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## Outbreak of Amoxicillin-Resistant *Haemophilus influenzae* Type b: Variable Number of Tandem Repeats as Novel Molecular Markers

ALEX VAN BELKUM,<sup>1\*</sup> WILLEM J. G. MELCHERS,<sup>2</sup> CATHELIJN IJSSELDIJK,<sup>1</sup>  
LIESEL NOHLMANS,<sup>3</sup> HENRI VERBRUGH,<sup>1</sup> AND JACQUES F. G. M. MEIS<sup>2</sup>

Department of Medical Microbiology and Infectious Diseases, University Hospital Rotterdam, 3015 GD Rotterdam,<sup>1</sup>  
Department of Medical Microbiology, University Hospital Nijmegen, 6500 HB Nijmegen,<sup>2</sup>  
and Department of Medical Microbiology, Rijnstate Hospital,  
6815 AD Arnhem,<sup>3</sup> The Netherlands

Received 27 December 1996/Returned for modification 12 February 1997/Accepted 27 February 1997

An outbreak caused by amoxicillin-resistant *Haemophilus influenzae* type b was noted among patients suffering from chronic obstructive pulmonary disease. Since infections were clustered in time and place, an ongoing outbreak was suspected. The spread of the strain and the course of the outbreak could be followed by random amplification of polymorphic DNA (RAPD) analysis of the different bacterial isolates. In addition, studies were aimed at the determination of length polymorphism in regions of repetitive DNA. By PCR-mediated amplification of variable number of tandem repeat regions (VNTRs), additional insight into the genome composition of the epidemic strain was gained. Our results show that VNTRs comprising repeat units that are 3, 5, or 6 nucleotides in length provided stable genetic markers that can be used for molecular typing of *H. influenzae* type b. VNTRs built from tetranucleotide units, however, appear to be hypervariable and not suited for epidemiological studies. The observed variability in this latter class of VNTRs might be reminiscent of the bacterium's capacity to deal with unfavorable host factors.

*Haemophilus influenzae* is a gram-negative bacterium well-known for its capacity to cause disease. Detailed studies have demonstrated that a large and genetically very diverse pool of *H. influenzae* strains circulate in the community, even to such an extent that genetically identical strains are hardly ever identified from unrelated individuals (18). On the other hand, transmission of strains among members of a single household has been documented (4). Among children nursed in day care centers, it has been demonstrated that considerable strain sharing can occur, whereas other bacterial types remain restricted to certain individuals (21). The pathogen can cause a broad spectrum of clinical syndromes, which may vary from pneumonia, septicemia, and meningitis to soft-tissue abscesses, lung abscesses, and peritonitis (13). Patients with exacerbations of chronic obstructive pulmonary disease (COPD) are mostly colonized with a particular strain of *H. influenzae* which can be a cause of respiratory infections. Cross infection between these patients can be suspected when in a limited period of time the rate of isolation of  $\beta$ -lactamase-producing strains increases. Normal rates for this type of strain are on the order of 5 to 7% in The Netherlands, whereas in the United Kingdom values of 10 to 14% are scored.

In order to effectively monitor the rise of new and successful clones of *H. influenzae* and to adequately monitor epidemics, effective tools for detailed molecular typing of *H. influenzae* have been developed (11, 14, 15, 19, 22). Most of these procedures generate complex DNA or protein banding patterns. Banding patterns are hard to reproduce precisely between laboratories (5, 23), and methods producing digital or binary output could be of additional value (26). In this report we

describe the application of an alternative typing procedure for *H. influenzae* strains (24). This novel approach is based upon the molecular characterization of discrete regions of repetitive DNA in the *H. influenzae* genome. The organization of these regions is simple and straightforward: short sequence motifs, ranging from 3 to 6 nucleotides in length, occur in tandem, strongly varying in overall numbers per locus (9, 24). Some of these regions have previously been studied at the level of gene expression modulation (9, 25, 27), but additional regions were identified with the help of the recently solved whole genome sequence of the Rd strain of *H. influenzae* (7, 9). The usefulness of tracking the different classes of variable number of tandem repeat regions (VNTRs) is demonstrated by follow-up of COPD patients involved in an outbreak caused by an amoxicillin-resistant strain of *H. influenzae* in a pulmonology department in a Dutch general hospital.

### MATERIALS AND METHODS

**Bacterial strains and cultivation.** *H. influenzae* strains were cultured from sputum samples obtained from patients admitted with acute exacerbations of COPD. All patients were over 55 years of age, and samples were obtained in the months of October and November 1994. Strains were cultured on chocolate agar. Strain identification and susceptibility testing was performed according to standard microbiological methods (10). Serotyping was done by the slide agglutination assay (Murex Diagnostics, Utrecht, The Netherlands).

**DNA isolation and PCR-mediated typing studies.** After overnight growth *H. influenzae* cells were harvested directly into a guanidinium isothiocyanate-containing lysis buffer (3, 17). The resulting lysate was processed for DNA purification. The DNA concentration was determined, and the solution was stored at a DNA concentration of 10 ng/ $\mu$ l at  $-20^{\circ}\text{C}$ . Random amplification of polymorphic DNA (RAPD) analyses were performed as described previously (22) with a combination of primers ERIC1 and ERIC2 (12). Amplification of VNTR regions was done by recently developed protocols (24). The tetranucleotide VNTR loci Hi 4-3, Hi 4-5, Hi 4-10, and Hi 4-11 were analyzed, together with the trinucleotide VNTR locus Hi 3-1, the pentanucleotide VNTR locus Hi 5-2, and the hexanucleotide VNTR loci Hi 6-1 and Hi 6-2. Some additional information on the repeat characteristics is given in Table 1.

The fragments obtained by RAPD analysis were analyzed by gel electrophoresis in ordinary 2 to 3% agarose gels (Hispanagar; Sphaero Q, Leiden, The

\* Corresponding author. Mailing address: Department of Medical Microbiology and Infectious Diseases, University Hospital Rotterdam, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands. Phone: 31-10-4635813. Fax: 31-10-4633875. E-mail: vanbelkum@bacl.azr.nl.

TABLE 1. Survey of characteristics of VNTR regions used for genome analysis of *H. influenzae*<sup>a</sup>

Repeat unit length	Repeat code	Repeat position	Unit sequence	No. of units
3	Hi 3-1	291617-291644	ATT	9
4	Hi 4-3	570800-570892	CAAT	23
4	Hi 4-5	705897-705979	TTGG	20
4	Hi 4-10	1543152-1543252	TTGC	25
4	Hi 4-11	1608031-1608099	CAAT	17
5	Hi 5-2	1368890-1368910	GTCTC	4
6	Hi 6-1	283097-283115	CTGGCT	4
6	Hi 6-2	296053-296071	GGCAAT	3

<sup>a</sup> Repeat positions, unit sequences, and numbers are identified on the basis of data presented elsewhere (7, 9).

Netherlands). Due to the small size of the VNTR amplicons and the required 3- to 6-nucleotide resolution, these DNA fragments were separated by length on 3% MetaPhor agarose (FMC Bioproducts, Biozym, Landgraaf, The Netherlands). Molecular sizes were determined with the help of a 10-bp ladder (Boehr-

inger Mannheim, Mannheim, Germany). Gels were run in 0.5× TBE (Tris-borate-EDTA) buffer at a constant voltage of 100 V (17). The gels were photographed with a charged coupled device camera equipped with a Fujinon zoom lens. Data were collected and thermoprinted by using the Visionary Photo Analyst system (FotoDyne, Progress Control, Waalwijk, The Netherlands).

## RESULTS

*H. influenzae* was retrieved from 27 patients admitted to a single pulmonary ward in a 3-month period (epidemic strains in Table 2). Nearly all strains (26 of 27; 96%) collected in a relatively short period of time appeared to be resistant to amoxicillin. Fifty-nine percent of these strains produced β-lactamase. This clearly suggested an outbreak of infections. All strains were of the b serotype, and RAPD analysis-mediated typing revealed that all of the resistant strains belonged to a single, genetically homogeneous type (Table 2). With respect to the data obtained by RAPD analysis, all of the outbreak-related strains were type A, and the eight reference strains (strains 4, 11, 13, 15, 17, 19, 29, and 30) were given genetic

TABLE 2. Survey of typing data obtained for control and outbreak-related strains of *H. influenzae*<sup>a</sup>

Strain type and no.	RAPD type	Serotype	Amx <sup>r</sup>	No. of repeats							
				Hi 4-3	Hi 4-5	Hi 4-10	Hi 4-11	Hi 3-1	Hi 5-2	Hi 6-1	Hi 6-2
Epidemic strains											
1	A	b	+	27	12	18	34	9	2	10	4
2	A	b	+	24	14	17	34	9	2	10	4
3	A	b	+	27	12	18	34	9	2	10	4
5	A	b	+	27	12	18	34	9	2	10	4
6	A	b	+	27	10	18	34	9	2	10	4
7	A	b	+	27	12	18	34	9	2	10	4
8	A	b	+	27	12	18	34	9	2	10	4
9	A	b	+	27	10	18	34	9	2	10	4
10	A	b	+	27	12	18	34	9	2	10	4
12	A	b	+	30	12	18	34	9	2	10	4
14	A	b	+	28	10	18	34	9	2	10	4
16	A	b	+	27	10	18	34	9	2	10	4
18	A		-	24	11	18	23	9	2	10	4
20	A	b	+	15	20	20	35	9	2	10	4
21	A	b	+	24	14	18	34	9	2	10	4
22	A	b	+	27	10	18	34	9	2	10	4
23	A	b	+	27	10	20	34	9	2	10	4
24	A	b	+	16	20/22	20	34	9	2	10	4
25	A	b	+	27	12	20	34	9	2	10	4
26	A	b	+	27	12	20	34	9	2	10	4
27	A	b	+	24	14	20	34	9	2	10	4
28	A	b	+	26	12	20	36	9	2	10	4
31	A	b	+	27	15 <sup>mb</sup>	18	34	9	2	10	4
32	A	b	+	27	12	20	±40	9	2	10	4
33	A	b	+	25	26 <sup>mb</sup>	19	33	9	2	10	4
34	A	b	+	28	12	18	34	9	2	10	4
35	A	b	+	26	12	21	34	9	2	10	4
Unrelated control strains											
4	B		-	33	16	3	23	9	2	8	4
11	C		-	22	16	19	23	9	3	10	2
13	D		-	17	4	3	14	9	1	9	2
15	E		-	19	28	18	34	9	7	10	4
17	F		-	12	16	30	19	5	2	9	10
19	G	b	-	20	mb	18	7	9	3	10	4
29	H		-	21	14	2	30	5	1	10	15
30	I		-	27	16	21	±40	9	5	8	1

<sup>a</sup> The numbers of repeats were calculated for the diverse loci present in all of the strains included in the present study. mb, multiple bands visible in the gel; in some cases the length of the most prominent band is included. All strains that are amoxicillin resistant produce β-lactamase. Average values and standard deviations of the lengths of the different VNTRs (epidemic versus unrelated strains, respectively) are as follows: Hi 4-3, 25.7 ± 3.2 and 21.4 ± 5.9; Hi 4-5, 13.0 ± 3.7 and 15.7 ± 6.5; Hi 4-10, 18.7 ± 1.1 and 14.3 ± 9.7; and Hi 4-11, 33.8 ± 2.5 and 23.8 ± 10.0. This shows that the epidemic strains differ to a lesser extent than could be expected on the basis of random variation.

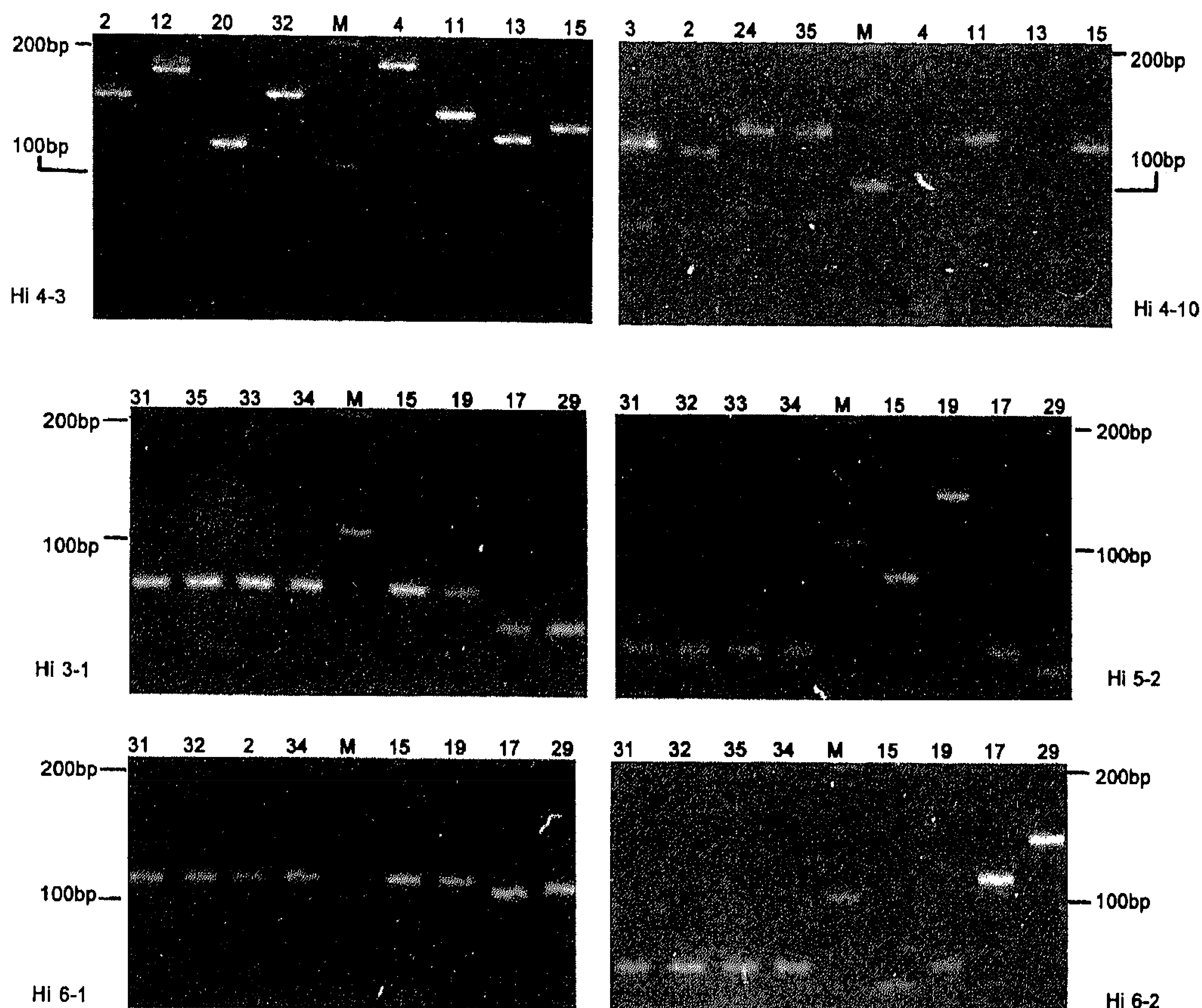


FIG. 1. VNTR polymorphisms in *H. influenzae* strains. Identification of the isolates involved is given by the numbering above the individual lanes (see also Table 2). Lane M, molecular length marker; the sizes of the DNA fragments are given alongside the different panels. The panels display the results obtained for the various VNTR loci (Hi 4-3, Hi 4-10, Hi 3-1, Hi 5-2, Hi 6-1, and Hi 6-2). The arrows in the panel at the upper right indicate the positions of weakly staining DNA fragments.

indices B through I, respectively. Note that all of the amoxicillin-resistant strains belonged to the A cluster.

Tracking VNTR length variability by PCR (for example, see Fig. 1) demonstrated that the cluster of outbreak-related strains was homogeneous, as long as only tri-, penta-, and hexanucleotide repeat regions were included in the analysis (Table 1). A digital code (9, 2, 10, and 4 for Hi 3-1, Hi 5-2, Hi 6-1, and Hi 6-2, respectively) was established for all strains. Inclusion of the Hi 3-1 repeat appeared to be irrelevant, since only two allelic variants were encountered (9 or 5 units). This degree of variability was too small to add to the overall differentiation. VNTR regions consisting of repeat units 4 nucleotides in length show apparent hypervariability. However, strains 1, 3, 5, 7, 8, and 10 were identical. Other highly homologous but smaller groups were formed as well; strains 6, 9, 16, and 22 were identical, as were strains 25 and 26. The mathematical spread in the number of repeats among epidemic strains versus that among the control strains is given in footnote *a* of Table 2. Note that the length deviation among the epidemic strains is less than that calculated for the incidentally isolated control strains.

## DISCUSSION

Incidences of up to 20% of all nosocomial cases of pneumonia have been attributable to *H. influenzae* (1). Nosocomial transmission of *H. influenzae*, although not a frequent phenomenon, is recognized regularly and can occur between clinically diverse patients. Outbreaks among pediatric patients (2) and

elderly people (20) have been described. The epidemiology of these outbreaks was studied by procedures as widely divergent as serotyping, biotyping, multilocus enzyme electrophoresis, major outer membrane protein profiling, and direct DNA-mediated typing procedures. Sometimes strains can be tracked down quite easily due to the presence of antibiotic resistance markers (8). We present here another example of an outbreak due to *H. influenzae* serotype b carrying a clear antibiotic resistance marker: amoxicillin resistance. In general, the incidence of amoxicillin resistance among *H. influenzae* strains in The Netherlands is low (5 to 7%), but during the outbreak period the percentage rose to 59. The spread of the strain was confined to the pulmonology ward, and a change of therapy to ofloxacin (200 mg twice daily) or amoxicillin-clavulanic acid (625 mg three times daily) soon led to the disappearance of the epidemic strain.

Molecular typing by RAPD analysis revealed the genetic homogeneity of the epidemic *H. influenzae* type b strain. It was recently shown that local variation in the *H. influenzae* genome can be determined at sites of repetitive DNA (9, 24, 25, 27). As has been demonstrated previously, the bacterium uses this type of variability to modulate gene expression, either by altering promoter activity (25) or by affecting protein reading frames (27). We show here that repeats from within the 4-nucleotide class are especially subject to highly frequent modification, even among epidemiologically closely related strains. The length range of PCR products is relatively large (Table 2). Highly mutable sites like the ones described here and previ-

ously (16) have been named contingency loci and are suggested to play a pivotal role in adaptive evolution. Although these regions are of extreme importance biologically, the usefulness of these regions for molecular typing is limited.

On the other hand, there exist other classes of VNTRs that seem to evolve at a lower speed. This enables their inclusion in epidemiological monitoring assays. The repeats named Hi 3-1, Hi 5-2, Hi 6-1, and Hi 6-2 are sufficiently stable to allow for the adequate discrimination of epidemiologically related strains from nonrelated ones. As such, the technically simple assays used here form a valuable addition to the spectrum of procedures available for typing *H. influenzae* isolates. The presence of potential VNTRs showing species specificity with respect to sequence motifs has been elucidated for a variety of other microorganisms as well (6). Consequently, VNTR analysis may in the future combine direct DNA diagnostics with molecular typing in a single DNA amplification reaction.

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