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Incidence and spread of *Haemophilus influenzae* on an Antarctic base determined using the polymerase chain reaction

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SUMMARY

A PCR-based method of detecting *Haemophilus influenzae* in cultures inoculated from throat swabs was evaluated using samples from groups of laboratory staff and medical students and then applied to samples originating from the closed human community of an Antarctic research station. Suitable PCR primers to an *H. influenzae* gene (*omp*P2) were used to amplify the gene from DNA preparations made from mixed growth on chocolate agar with added vancomycin. PCR product was reamplified and subjected to restriction endonuclease digestion to allow temporal and spatial mapping of strains over an 8-month period. Eleven different strains of *H. influenzae* were detected. One particular strain was detected in a third of the base members.

INTRODUCTION

The closed communities of the British Antarctic Survey's four permanently manned research stations provide excellent opportunities for studies into the population dynamics of human commensal bacteria. These small human populations are subject to periods of isolation from outside contact for up to 8 months at a time. These factors have previously been utilized in the study of carriage and transmission of human commensals such as *Escherichia coli* [1], staphylococcus [2] and enterococcus [3]. The most recent of such studies was that of Kerr and colleagues (4), who investigated the carriage and transmission of *Haemophilus parainfluenzae* in the inhabitants of Signy base in the South Orkney Islands. In this paper we report the results of a study which used the polymerase chain reaction (PCR) and DNA restriction enzyme analysis to investigate the carriage and spread of *Haemophilus influenzae* in contemporaneous samples from the subjects of Kerr and colleagues [4].

Haemophilus influenzae is a pathogen and a commensal of human hosts. Capsule type b *H. influenzae* (Hib) is a major cause of systemic infections including meningitis and epiglottitis [5]. Non-capsulate *H. influenzae* and other capsulate strains commonly cause non-invasive infections of the upper and lower respiratory tract, sinuses and middle ear, and occasionally cause invasive disease in susceptible groups [5–7]. *H. influenzae* is traditionally detected by cultural techniques. Isolation from the throats of healthy adults, however, may be difficult as it represents a very small component of the total haemophilus flora.

Only 2·3% (2/88) of the haemophilus isolates of Kerr and colleagues (4) were *H. influenzae*, the majority (82%) being *H. parainfluenzae*. Using a similar subculture based method to investigate the carriage and spread of *H. influenzae* within the same population would have presented considerable difficulties due to the large number of cultures required. Instead, we developed a technique which used DNA amplification in the polymerase chain reaction (PCR) to detect small numbers of *H. influenzae*, and enzyme restriction to further characterize the strains encountered.

Alleles of the gene coding for the major outer membrane porin protein P2 of *Haemophilus influenzae* (*omp*P2) have been sequenced [8–11] and found to be 970–1155nt in length and to consist of interspersed conserved and hypervariable regions. Forbes and colleagues [9] showed that *omp*P2 could be amplified by PCR from both capsulate and non-capsulate *H. influenzae* by using 21-mer oligonucleotide primers derived from conserved regions, and it was on this observation that our detection method was based.

PCR has been shown to be useful in the detection of micro-organisms in many other situations where extraction, culture and identification are difficult or time consuming, examples including *Mycobacterium tuberculosis* [12, 13], *Chlamydia trachomatis* [14], *Pneumocystis carinii* [15], *Mycoplasma pneumoniae* [16], *Helicobacter pylori* [17], *Salmonella typhi* [18] and *Toxoplasma gondii* [19]. Although it has previously been used to detect *H. influenzae* in infected cerebrospinal fluid [20] in which it would usually be the only organism present, its use in the detection of this species from a mixed bacterial culture represents a novel approach which may in future prove helpful in both clinical and epidemiological settings.

METHODS

Bacterial strains

Haemophilus parainfluenzae NCTC 7857, NCTC 10665 and NCTC 11607, *H. parahaemolyticus* NCTC 8479, *H. paraphrophilus* NCTC 10557, *H. paraphrohaemolyticus* NCTC 10670, *H. segnis* NCTC 10977, *H. haemolyticus* NCTC 10659 and *H. aphrophilus* NCTC 5906 were obtained from the National Collection of Type Cultures, London. Hib and non-capsulate *H. influenzae* were obtained from Dr K. Forbes, Aberdeen. Six different strains of *H. parainfluenzae* were obtained from Dr G. Kerr, Aberdeen. *Neisseria meningitidis* was obtained from Dr F. Abbadi, Aberdeen. One of each of the following clinical isolates was obtained from the Medical Microbiology diagnostic laboratory, Aberdeen Royal Hospitals Trust, Scotland: *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, alpha-haemolytic streptococcus, coagulase-negative staphylococcus and unspeciated corynebacterium. All were grown for 24 h on chocolate agar in air + 5% CO_2 at 37 °C. Chromosomal DNA was extracted by the technique of Forbes and colleagues [21] from all the above haemophilus strains and previously extracted DNA was obtained from a further 28 strains of *Haemophilus parainfluenzae*.

Subjects

Three human populations were studied. In the UK 2 groups comprising 6 microbiologists and 49 medical students served to establish the methodologies. The Antarctic subjects were 15 overwintering male base-members (identified

below as subjects A–O) whose average age was 26. For eight of these (B, C, D, F, H, I, J and N) this was their second consecutive winter together. With the exception of subject O the remainder arrived in November, 3 months prior to the start of bacteriological sampling. Subject O arrived 4 months later, in mid March. Only subjects E, J, L and N were smokers.

Bacteriological samples

UK throat swab samples were obtained by rubbing a sterile cotton wool swab over one or other tonsillar fossa, inoculating it on chocolate agar supplemented with 5 μg/ml vancomycin hydrochloride (Sigma) and culturing for 24 h in air + 5% CO_2 at 37 °C. Each of the samples from the microbiologists was plated out onto two agar plates, one of which was 'spiked' by the addition to its inoculum of a loopful of Hib bacteria from a pure culture before spreading and incubation. Antarctic samples were the duplicate freeze-dried samples of Kerr and colleagues [4], which had been obtained from the left tonsillar fossae of the Antarctic subjects during the first week of each month between February and September 1990 and cultured on non-selective media prior to freeze drying. Freeze-dried material was rehydrated with 200 μl sterile distilled water and inoculated on vancomycin supplemented chocolate agar plates for 24 h culture. Out of 104 freeze-dried samples received 28 (27%) failed to produce any growth. The samples which failed to grow were from the following subjects: I/February; A, B, D, E, F, G, I, K, L, N and O/June; all subjects/August; and M/September. The sporadic nature of these failures would suggest some aberration in the freeze-drying process during June and August. All June and August samples have been excluded from interpretation as they provided no useful data.

Oligonucleotides

The *omp*P2 primers and probe were those described by Forbes and colleagues [9]: Primers O_1 (5′-ATA ACA ACG AAG GGA CTA ACG) at the 5′ end of the gene and O_3 (5′-ACC TAC ACC CAC TGA TTT TTC) at the 3′ end. Hybridization probes were made by PCR amplification according to the protocol below incorporating digoxygenin-labelled dUTP (DIG-labelling kit, Boehringer Mannheim) using Hib DNA with *omp*P2 primers O_1 and O_3 or the 16S rDNA primers pA and pH [22].

Polymerase chain reaction

Half a plate of bacterial growth was harvested into 1 ml sterile distilled water and washed twice by vortexing, centrifugation at 14000 g and removal of the supernatant. Cells were resuspended in 1 ml sterile distilled water and lysed by heating at 100 °C for 5 min. These suspensions were diluted to 1/500 (pure culture samples) or 1/25 (throat swab samples) in sterile water to make working DNA suspensions. Amplification reactions took place under sterile mineral oil in a total volume of 20 μl containing 2 μl of target DNA suspension, 0·5 units Taq DNA polymerase and PCR reagents at the following final concentrations: 250 nM oligonucleotide primers (each), 200 μM-dNTPs (each), 1·5 mM-$MgCl_2$, 75 mM-Tris-HCl pH 9·0, 20 mM-$(NH_4)_2SO_4$, 0·01% Tween 20. Amplification conditions were: 94 °C (4 min), then 30 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (2 min), then 72 °C (8 min). To check for contamination at least one negative control reaction mix containing all the reaction components except template DNA was included in

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each PCR batch. If any product was amplified from this blank the whole batch was discarded. The sensitivity of detection was determined by preparing serial dilutions from 10^{-1} to 10^{-16} of pure culture sample, determining the viable count and using these dilutions as target DNA in PCR reactions. PCR product was loaded onto 1% agarose gels alongside a DNA size standard (1 kb ladder, Gibco BRL) and electrophoresed for 1–2 h at 5 V cm^{-1} in a Tris-borate, EDTA buffer system [23]. Gels were stained with ethidium bromide (0·05 μg ml^{-1}) for 1 h, after which amplified *omp*P2 DNA was visible with UVB transillumination as bands of approximate length 1 kb.

Further amplification of PCR products

*Omp*P2 detected on agarose gels was reamplified using a 'band-stab' technique [24], in which small amounts of PCR product were transferred from gels into fresh reaction mixes on sterile syringe needles and used as template DNA for further PCR.

Southern hybridisation

The restriction endonuclease *Eco*RI was used to digest 2–12 μg samples of haemophilus DNA. Restriction fragments were separated on 0·8% agarose gels as above and Southern-blotted according to the method of Sambrook and colleagues [23]; 0·2–9 μg genomic DNA (from known organisms), or 3–4 μl PCR product was dot-blotted onto nylon membranes (Hybond-N), air-dried and bound to the membrane by u.v. cross-linking (Spectrolinker XL 1000 UV Crosslinker). All membranes were subjected to two 5 min washes in 2 × SSC, 0·1% sodium dodecyl sulphate (room temperature) and two 15 min washes in 0·1 × SSC, 0·1% sodium dodecyl sulphate (68 °C). Detection was by the method recommended in the DIG labelling kit using a Lumigen-PPD chemiluminescent EIA (Boehringer Mannheim). Probe was stripped from membranes by washing twice for 15 min in 0·2 M-NaOH, 0·1% sodium dodecyl sulphate at 45 °C.

Restriction endonuclease digestion

The restriction endonucleases *Alu*I and *Dra*I were used to digest 1–2 μl samples of each reamplified PCR product. Restriction fragments were electrophoresed on 1·5% agarose gels, stained with ethidium bromide and visualized by UVB transillumination. Fingerprints from each sample were recorded digitally and approximate DNA fragment lengths determined using CAM software (Cybertech, Berlin) to enable preliminary comparison of alleles. *Alu*I and *Dra*I restriction fragments from PCR products which produced similar patterns were confirmed as indistinguishable or not by electrophoresing them in adjacent lanes.

RESULTS

Specificity of ompP2 to H. influenzae

The specificity of the PCR primers was exhaustively tested. No visible PCR products were detected using O_1 and O_3 primers when amplifying genomic DNA from type strains of *H. paraphrophilus*, *H. paraphrohaemolyticus*, *H. haemolyticus*, the type strains and six other strains of *H. parainfluenzae*, *Neisseria meningitidis*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, alpha-hae-

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Table 1. *Organisms tested for the presence of ompP2*

	PCR*	Southern hybridization
H. influenzae	+	+
H. parainfluenzae	−(6st)	−(34st)
H. parainfluenzae 7857	−	−
H. parainfluenzae 10665	−	−
H. parainfluenzae 11607	−	−
H. paraphrophilus	−	−
H. paraphrohaemolyticus	−	−
H. haemolyticus	−	−
H. aphrophilus	n/t	−
H. parahaemolyticus	n/t	−
H. segnis	n/t	−
Neisseria meningitidis	−	n/t
Escherichia coli	−	n/t
Corynebacterium sp.	−	n/t
Staphylococcus aureus	−	n/t
Staphylococcus (coagulase negative)	−	n/t
Streptococcus pneumoniae	−	n/t
Streptococcus (alpha-haemolytic)	−	n/t

* +, tested positive by this method; −, tested negative by this method; n/t, not tested by this method; st, strains.

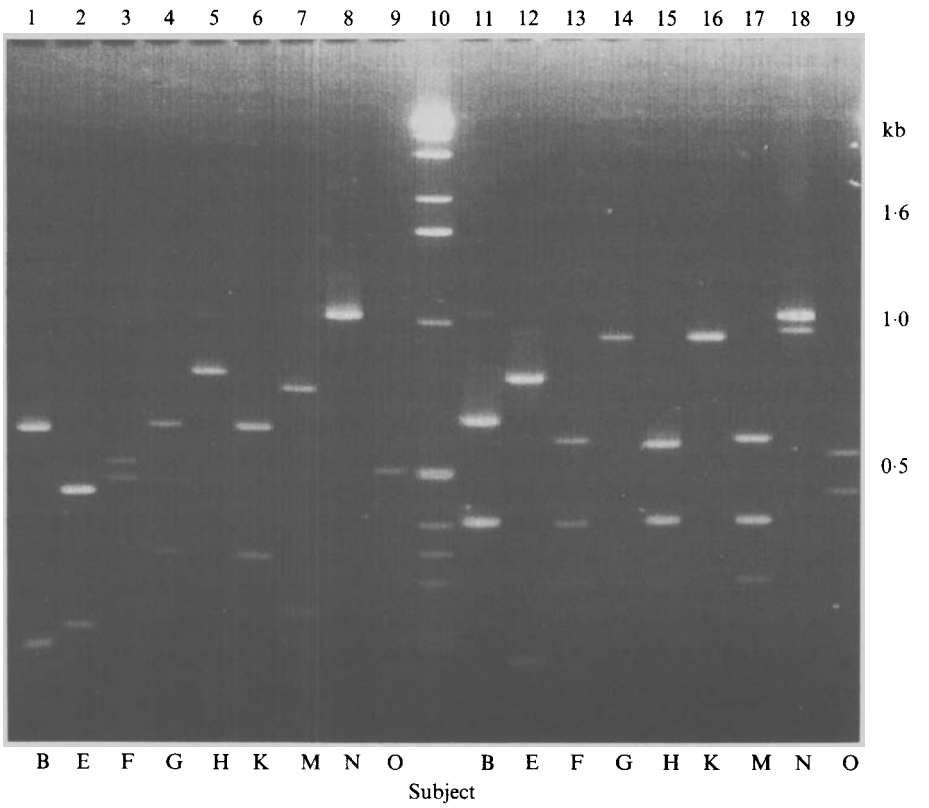

Fig. 1. Restriction digests of PCR products from April samples. Lanes 1–9 *Alu*I digests; lane 10 size standard; lanes 11–19 *Dra*I digests.

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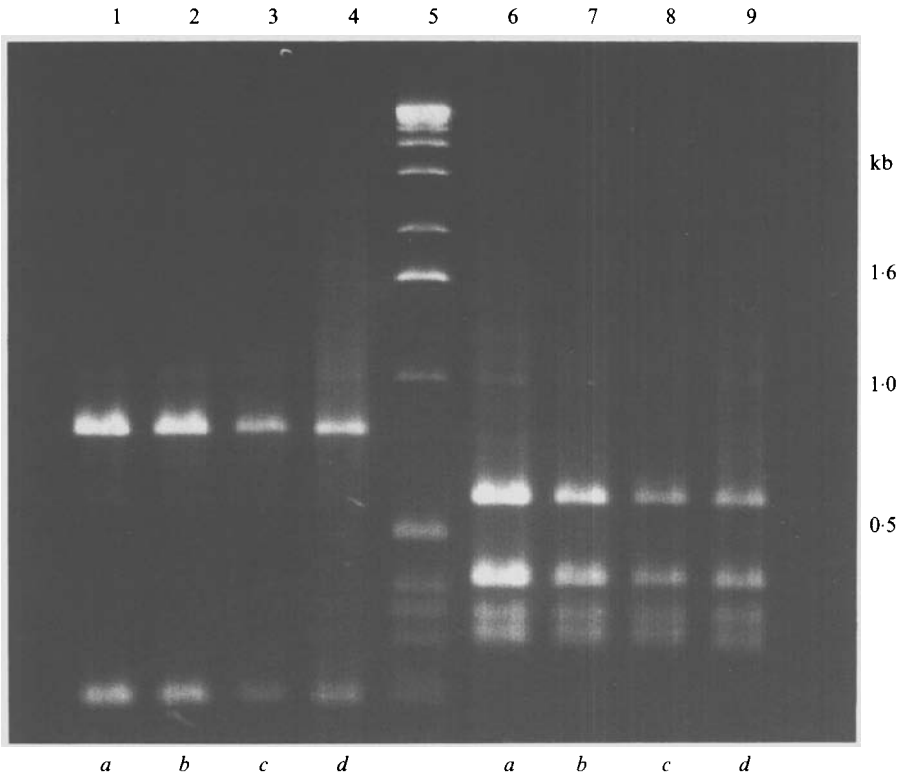

Fig. 2. Restriction digests from subject H (strain i5). Lanes 1–4 *Alu*I digests; lane 5 size standard; lanes 6–9 *Dra*I digests. *a*, February; *b*, March; *c*, April; *d*, July.

molytic streptococcus, coagulase-negative staphylococcus and unspeciated corynebacteria. In addition, the *omp*P2 probe did not hybridize with genomic DNA from the type strains of *H. parahaemolyticus*, *H. paraphrophilus*, *H. paraphrohaemolyticus*, *H. segnis*, *H. haemolyticus*, *H. aphrophilus* or the 3 type strains and 11 other strains of *H. parainfluenzae* after Southern hybridization of electrophoresed *Bam*HI fragments or with dot blotted genomic DNA from all these and a further 23 *H. parainfluenzae* strains. The probe hybridized with the positive control (Hib or non-capsulate *H. influenzae* genomic DNA) on each membrane. All dot blotted samples subsequently hybridized with the 16S rDNA probe, confirming the presence of detectable quantities of membrane-bound genomic DNA. These results are summarized in Table 1. These findings concurred with the reports of Hansen and colleagues [10] that the amino acid sequence of Hib-*omp*P2 shares only 23–25% homology with the *omp*F porin of *Escherichia coli*, and of Munson and colleagues [11], who found less than 20% homology between the protein sequence of *omp*P2 and other porin genes.

Detection and differentiation of ompP2 in PCR products from throat swab samples

The concentration of *H. influenzae* required to produce a visible *omp*P2 PCR product was calculated as 200 bacteria per 20 μl reaction mix. *Omp*P2 was amplified from two out of the six unspiked microbiologists' throat swab cultures and all of those spiked with *H. influenzae*. The two unspiked samples which

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Table 2. Haemophilus influenzae *strains detected*

	Feb.	Mar.	April	May	July	Sept.
A	–	–			–	+
B*	i1	i1	i1		i1	i1
C*	i2	i2, i5	–		–	–
D*	i2	–		–	–	
E	i3	–	i3		i3	
F*	–	–	i4	–	–	–
G	i2	–	i2, i11	–	–	–
H*	i5	i5	i5		i5	
I*	i2	i2		–	–	
J*	–	–	–		+	i6
K		i2, i6	i2	i2	–	–
L	–	–	–	+	–	–
M	i7	i7	i7		i7	
N*	i8, i9	i8, i9	i8	i8	i8, i9	i8, i9
O			i10	–	i10	–

* Subject's second-consecutive winter on the base. +, *omp*P2 allele amplified but not identifiable. *omp*P2 allele not amplified.

Subjects are designated A–O. Alleles are numbered from i1 to i10 in the order in which they were first encountered. A blank space indicates a freeze-dried sample which either was missing or failed to produce any growth.

amplified *omp*P2 came from subjects who were engaged in research involving *H. influenzae*. *Omp*P2 was amplified from 48% (23/49) of the medical student samples. One of these produced an additional 250 bp PCR product suggesting either the existence of a duplicated primer site within the gene, or an *omp*P2 allele with an internal portion deleted. Samples from neither of these two groups produced more than one visible product in the 1 kb region.

Of the Antarctic throat swab samples which were successfully recovered *omp*P2 was amplified from 54% (39/72). After reamplification and enzyme restriction 11 different alleles were obtained from 36 of these. Seven of the *omp*P2 positive samples produced two separate alleles, and three either failed to reamplify after band stabbing or produced such a small amount of reamplified DNA that enzyme restriction was not possible. Examples of each of the 11 *omp*P2 alleles hybridized to the *omp*P2 probe. Figure 1 shows the restriction digests of all PCR products amplified from April samples, demonstrating the diversity typically encountered. Figure 2 shows the restriction digests of all PCR products amplified from subject H, demonstrating that the same allele was amplified on each occasion. The overall results are summarized in Table 2.

DISCUSSION

Preliminary results with *H. influenzae* spiked and unspiked throat swab samples from microbiologists and students showed that detection of *H. influenzae* by our PCR-based method was possible, and we subsequently demonstrated by PCR amplification of DNA from control organisms that the method was specific to this species.

No surveys of *omp*P2 diversity by restriction mapping or nucleotide sequencing [8, 9, 25] have found an identical *omp*P2 allele in different strains of *H. influenzae*

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It is therefore likely that carriage of *H. influenzae* by adults is much higher than our results or those of previous studies suggest.

In those subjects with resident strains (B, E, H, M and N) the host has clearly not been able to eradicate *H. influenzae* successfully despite an ample period for a suitable immune response to develop. It is possible that this colonization could be symbiotic, conferring advantage to the bacterium in the provision of a favourable habitat, and to the host through the exclusion of such a niche to other, perhaps more pathogenic, strains of *H. influenzae*.

Subject N was colonized with two distinct, equally detectable resident strains. These showed up initially as *omp*P2 fragments of different lengths, and were confirmed as different by their restriction patterns after reamplification from the separate bands. Subsequent subculture and identification of 14 haemophilus like colonies from this subject (N/September) resulted in the detection of 13 *H. parainfluenzae* isolates and one *H. influenzae*, the *omp*P2 restriction pattern of which was recognizable as that of allele i8. A *H. influenzae* strain with an i9 *omp*P2 pattern was not found in this sample, presumably because it was proportionately much rarer than i8. Although the UK samples were not subjected to endonuclease restriction, none of them was seen to produce more than one 1 kb *omp*P2 band. Kuklinksa and Kilian [30] found pharyngeal carriage of more than one *H. influenzae* strain to be common in children but not to occur in adults. Our failure to detect more instances of multiple strain carriage reflects either the rarity of this phenomenon or a lack of sensitivity in the method. Given that other PCR based detection methods are able to amplify template DNA from as few as 1–10 organisms [12, 16–18], we are currently investigating measures to increase sensitivity such as the use of internal primers (nested PCR) and DNA extraction by chemical or enzymatic methods.

During the study we attempted to use uncultured throat swab material and unreconstituted freeze-dried cultures to provide target DNA for *omp*P2 detection, neither of which produced useful results. It is assumed that the quantity of *H. influenzae* taken onto a throat swab was inadequate for PCR amplification, and that the freeze-drying medium contained PCR inhibitors which we were unable to remove.

We also attempted to gain more precise information about both the *H. influenzae* strains encountered and the *omp*P2 alleles amplified. Firstly we synthesized oligonucleotide primers to *H. influenzae* outer membrane protein P1 gene (*omp*P1) conserved regions. Although these primers amplified differentiable *omp*P1 alleles from *H. influenzae* they were not species-specific, also amplifying DNA from *H. parainfluenzae*. Secondly we assessed the practicability of nucleotide sequencing with a small pilot study, in which three isolates of the i1 *omp*P2 allele were found to contain an identical 300 nt sequence at the 3′ end.

The closed human community is an ideal environment in which to study the transfer of bacteria from one subject to another. The further refining of PCR-based detection techniques such as this along the lines discussed will prove to be of great value in future epidemiological investigations of a similar nature.

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