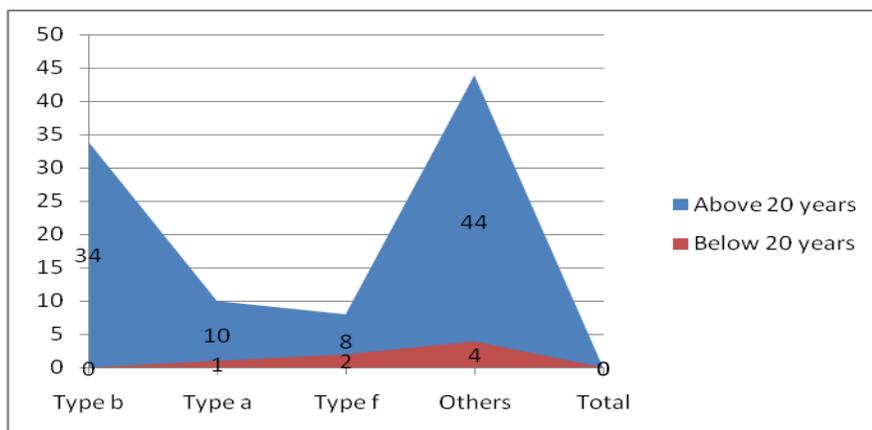
Haemophilus influenzae isolates, strains belonging to all 8 biotypes were detected and 47 strains belonged to biotype I (Table 1).

Fig.1 Seasonal variation of *Haemophilus influenzae*



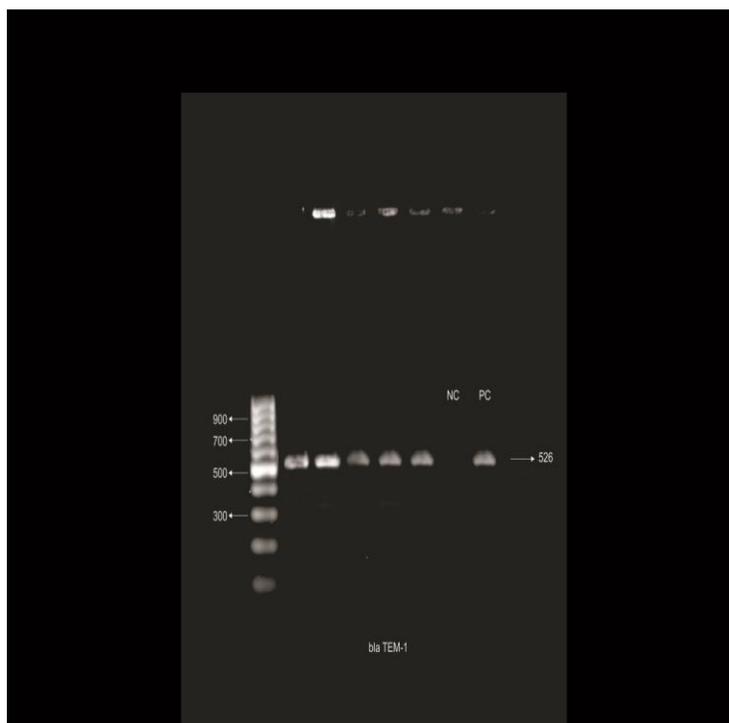
The strains isolated were more during winter months of October to January

Fig.2 Serotypes in different age groups



No type b was isolated from below 20 years and this is statistically significant ($p < 0.05$)

Fig.3 TEM-1 gene detection by PCR



Beta lactamase mediated resistance was seen in 14.6% isolates in this study, whereas in multicentre studies done in US between 1984-1999 it was 15% to 36 % (Nag *et al.*, 2001) and in recent studies done in China, the percentage of Ampicillin resistant strains progressively increased from 4% in the year 2000 to 29% in 2012 (Zhu *et al.*, 2015). The resistance detected was higher among type b

(60%) strains when compared to non type b strains (40%) which is similar to study by Nag *et al.*, (30% and 20%) suggesting that the strains that are invasive is showing a higher ampicillin resistance (Nag *et al.*, 2001).

All the ampicillin resistant isolates produced beta-lactamase enzyme when screened by the phenotypic acidimetric method, but when

analyzed by molecular method, only 13 isolates (86%) were positive for TEM-1 gene which is similar to many studies where the detection of TEM-1 gene (93%) is more when compared to the ROB-1 gene (5%) detection (Hasegawa *et al.*, 2003; Farrell *et al.*, 2005 and Kuvat *et al.*, 2015).

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