SHORT REPORT

Antimicrobial susceptibility and genetic characteristics of *Haemophilus influenzae* isolated from patients with respiratory tract infections between 1987 and 2000, including β-lactamase-negative ampicillin-resistant strains

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(Submitted 27 May 2006; first published online 6 September 2006)

SUMMARY

The minimum inhibitory concentration (MIC) of five antibiotics and the presence of resistance genes was determined in 163 *Haemophilus influenzae* isolates collected over 13 years (1987–2000) in four two-yearly sampling periods from patients with respiratory tract infections. The prevalence of β-lactamase-negative ampicillin-susceptible strains was approximately 80% over the sampling period although fewer strains (65.9%) were recovered in the period 1995–1997. TEM-1 type β-lactamase-producing strains were less frequent starting at 15.6% and declining to 2.2% in the final sampling period. Low-β-lactamase-negative ampicillin-resistant (BLNAR) strains were uncommon in 1987–1989 (2.2%), peaked to 19.5% in 1995–1997, but fell back to 11.1% by 2000. Fully BLNAR strains were not detected until the last sampling period (6.7%). The MICs of ampicillin, levofloxacin, cefditoren and ceftriaxone remained stable but there was an eight-fold increase in the MIC of cefdinir over the sampling period. Pulsed-field gel electrophoresis of DNA digests showed that three representative BLNAR strains were genetically distinct and 11 DNA profiles were identified among 17 low-BLNAR strains. These data suggest that the number of genetically altered BLNAR and low-BLNAR strains are increasing in Japan.

*Haemophilus influenzae* can cause a variety of infections, including otitis media, bronchitis, pneumonia and meningitis [1, 2]. In the past, the activity of two β-lactamases, TEM-1 and ROB-1, accounted for almost all isolates with decreased susceptibility to ampicillin [3]. At present, the global prevalence of β-lactamase-negative ampicillin-resistant (BLNAR) *H. influenzae* remains low [4, 5], but the proportion of clinical BLNAR isolates is rapidly increasing, and has now reached more than 20% in Japan [6]. The characteristics of antimicrobial resistance of these strains are a serious concern for clinical prescribing. BLNAR strains have a resistance mechanism that decreases the affinity of ampicillin for penicillin-binding proteins (PBPs) [7]. The resistance phenotypes are classified according to substitutions at three positions of the *ftsI* gene which mediates septal peptidoglycan synthesis allowing the classification of strains as BLNAR or low-BLNAR by PCR [8].

In total, 163 strains of *H. influenzae* were isolated from sputum from patients with respiratory tract infections in Nagasaki University and its affiliated hospitals. These strains were selected at random and were divided into four groups, 45 strains between 1987 and 1989, 42 between 1991 and 1993, 41 between 1995 and 1997, and 45 between 1998 and 2000. Strains were capsule typed by slide agglutination with antisera (DiVco Laboratories, Detroit, MI, USA) and
-lactamase production was detected using a nitrocefin-impregnated disk (Becton Dickinson, Sparks, MD, USA). The minimum inhibitory concentration (MIC) of five antibiotics was determined by the agar dilution method according to the guidelines of the National Committee for Clinical Laboratory Standards [9]. The antibiotics were: ampicillin (Meiji Seika Kaisha, Tokyo, Japan), levofloxacin (Daiichi Pharmaceutical Co., Tokyo), cefditoren (Meiji Seika Kaisha), cefdinir (Astellas Pharma Inc., Tokyo) and ceftriaxone (Chugai Pharmaceutical Co., Tokyo).

PCR was performed to identify resistance genes using a multiplex assay as described previously [8]. Four sets of primers were obtained from Wakunaga Pharmaceutical Co. (Hiroshima, Japan): P6 primers to amplify the P6 gene which encodes the P6 membrane protein specific for *H. influenzae*; TEM-1 primers to amplify a part of the *bla*TEM-1 gene; PBP3-S primers to identify an Asn526→Lys amino-acid substitution in the *ftsI* gene; and PBP3-BLN primers to identify an Asn526→Lys and Ser385→Thr amino-acid substitution in the *ftsI* gene.

Pulsed-field gel electrophoresis (PFGE) was performed as described previously [10] using *SmaI* digestion (Takara Shuzo Co., Shiga, Japan) and electrophoresis in a CHEF Mapper PFGE system (Bio-Rad Life Science Group, Hercules, CA, USA) was carried out at 6 V/cm with switch times of 0.47 and 63 s, and a run-time of 20 h. After staining with ethidium bromide, the interpretation of PFGE patterns was based on the criteria described by Tenover et al. [11]. Briefly, PFGE patterns were classified into four groups: identical in profile = indistinguishable; 1–3 bands difference = closely related; 4–6 bands difference = possibly related; and > 7 bands difference = different.

The Table shows that of the 45 strains isolated from 1987 to 1989, 37 (82.2%) were classified as -lactamase-negative ampicillin-susceptible (BLNAS) strains, seven strains produced TEM-1-type -lactamase and were ampicillin resistant (BLPAR) and one strain was classified as low-BLNAR by PCR.

The proportion of BLNAS strains fell in the ensuing two sampling periods but recovered in the final time period to 80%. The frequency of BLPAR strains fluctuated from 15.6% in the initial period through 6.3% and 12.2% to 2.2% in the final sampling period. Similar variation was observed for low-BLNAR strains with just 2.2% of strains expressing this phenotype in the first period but rising to almost 20% in the third period before falling back to 11%. Three BLNAR strains (6.7%) were detected only in the fourth sampling period. The respective MIC\textsubscript{80} values (μg/ml) for the four periods against the strain collection was

<table>
<thead>
<tr>
<th>Year</th>
<th>BLNAS\textsuperscript{a}</th>
<th>BLPAR\textsuperscript{b}</th>
<th>Low-BLNAR\textsuperscript{c}</th>
<th>BLNAR\textsuperscript{d}</th>
<th>MIC\textsubscript{80} of five antibiotics (μg/ml)</th>
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<td>AMP\textsuperscript{e}</td>
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<tr>
<td>A 87–89</td>
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<td>BLNAS</td>
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<td>Low BLPAR</td>
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<td>B 91–93</td>
<td>78.1</td>
<td>6.3</td>
<td>15.6</td>
<td>0</td>
<td>Total</td>
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<td>BLNAS</td>
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<td>Low BLPAR</td>
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<td>C 95–97</td>
<td>65.9</td>
<td>12.2</td>
<td>19.5</td>
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<td>BLNAR</td>
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\textsuperscript{a}β-lactamase-negative ampicillin susceptible strains. \textsuperscript{b}β-lactamase-producing ampicillin-resistant (TEM-1 type) strains. \textsuperscript{c}Low β-lactamase-negative ampicillin-resistant strains. \textsuperscript{d}β-lactamase-negative ampicillin-resistant strains. \textsuperscript{e}Ampicillin. \textsuperscript{f}Ceftriaxone. \textsuperscript{g}Cefdinir. \textsuperscript{h}Cefditoren. \textsuperscript{i}Levofloxacin.
is also shown in the Table. The NCCLS susceptibility/resistant break-points for *H. influenzae* are 1 μg/ml for ampicillin, 2 μg/ml for levofloxacin, 1 μg/ml for cefdinir, and 2 μg/ml for ceftriaxone. The MIC of ampicillin, ceftriaxone, cefditoren and levofloxacin remained constant or within one doubling concentration over the years but resistance to cefdinir increased by eight-fold over the sampling period (Table).

The three BLNAR strains gave distinct DNA profiles in PFGE while 11 profiles were distinguished among the 17 low-BLNAR strains. Two of these profiles exhibited some similarities to profiles found in fully BLNAR strains. Three pairs of strains each exhibited similar patterns and four strains were grouped within the same pattern (Fig.). All but two of the 20 strains were non-typable with capsular antisera.

*H. influenzae* is one of the important pathogens associated with respiratory tract infections and thus acquisition of antimicrobial resistance raises concern. The prevalence of BLNAR strains was reported to be 2.4% in the United States between 2002 and 2003 [12], 1.3% in France in 1999 [13], and 9.3% in Spain between 1998 and 1999 [14]. Nevertheless, their global prevalence remains relatively low. However, BLNAR strains are spreading rapidly with increasing frequency in Japan with reported prevalence rates of 14.9% between 1996 and 1997 [15], and 23.1% between 1998 and 1999 [6], although BLNAR was identified by only MIC in these reports. We report here a prevalence of BLNAR strains of 6.7% by PCR between 1998 and 2000. An understanding of the characteristics of antimicrobial resistance of *H. influenzae*, especially BLNAR strains, is important not only for prescribing clinicians but also for formulation of practicable chemotherapy guidelines.

Although *H. influenzae* strains are generally susceptible to the early cephalosporins [5, 12], the BLNAR and low-BLNAR strains recovered here showed a marginal increase in MIC to two of the three cephalosporins tested and an eight-fold increase in MIC of cefdinir which is consistent with a previous report from Japan [6].

PFGE of DNA macrorestriction fragments is a sensitive fingerprinting method for *H. influenzae* and this method was used by Karlowsky *et al.* [4] to demonstrate clonal dissemination of BLNAR strains in the United States between 2000 and 2001. However, the BLNAR and low-BLNAR strains found here displayed a variety of genetic backgrounds. It has previously been reported that *H. influenzae*, including resistant strains, can be transmitted at day-care centres or in the home [13, 16], and this may
be one reason for the spread of BLNAR strains in Japan. We did observe that some low-BLNAR strains isolated from different patients had similar PFGE patterns and therefore must consider that such strains could potentially spread in a community. Ongoing monitoring of H. influenzae resistance determinants is thought to be important and may help to predict how this organism responds to current antimicrobial regimens [17]. Despite the limitations of this pilot study, particularly the small sample size, it shows the value of surveillance of antimicrobial resistance levels and their genetic determinants in H. influenzae and further surveys specifically of BLNAR strains in the wider Japanese community should be undertaken to inform antimicrobial prescribing policy.

ACKNOWLEDGEMENTS

We thank Akihiro Wada (Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Japan), Chieko Shimauchi (Miyazaki Prefectural Nursing University, Japan) and Matsuhis Inoue (Kitasato University School of Medicine, Japan) for their help in completing the PFGE studies. The study was supported by U.S.–Japan Cooperative Medical Science Program, Acute Respiratory Infections Panel.

DECLARATION OF INTEREST

None.

REFERENCES