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Carriage of multiple ribotypes of non-encapsulated *Haemophilus influenzae* in Aboriginal infants with otitis media

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SUMMARY

Ribotyping with the restriction enzyme *Xba*I was used to study the dynamics of carriage of non-encapsulated *Haemophilus influenzae* (NCHi) in Aboriginal infants at risk of otitis media. Carriage rates of NCHi in the infants in the community were very high; the median age for detection was 50 days and colonization was virtually 100% by 120 days of age and persisted at a high level throughout the first year of life [1]. Eighteen different ribotypes of NCHi were identified from 34 positive swabs taken from 3 infants over a period of 9 months. The same ribotypes were recovered for up to 3 months from consecutive swabs of individual infants, and 12 of 27 swabs (44·4%) yielded two ribotypes from four colonies typed. Statistical analysis suggested that most swabs would have been positive for two ribotypes if enough colonies had been typed although the second most frequent ribotype was detected on average in only 13% of strains. Early colonization and carriage of multiple ribotypes of NCHi may help to explain the chronicity of carriage and thus the persistence of otitis media in Aboriginal infants.

INTRODUCTION

Otitis media is endemic in Aboriginal infants in the Northern Territory of Australia; in one rural community all infants were affected within several weeks of birth [1]. Nasopharyngeal colonization with multiple species of respiratory bacterial pathogens (*Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*) occurs within weeks of birth and predicts the subsequent onset of otitis media in Aboriginal infants [1]. Aboriginal people are also subject to high rates of acute respiratory infection requiring antibiotic treatment or hospitalization [2], as are other disadvantaged populations [3]. It is also plausible that the high rates of chronic bronchitis and respiratory failure in Aboriginal adults [2] are related to the early age of bacterial infection and to repeated exposure to bacterial pathogens through childhood. Chronic bacterial exposure and bacterial endemicity from such an early age are themselves consequences of poor social conditions, overcrowding, and poor hygiene. We previously suggested that such early exposure to multiple pathogens, before maturation of the immune system, could result in partial immune tolerance or suppression and thus to prolonged carriage [1]. This problem may be compounded because of concurrent colonization by multiple strains of the bacterial pathogens. The multiplicity of strains could contribute to the duration and chronicity of carriage, and thus to the persistence of otitis media and other respiratory problems in this population.

Non-encapsulated *Haemophilus influenzae* (NCHi) are major agents in the aetiology of otitis media [1] and chronic bronchitis [4]. Techniques applied to the characterization of isolates of NCHi for epidemiological studies include biotyping, SDS-PAGE of outer membrane and whole-cell proteins, multilocus enzyme electrophoresis, analysis of lipopolysaccharides, re-

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striction endonuclease analysis and PCR typing [5–7]. Ribotyping using the restriction enzyme *Eco*R I has been applied also to isolates of NCHi [6]. Here we have developed this technique further, using the enzyme *Xba* I, to explore the dynamics of NCHi carriage in infants with otitis media from whom nasopharyngeal swabs had been regularly collected. We show that within the one small community over a period of 9 months at least 18 different strains of NCHi had colonized 3 infants.

MATERIALS AND METHODS

Community

The Aboriginal community is situated on an island located 60 km north of Darwin, with a total population of 1100, and an annual birth rate of 30. Despite recent improvements, Aboriginal families still live in poor social circumstances in overcrowded housing, and with inadequately resourced health services.

Subjects

The longitudinal study of otitis media in Aboriginal infants has been previously reported [1]. Maternal consent for ear screening and collection of nasopharyngeal swabs was sought before each examination and the protocol was approved by an Institutional Ethics Committee working within the guidelines of the NHMRC. Three infants born in April and May 1993 and seen every 2–3 weeks until January 1994 were selected retrospectively for further study of bacterial isolates from nasopharyngeal swabs. Otitis media had been diagnosed in each infant within 1 month of birth following nasopharyngeal colonization with respiratory bacteria [1], and in other respects the infants were typical of those in the study. The 43 nasopharyngeal swabs collected during the study period from the three infants (940, 946 and 947) provided the sampling frame for the NCHi isolates in this study.

Treatment and outcome

Infants found to have otitis media were enrolled in a pilot study of antibiotic treatment which has been described previously [8]. Details of antibiotics taken by the three infants as part of the pilot study and antibiotics prescribed by the community clinic are summarized in Table 1. Resolution of otitis media was not observed during the study period in any of these infants.

Isolates

Microbiologic methods were as described previously [1]. Briefly, nasopharyngeal swabs were smeared for Gram staining and frozen in 1·0 ml of transport broth. Thawed broth was well mixed and 10 μl aliquots were cultured on 7% chocolate agar and chocolate agar plus bacitracin, vancomycin and clindamycin [9]. From most swabs there was a heavy growth of *H. influenzae* and four colonies were sampled from each plate, taking care to select any colonies that were morphologically distinct. Isolates of *H. influenzae* were identified by their requirement for X and V factors. NCHi were recognized by their lack of reaction with antisera against capsular antigens, b or a, c–f (Phadebact, KaroBio Diagnostics AB, Huddinge, Sweden).

DNA preparation

Total chromosomal DNA was extracted by a modification of the method described by Pitcher and colleagues [10]. Bacterial colonies from an overnight culture were suspended in 200 μl 10 mM-Tris-HCl 1 mM-EDTA pH 8·0 (TE) and pelleted by centrifugation. Pelleted cells were washed twice in this manner. Lysozyme was added to 200 μl of the resuspended pellet at 100 μg/ml and incubated at 4 °C for 15 min. This was followed by the addition of RNaseA at 150 μg/ml and a 30 min incubation. To this, proteinase K (100 μg/ml) was added, and incubated at 65 °C for 2 h, followed by 500 μl of 5 M guanidinium thiocyanate, 100 mM-EDTA and 0·5% v/v sarkosyl. This mixture was placed on ice and 250 μl 7·5 M ammonium acetate was added. After extraction once with chloroform/isoamyl alcohol the DNA was precipitated with isopropanol (0·54 volume). Following centrifugation, the pellet was washed three times with 70% ethanol and resuspended in TE.

Restriction enzyme digestion

Approximately 1 μg DNA was digested in a reaction mixture of 10 μl that contained in addition 1 μl One-Phor-All buffer, 1 μl *Xba* I (15 units/μl, Pharmacia LKB Biotechnology, Uppsala, Sweden) and 3 μl H_2O. The mixture was incubated at 37 °C for 2 h. The same

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Table 1. *Antibiotics prescribed for acute otitis media or otitis media with effusion, and compliance achieved in each of the three infants during the study period*

		August		September		October	
Infant	July 28 1993	9	24	6, 7	20, 21	4, 5	18, 19
940	Bicillin I.M.	Bicillin	Amoxycillin	Amoxycillin	Bicillin*	Cefaclor	Cefaclor
Compliance			80–100%	80–100%		80–100%	50–80%
946	Amoxycillin	Amoxycillin	Cefaclor	Bicillin*	Cefaclor	—	—
Compliance	80–100%	80–100%	< 50%		80–100%		
947	—	—	Amoxycillin	Amoxycillin	Cefaclor	Cefaclor	Cefaclor
Compliance			80–100%	80–100%	80–100%	80–100%	80–100%

* Antibiotic prescribed by the community clinic.

Table 2. *Frequency distribution of different ribotypes amongst samples of four colonies from 27 different swabs in three infants*

No. of isolates of		Infant			
Most frequent ribotype	Less frequent ribotype	940	946	947	Total number of swabs
4	0	4	6	5	15
3	1	4	6	0	10
2	2	2	0	0	2
		10	12	5	27

Probability of 'less frequent' ribotype $= (10+2\times2)/(4\times27)$
$= 0{\cdot}13$.

procedure was used to digest 1 μg DNA with *Eco*R I, *Sac* I, *Nco* I, *Pst* I, *Nhe* I, *Apa* I, *Stu* I, and *Bcl* I.

Gel electrophoresis and Southern transfer

DNA restriction fragments were separated by electrophoresis for 16 h at 1·1 V/cm on a 0·8% agarose gel in 1X TAE (4 mM tris acetate, 0·2 mM EDTA pH 8·0). DNA was transferred to a positively charged membrane (Hybond N+, Amersham) in 0·4 M-NaOH and neutralized in 2X SSC (0·3 M-NaCl, 30 mM tri-sodium citrate) substantially as described in [11].

Ribosomal DNA probe

The probe was prepared by random primer labelling with [α-^{32}P]dATP of the insert from plasmid pKK3535 [12], containing an *Escherichia coli* ribosomal RNA operon. The Gigaprime DNA labelling kit (Bresatec) was used.

DNA hybridization

The blot was pre-hybridized for 4 h at 65 °C before addition of the labelled rDNA probe. The pre-hybridization mixture contained 0·3 M-NaCl, 20 mM NaH_2PO_4, 2 mM-EDTA, 1% SDS, 0·5% non-fat skim milk powder and 0·5 mg/ml herring sperm DNA. Hybridization was for 16 h at 65 °C and post-hybridization washes were at 65 °C with 0·1% SDS/1XSSC. Membranes were then exposed to Kodak X-Omat AR film and the resulting patterns compared.

Statistical methods

To assess whether the same ribotypes were more likely to be isolated from the same children from successive swabs (occasions), we counted the number of occasions when the same ribotype was observed in the next swab from the same child, calculated the number *expected* (on the assumption that ribotypes were randomly distributed in time), and used the Poisson distribution [13] to calculate the probability that the excess of *observed* over *expected* could have occurred by chance.

To assess whether the same ribotypes were more likely to be isolated from different children over the same period of time, we counted the observed number

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of occasions when the same ribotype was isolated from two different children at intervals of 1 month or less. We compared the observed and expected numbers using the Poisson distribution [13].

To estimate the average frequency of any second ribotype, we assumed that, any such ribotype would constitute a proportion (p) of colonies from each swab; we then applied the binomial distribution [14] to the observed frequency of recovery of more than one ribotype (Table 2), and estimated the proportion, p, by maximum likelihood [14].

Results

Preliminary experiments suggested that *Xba* I had the capacity to discriminate more ribotypes than *Eco*R I when used with the probe pKK3535. To compare the 2 enzymes, 13 isolates of NCHi were ribotyped using both *Eco*R I and *Xba* I restriction enzymes. *Xba* I generated 7 distinct ribotypes while *Eco*R I generated 6 ribotypes that were difficult to distinguish (not shown). As well as discriminating between ribotypes at a more informative level, this alternate method using *Xba* I generated fewer bands in the 2–10 kb size range allowing easier interpretation of the patterns. Seventeen representative *Xba* I ribotypes are shown in Figure 1. Restriction enzymes *Sac* I, *Nco* I, *Pst* I, *Nhe* I, *Apa* I, *Stu* I, *Bcl* I were less discriminatory (not shown).

Of 43 nasopharyngeal swabs collected from 3 Aboriginal infants over a period of 9 months, 34 swabs grew NCHi. None of the infants had positive nasopharyngeal cultures in the few days after birth; the average age of NCHi acquisition was 23 days. Sporadic negative cultures at later times were most likely the result of swabs which collected insufficient sample, evident from the presence of mucus and very few epithelial cells on the Gram stain, and failure to culture other respiratory bacteria. The dates of these sporadic negative swabs were not correlated with the dates on which antibiotics were prescribed.

The striking result was that each of the infants was multiply colonized with NCHi. A total of 18 distinct ribotypes were present among the isolates in this study and the 3 infants (940, 946 and 947) were seen to carry 9, 9 and 4 ribotypes, respectively, over this period.

Capsulated strains of *H. influenzae* were isolated on several occasions, either as the dominant organism or carried together with non-capsulate strains. However, colonization with NCHi far outweighed colonization with capsulated strains. The distribution of capsulate

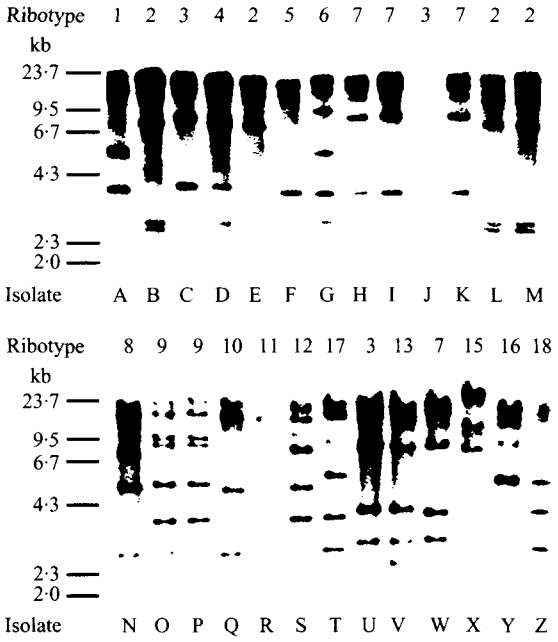

Fig. 1. Autoradiograms displaying 17 representative *Xba* I ribotypes of NCHi from 26 isolates (denoted A to Z) collected from the three infants studied.

strains, and the distribution of ribotypes of the non-capsulate isolates is represented in Figure 2. The dates on which particular ribotypes appeared or disappeared were not correlated with the dates on which antibiotics were prescribed.

Clustering of ribotypes in time indicated that recovery of types was not a random process. The same ribotype tended to be recovered on successive occasions from each infant; for example, infant 940 carried ribotype two at five successive examinations. There were 16 instances where the same ribotype was recovered from the same child at successive examinations, compared with 4·6 expected by chance ($P < 0{\cdot}001$ by Poisson distribution). There was also a tendency for the same ribotype to be isolated from different infants over the same interval of time; for example, ribotype six was isolated from infants 940 and 946 at the same examination date. There were only 4 ribotypes which occurred in more than 1 infant (Fig. 2), and in 3 of these 4 instances, compared with 1·56 expected, dates of examinations were within 1 month of each other. However, because of the small numbers, this difference was not statistically significant.

However, an important observation was the high incidence of simultaneous carriage of two ribotypes in the same infant. Co-colonization was evident on 12 occasions from 27 swabs for which four colonies were

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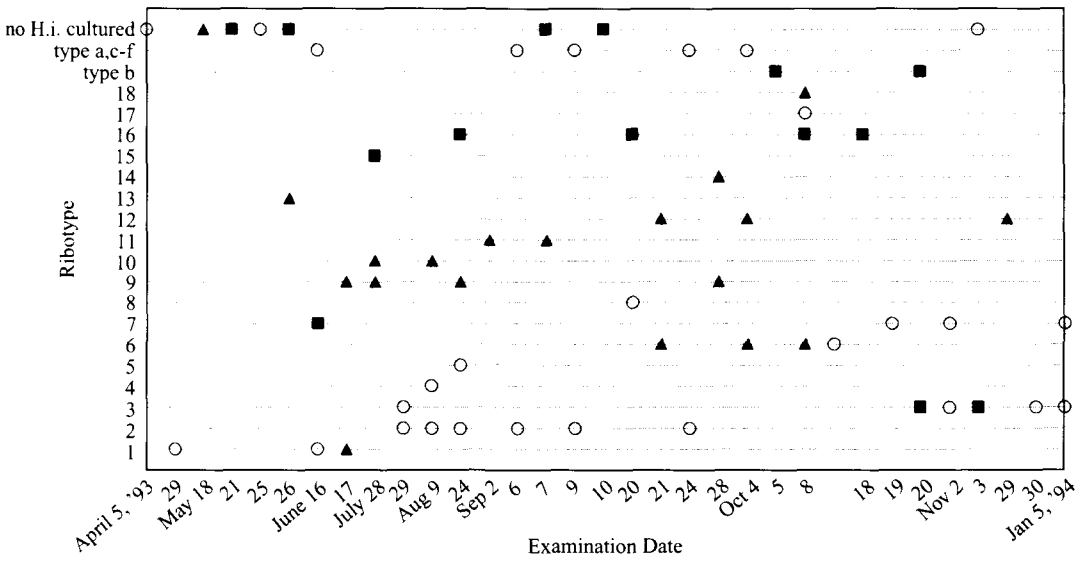

Fig. 2. NCHi ribotypes identified in three infants over time from one rural Aboriginal community. Isolates from the different infants are characterized by: Infant ID – 940 ○ born, 1/4/93; 946 △, 16/5/93; 947 □, 17/5/93.

typed. Another seven swabs positive for NCHi yielded only a single ribotype, but in each case fewer than four colonies could be typed. The frequency distribution of different ribotypes was consistent with the assumption of a dominant strain with a second strain colonizing concurrently at a lower rate. The average frequency of the second ribotype was estimated as 13%. To test the assumption that the frequency of detection of a second ribotype is a function of the number of colonies typed from each swab, and to get an independent estimate of the frequency of any second ribotype, we ribotyped 30 colonies of NCHi from a single swab in a separate experiment. The 30 colonies were picked from the original swab (stored at −70 °C) collected from infant 940 on 29 July 1993. Of the 30 colonies, 26 (86·6%) were of one ribotype (ribotype 3), and four (13·3%) were of a second (ribotype 2). From these data and those of Table 2, we estimated the average frequency of colonies of a second ribotype to be 13% with 95% confidence limits of 7·5–29%. A third ribotype was never detected, even in the sample of 30 colonies. Based on these data, there is a 95% probability that if a third ribotype was present, the average frequency was less than 4·5%.

A preliminary analysis of similarities between ribotypes, using a clustering algorithm [15], showed that when a new ribotype appeared, it was no more likely to resemble the immediately preceding ribotype than any other ribotype. This confirms our expectation that the observed changes in ribotypes over time are due to new colonizations or to differential selection of pre-existing but previously undetected ribotypes, and not due to evolutionary change in ribotypes during the short time course of our study.

DISCUSSION

The high carriage rates of NCHi in Aboriginal infants in this population have been previously reported; in conjunction with high carriage rates of *Streptococcus pneumoniae* and *Moraxella catarrhalis*, these pathogens contribute to high rates of otitis media [1]. Rates of invasive disease due to encapsulated strains of *H. influenzae* have also been unacceptably high in Aboriginal populations [16], although rates of disease due to *H. influenzae* type b have decreased greatly since the introduction of Hib vaccine [17].

Ribotyping, using the restriction enzyme *Xba* I in combination with the probe pKK3535, which contains the entire rRNA operon (5S, 16S, 23S and spacers), was found to be optimal for studying the carriage of multiple ribotypes of NCHi in Aboriginal infants. In earlier work [6], NCHi was ribotyped using the restriction enzyme *Eco*R I and a probe containing 16S and 23S rRNA genes; however, in our hands and with a more discriminatory probe, *Xba* I was found to be more informative than *Eco*R I.

The additional discrimination afforded by ribotyping showed that carriage of NCHi in the three infants was persistent with dense colonization throughout the 9-month study period and 18 different ribotypes were detected. However, it appears (Fig. 2) that each ribotype can persist for a period of weeks or

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months as a dominant ribotype before being replaced by another, possibly because of a type-specific host immune response to the NCHi [18]. There was no evidence that the appearance or disappearance of any ribotype was systematically related to the date of prescription of antibiotic treatment; the lack of such a correlation could be due to antibiotic resistance, the small number of time points, or to other unknown factors.

Concurrent colonization with two strains within a single infant was detected on 12 of 27 (44%) separate occasions. The frequency of detection of a second ribotype when few colonies were ribotyped (Table 2), is consistent with the presence of a second ribotype with an average frequency of 13%. We suggest that a second ribotype would be detected on average, in at least 13% of all colonies typed in most Aboriginal infants; however, as no ribotype remains as the dominant ribotype for ever (Fig. 2), it must be the case that when a previously dominant ribotype is becoming less frequent, two ribotypes could co-exist in more equal proportions for at least a short period of time. More data are needed to assess the dynamics of changes in ribotype frequency over time in individual children. The development of a new rapid technique for typing NCHi, PCR-ribotyping [19], will facilitate future work.

Carriage of multiple types of NCHi has been documented in two previous reports. One study of healthy children in day care, where the carriage rate of NCHi was 39%, reported 8 occasions of concurrent carriage of 2 types (characterized by multilocus enzyme electrophoresis and biotype) among 10 colonies analysed for each positive sample, in 41 cultures collected from 21 children [20]. In another study, in which 6 separate colonies from each of 6 unrelated individuals with otitis media were examined, simultaneous colonization by NCHi of more than one outer membrane protein type was observed on one occasion [5]. These observations confirm the multiplicity of strains carried, often concurrently, and support our conjecture that multiple strains may help explain the duration and chronicity of carriage, and thus the persistence of otitis media and other respiratory problems in Aboriginal infants [1].

Because of the ubiquity of otitis media and bacterial colonization in Aboriginal infants, and because of our evidence showing a temporal relationship between colonization and onset of otitis media [1], we are currently commencing a controlled trial of long-term antibiotic treatment, with particular attention to compliance, to evaluate its effects on both otitis media and colonization.

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REFERENCES

1. Leach AJ, Boswell JB, Asche V, Nienhuys TG, Mathews JD. Bacterial colonization of the nasopharynx predicts very early onset and persistence of otitis media in Australian Aboriginal infants. Pediatr Infect Dis J 1994; **13**: 983–9.
2. Plant AJ, Condon JR, Durling G. Northern Territory health outcomes. Morbidity and mortality 1979–1991. NT Department of Health and Community Services, 1995; 123–37.
3. Bale JR, ed. Etiology and epidemiology of acute respiratory tract infection in children in developing countries. Rev Infect Dis 1990; **12**: S861–6.
4. Turk DC. The pathogenicity of *Haemophilus influenzae*. J Med Microbiol 1984; **18**: 1–16.
5. Murphy TF, Bernstein JM, Dryja DM, Campagnari AA, Apicella MA. Outer membrane protein and lipooligosaccharide analysis of paired nasopharyngeal and middle ear isolates in otitis media due to nontypable *Haemophilus influenzae*: Pathogenetic and epidemiological observations. J Infect Dis 1987; **156**: 723–31.
6. Bruce KD, Jordens JZ. Characterization of noncapsulate *Haemophilus influenzae* by whole-cell polypeptide profiles, restriction endonuclease analysis, and rRNA gene restriction patterns. J Clin Microbiol 1991; **29**: 291–6.
7. Jordens JZ, Leaves NI, Anderson EC, Slack MPE. Polymerase chain reaction-based strain characterization of noncapsulate *Haemophilus influenzae*. J Clin Microbiol 1993; **31**: 2981–7.
8. Leach AJ, Shelby-James T, Mayo M, Gratten M, Mathews JD. Report of a multidrug resistant clone of *Streptococcus pneumoniae* (MR*spn*) in Aboriginal infants in the Northern Territory. Commun Dis Intell 1995; **19**: 134–7.
9. Chapin KC, Doern GV. Selective media for recovery of *Haemophilus influenzae* from specimens contaminated with upper respiratory tract microbial flora. J Clin Microbiol 1983; **17**: 1163–5.
10. Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 1989; **8**: 151–6.
11. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
12. Brosius J, Ullrich A, Raker MA, Gray A, Dull TJ,

Gutell RR, Noller HF. Construction and fine mapping of recombinant plasmids containing the *rrn* B ribosomal RNA operon of *E. coli*. Plasmid 1981; **6**: 112–8.
13. Moroney MJ. Facts and figures, 2nd ed. London: Penguin 1953: 105.
14. Armitage P, Berry G. Statistical methods in medical research, 2nd ed. Oxford: Blackwell Scientific, 1987: 56–60, 139.
15. Felsenstein J. Phylip-Phylogeny inference package (Version 3.2). Cladistics 1989; **5**: 164–6.
16. Hanna JN. The epidemiology of invasive *Haemophilus influenzae* infections in children under five years of age in the Northern Territory: a three year study. Med J Aust 1990; **152**: 234–40.
17. Hanna J, Messer R. *Haemophilus influenzae* type b meningitis in far North Queensland, 1989 to 1994. Commun Dis Intell 1995; **19**: 91–3.
18. Faden H, Bernstein J, Brodsky L, et al. Otitis media in children. I. The systemic immune response to non-typable *Haemophilus influenzae*. J Infect Dis 1989; **160**: 999–1004.
19. Smith-Vaughan H, Sriprakash KS, Mathews JD, Kemp DJ. Long PCR-ribotyping of non-typable *Haemophilus influenzae*. J Clin Microbiol 1995; **33**: 1192–5.
20. Trottier S, Stenberg K, Svanborg-Eden C. Turnover of nontypable *Haemophilus influenzae* in the naso-pharynges of healthy children. J Clin Microbiol 1989; **27**: 2175–9.