High ampicillin resistance in different biotypes and serotypes of \textit{Haemophilus influenzae} colonizing the nasopharynx of healthy school-going Indian children

Amita Jain,\textsuperscript{1} Pradeep Kumar\textsuperscript{1} and Shally Awasthi\textsuperscript{2}

Departments of Microbiology\textsuperscript{1} and Pediatrics\textsuperscript{2}, King George’s Medical University, Lucknow, UP, 226003, India

\textit{Haemophilus influenzae} is one of the main causes of otitis media, sinusitis, meningitis, pneumonia and septicaemia in children, and the development of ampicillin resistance in \textit{H. influenzae} is a cause of serious concern. The aim of the present study was to determine the prevalence of ampicillin resistance in \textit{H. influenzae} colonizing the nasopharynx of school-going healthy North Indian children, and to compare the distribution of different biotypes and serotype b in this population. A total of 2400 school-going healthy children from 45 rural and 45 urban schools were enrolled.

Nasopharyngeal swabs were collected from the children and cultured. \textit{H. influenzae} was isolated from 1001 (41.7\%) of the 2400 nasopharyngeal swabs collected. All these \textit{H. influenzae} isolates were biotyped and serotyped, and their antibiotic susceptibility tested. All eight biotypes were present in this population. The most prevalent biotypes were I (19.6\%), II (16.8\%) and III (25.0\%). Of the 1001 isolates, 316 (31.6\%) were \textit{H. influenzae} type b and 685 (68.4\%) were non-type b \textit{H. influenzae}, and 22.9\% were resistant to ampicillin, 41.9\% to chloramphenicol, 27.5\% to erythromycin and 67.3\% to co-trimoxazole. Of the 316 \textit{H. influenzae} type b isolates, 44.0\% were ampicillin resistant, while only 13.1\% non-type b \textit{H. influenzae} isolates were ampicillin resistant. Of the 229 ampicillin-resistant \textit{H. influenzae} isolates, 196 (85.6\%) were positive for \(\beta\)-lactamase; 93.4\% (214/229) were biotypes I, II and III, of which 49\% were biotype I, 27.9\% were type II and 16.6\% were type III. Most of the strains belonging to biotypes III–VIII were ampicillin sensitive. Ampicillin resistance is significantly more common in biotype I and serotype b than in other biotypes and serotypes.

\textbf{INTRODUCTION}

\textit{Haemophilus influenzae} asymptomatically colonizes the nasopharynx of healthy individuals, and causes systemic disease and mucous membrane infections. Eight biotypes and six serotypes are used as epidemiological markers for studying the pattern of colonization of \textit{H. influenzae} and to identify the strains of bacterium commonly known to be pathogenic (Alrawi \textit{et al.}, 2002). For instance biotype I and serotype b are commonly associated with meningitis in children, and biotypes II and III are commonly associated with upper respiratory tract infections (Pittman, 1931; Kilian, 1976; Gratten, 1983).

Ampicillin/amoxyccillin was the empirical treatment for \textit{Haemophilus} disease until the recent past. Development of ampicillin resistance in causative organisms led to use of third-generation cephalosporins (e.g. ceftixime) as empirical drugs. Resistance to ampicillin results from the production of a \(\beta\)-lactamase and alteration of the antibiotic target, penicillin-binding protein (Markowitz, 1980; Mendelman \textit{et al.}, 1984). Treatment problems linked to the prevalence of \(\beta\)-lactam resistance are compounded by the frequency of cross resistance to many other antibiotics (Talon \textit{et al.}, 2000). The high cost of cephalosporins and the development of drug resistance due to irrational use of antimicrobials are two important limiting factors in the use of cephalosporins in the peripheral level health care system, in developing countries. Currently, in Indian hospitals co-trimoxazole is recommended for the treatment of children with respiratory tract infection at primary health centres. Children presenting with meningitis are treated with third-generation cephalosporins (e.g. ceftixime). Continuous monitoring of the antimicrobial resistance patterns of \textit{H. influenzae} is recommended for cost-effective treatment of these infections. Vaccination against \textit{H. influenzae} is usually not carried out for children in our setting. Isolation of \textit{H. influenzae} from cerebrospinal fluid and blood in peripheral hospitals of developing countries may not be possible due to the lack of adequate lab facilities at these hospitals. Hence, the monitoring of nasopharyngeal \textit{H. influenzae} as a
surrogate marker for invasive and locally invasive *H. influenzae* seems an attractive option (Das *et al.*, 2002). In the present study, we aimed to characterize the *H. influenzae* serotypes and biotypes, and looked for their association with ampicillin resistance. An effort was also made to decipher the role of β-lactamase in the development of ampicillin resistance.

**METHODS**

**Study population.** From July 2000 to November 2002, a total of 2400 nasopharyngeal specimens were collected from normal school-going children aged between 5 and 10 years, after obtaining an informed written consent from the parents of the child. The mean age of enrolled children was 6.72 years. A total of 90 randomly selected (45 rural and 45 urban) government primary schools, from the Lucknow district of Uttar Pradesh, a Northern state of India, were enrolled. A uniform distribution of rural and urban, and male and female population was ensured.

**Sample collection and transport.** A calcium alginate tipped swab on a flexible aluminium wire (Hardwood Product Company) was used for the collection of nasopharyngeal specimens. Swabs were transported in STGG (skimmed milk, tryptone, glycerol and glucose) transport medium to the microbiology lab within a few hours.

**Bacterial isolation and antibiotic susceptibility test (AST).** Nasopharyngeal swabs were plated on chocolate agar with bacitracin (300 μg ml⁻¹). The plates were incubated at 37°C in a candle extinction jar, and growth was examined after 24 h. *H. influenzae* isolates were identified by their typical colony morphology, and X and V factor requirement, as described by Jain *et al.* (2005) and Doern (1995). AST was carried out on Haemophilus test medium by a disc diffusion method as per National Committee for Clinical Laboratory Standards (NCCLS) guidelines (NCCLS, 2000). Four drugs were tested: chloramphenicol (30 μg disc), erythromycin (15 μg disc), co-trimoxazole (2/5 μg trimethoprim and 23.5 μg sulfamethoxazole discs) and ampicillin (30 μg disc).

**Determination of β-lactamase.** All the ampicillin-resistant strains and 100 randomly selected ampicillin-sensitive strains were tested for β-lactamase production by a nitrocefin disc test (cefinate; Becton Dickinson Microbiology).

**RESULTS**

A total of 1001 (41.7%) isolates of *H. influenzae* were present in 2400 nasopharyngeal specimens from healthy school-going children. Of these isolates, 22.9% were resistant to ampicillin, 41.9% to chloramphenicol, 27.5% to erythromycin and 67.3% to co-trimoxazole. Of the 229 ampicillin-resistant *H. influenzae* isolates 196 (85.6%) were positive for β-lactamase. None of the ampicillin-sensitive strains were positive for β-lactamase. Co-resistance with other antimicrobials in β-lactamase positive and β-lactamase negative, ampicillin-resistant and ampicillin-sensitive strains is shown in Table 1. Co-resistance with other antimicrobials was significantly higher in β-lactamase-producing strains (Table 1).

The distribution of all biotypes, stratified as type b and ampicillin resistant, is shown in Table 2. All eight biotypes were found to be present in our population. Biotype III was most common (25.0%), followed by biotype I (19.6%). A strong correlation was seen between biotypes and *H. influenzae* isolates.

A strong correlation was seen between biotypes and *H. influenzae* isolates.

**Table 1.** Co-resistance in β-lactamase positive and β-lactamase negative, ampicillin-resistant and ampicillin-sensitive *H. influenzae* isolates

<table>
<thead>
<tr>
<th>Co-resistance to antimicrobials</th>
<th>Ampicillin sensitive (n = 772)</th>
<th>Ampicillin resistant (n = 229)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>β-Lactamase positive (n = 196)</td>
</tr>
<tr>
<td>None*</td>
<td>166 (21.5%)</td>
<td>15 (6.5%)</td>
</tr>
<tr>
<td>Co-trimoxazole* alone</td>
<td>237 (30.7%)</td>
<td>32 (13.9%)</td>
</tr>
<tr>
<td>Erythromycin alone</td>
<td>32 (4.1%)</td>
<td>11 (4.8%)</td>
</tr>
<tr>
<td>Chloramphenicol alone</td>
<td>62 (8.0%)</td>
<td>15 (6.5%)</td>
</tr>
<tr>
<td>Co-trimoxazole + erythromycin</td>
<td>71 (9.2%)</td>
<td>18 (7.8%)</td>
</tr>
<tr>
<td>Co-trimoxazole + chloramphenicol*</td>
<td>133 (17.2%)</td>
<td>65 (28.4%)</td>
</tr>
<tr>
<td>Chloramphenicol + erythromycin*</td>
<td>14 (1.8%)</td>
<td>13 (5.7%)</td>
</tr>
<tr>
<td>Chloramphenicol + erythromycin + co-trimoxazole*</td>
<td>57 (7.4%)</td>
<td>57 (25.0%)</td>
</tr>
</tbody>
</table>

*The difference between the groups ampicillin sensitive and total ampicillin resistant is significant.

**Serotyping.** The strains were serotyped by a slide agglutination test, using monospecific antisera for type b (Denka Seiken). Serotyping of all the isolates was done and strains were divided in two groups: *H. influenzae* type b and non-type b *H. influenzae*.

**Biotype analysis.** Biotypes were assigned to all the *H. influenzae* isolates by assessing their ability to produce urease, indole and ornithine decarboxylase using locally prepared media (Kilian, 1976; Gratten, 1983; Sottnek & Albritton, 1984). Briefly, the indole test was performed by growing each isolate in trypticase soy broth supplemented with NAD (5 μg ml⁻¹) and haemin (5 μg ml⁻¹). After 18 h growth at 37°C, Kovac reagent was added and the reaction was interpreted as described by Kilian (1976). The urease test was performed on Christensen’s urea agar supplemented with NAD (5 μg ml⁻¹) and haemin (5 μg ml⁻¹). Ornithine decarboxylase activity was assessed on Moeller decarboxylase base supplemented with NAD (5 μg ml⁻¹) and haemin (5 μg ml⁻¹).

**Statistical analysis.** Statistical analysis was performed using the STATA 8.2 package, version 6.0.
chloramphenicol was significantly more associated with significantly more resistant to ampicillin as compared to other biotypes. Ampicillin resistance was noted. The majority of ampicillin-resistant isolates were type b. All the biotype VIII isolates were non-type b H. influenzae.

Of the 229 ampicillin-resistant H. influenzae, 93·4 % (214/229) were biotypes I, II and III, of which 48·9 % were biotype I, 27·9 % were biotype II and 16·6 % were biotype III. Only 6·6 % (15/229) of the strains belonging to biotypes III–VIII were ampicillin resistant. Of the 316 H. influenzae type b, 44 % (139/316) were ampicillin resistant, while only 13·1 % (90/685) non-type b H. influenzae isolates were ampicillin resistant.

DISCUSSION

This study reports a high nasopharyngeal carriage of H. influenzae (41·7 %), especially serotype b (13·2 %) and biotypes I–III (61·3 %). The overall frequency of ampicillin-resistant H. influenzae was 22·9 %, of which 85·6 % were β-lactamase producing. Of the isolates, 31·6 % (316/1001) were type b, 44 % of which were ampicillin resistant. A strong correlation between H. influenzae biotypes and ampicillin resistance was noted. The majority of ampicillin-resistant isolates were type b, forming 49 % of strains. The number of ampicillin-resistant isolates of biotypes II and III is significantly lower as compared to biotype I. Ampicillin resistance is very low amongst biotypes IV–VIII, which also have low pathogenicity. Biotypes I, II and III were significantly more resistant to ampicillin as compared to other biotypes. Co-resistance with co-trimoxazole, erythromycin and chloramphenicol was significantly more associated with β-lactamase-positive organisms.

Reports from different parts of the world describe the carriage rate of H. influenzae as ranging from 11·6 to 76 % (Talon et al., 2000; Uraz et al., 2000; Josette et al., 2001; Das et al., 2002). A study from India has also reported a high H. influenzae type b carriage rate (57 %) in healthy school-going Indian children aged 5–10 years (Das et al., 2002). Weather conditions like cold and heavy rainfall, along with the low socio-economic status of the children, may be contributing factors resulting in the high prevalence. Some studies describe the H. influenzae type b carriage rate in healthy children to be around 10 %; the carriage rates in children were reported to be 9·6 % in day care centres and 9·1 % in elementary schools in Turkey (Bakir et al., 2002), 7 % in Thailand, and 12 % in Gambia and Venezuela (Olsen et al., 2005; Adegbola et al., 2005; Castillo-Febres et al., 2005). Published studies have reported a high prevalence of ampicillin-resistant H. influenzae in India (15·8 %, Ayyagari et al., 1985; 20 %, Patwari et al., 1996; 46 %, IBIS, 2002; 21·1 %, Nag et al., 2001 and 79 %, Das et al., 2002). The PROTEKT study (Kohno et al., 2003) has reported a carriage rate of 64·7 % for ampicillin-resistant H. influenzae in South Korea, 17·1 % in Hong Kong and 8·5 % in Japan. A study done in 1994–1995 of 1357 clinical isolates obtained from 30 medical centres in the United States reported the prevalence of ampicillin-resistant β-lactamase-positive H. influenzae isolates to be 36·4 % (Doern et al., 1997). Doern et al. (1999) reported the prevalence of ampicillin-resistant β-lactamase-producing H. influenzae isolates in the United States to be 34·2 % as a part of the SENTRY program. They further commented that the incidence of ampicillin-resistant β-lactamase-producing H. influenzae strains in North America levelled off at approximately 30 %. Gordon et al. (2003) reported a high prevalence of ampicillin-resistant isolates of H. influenzae in North America (27·9 %). The PROTEKT study (a worldwide study) reported the highest prevalence of ampicillin-resistant H. influenzae from South Korea (64·7 %) and the lowest from Italy (1·8 %) (Kohno et al., 2003).

Serotype b is the pathogenic H. influenzae type and biotype I causes mostly invasive diseases like meningitis in children, while biotypes II and III are more commonly associated with

<table>
<thead>
<tr>
<th>Biotypes</th>
<th>No. (%) of isolates of each biotype</th>
<th>Type b isolates</th>
<th>Ampicillin-resistant isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>95 % CI</td>
<td>No. (%)</td>
</tr>
<tr>
<td>I</td>
<td>196 (19·6 %)</td>
<td>110 (56·1 %)</td>
<td>(0·48–0·63)</td>
</tr>
<tr>
<td>II</td>
<td>168 (16·8 %)</td>
<td>71 (42·3 %)</td>
<td>(0·34–0·50)</td>
</tr>
<tr>
<td>III</td>
<td>250 (25·0 %)</td>
<td>57 (22·8 %)</td>
<td>(0·56–0·68)</td>
</tr>
<tr>
<td>IV</td>
<td>94 (9·4 %)</td>
<td>19 (20·1 %)</td>
<td>(0·12–0·29)</td>
</tr>
<tr>
<td>V</td>
<td>105 (10·5 %)</td>
<td>28 (26·7 %)</td>
<td>(0·18–0·36)</td>
</tr>
<tr>
<td>VI</td>
<td>51 (5·1 %)</td>
<td>14 (27·5 %)</td>
<td>(0·16–0·41)</td>
</tr>
<tr>
<td>VII</td>
<td>106 (10·6 %)</td>
<td>17 (16·0 %)</td>
<td>(0·15–0·24)</td>
</tr>
<tr>
<td>VIII</td>
<td>31 (3·1 %)</td>
<td>0 (0·0 %)</td>
<td>(0·0–0·11)</td>
</tr>
<tr>
<td>Total</td>
<td>1001</td>
<td>316 (31·6 %)</td>
<td>–</td>
</tr>
</tbody>
</table>
upper respiratory tract infections like otitis media and conjunctivitis (Kilian, 1976; Gratten, 1983; Alrawi et al., 2002). Das et al. (2002) found that the most common biotype was biotype I (36%), followed by II (18%) and III (18%). However, we observed that biotype III was most common (25%), followed by biotypes I (19-6%) and II (16-8%). Another study from North India based on invasive H. influenzae isolates reported that biotypes I, II & III were common among both serotype b and non-typable isolates. Biotype I was predominant (40%) among the typable strains, although most of the non-typable isolates (39%) were biotype II (Sharma et al., 2002). Teng et al. (1989) reported that biotype III has a higher incidence of ampicillin resistance among the different biotypes. Dabernat et al. (1988) reported that 50% of encapsulated-type strains were biotype I, and often resistant to ampicillin (38-5%), while 42% of non-encapsulated strains were of biotype II with 10-6% resistant to ampicillin. Granato et al. (1983) also reported that encapsulated strains of biotype I had the highest frequency of ampicillin resistance. Among H. influenzae isolates, serotype b strains predominated (83%), and most (96%) belonged to biotypes I or II (Gratten et al., 1985). A study of isolates of H. influenzae recovered from meningitis (Kilian et al., 1979) reported that 121 (93%) isolates belonged to biotype I. Kilian (1976) reported that the normal commensal flora of H. influenzae from the upper respiratory tract belonged to biotypes II and III. This observation suggests a possible additional virulence factor associated with biotype I organisms. In direct conflict to our findings, Long et al. (1983) suggested a tendency for less antibiotic resistance among serotypable versus non-serotypable isolates, and significantly less resistance among biotype I versus other biotype isolates. Albritton et al. (1978) reported significantly greater antibiotic resistance among biotype I and II. Gratten et al. (1985) reported that the carriage of non-serotypable biotype I and III organisms in healthy and sick children in Papua New Guinea does not differ significantly from that of serotypable biotype I and III strains.

Two mechanisms reported for the development of resistance to β-lactams in H. influenzae are (a) the production of β-lactamase, and (b) altered penicillin-binding-protein target sites (Mendelman et al., 1984). The carriage of a high molecular mass conjugative plasmid in H. influenzae type b isolates constitutes the genetic basis of resistance to ampicillin, chloramphenicol and tetracycline (Campos et al., 1989; Levy et al., 1993). Campos et al. (2003) reported that at a remarkably high level, 24-5%, H. influenzae type f isolates produced β-lactamase, and showed resistance to other antibiotics like co-trimoxazole, chloramphenicol and tetracycline.

A high carriage of H. influenzae isolates of type b and certain biotypes (I, II and III) was found in the nasopharynx of healthy school-going children in North India. Ampicillin resistance is significantly more common in biotype I, II and III and serotype b isolates than in isolates of other biotypes and non-serotype b.

**ACKNOWLEDGEMENTS**

The authors acknowledge the International Clinical Epidemiology Network (INCLLEN) and the United States Agency for International Development (USAID) for partial financial aid, and Community Antimicrobial Resistance Study Group (CAMR) members for their support.

CAMR group: M. K. Lalitha MD, Kurien Thomas MD, Anuradha Perakat DCH MRCP, Mary Kurien MS, Vinohar Balraj MD, Prakash Thoppuram MD and Jayaseelan MSc PhD (Christian Medical College and Hospital, Vellore, India); Mark Steinhoff MD (Johns Hopkins Medical Institute, Baltimore, MD, USA); Narendra Arora MD, Bimal Das MD and Arti Kapil MD (All India Institute of Medical Sciences, New Delhi, India); Shally Awasthi MD and Amita Jain (King George’s Medical University, Lucknow, India); A. K. Niswade MD, A. A. Pathak MD and Chaya MD (Government Medical College, Nagpur, India); Narendranathan MD and Kavitha Raja MD (Medical College, Trivandrum, India).

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Carriage of ampicillin-resistant H. influenzae


