Identification of *Bordetella pertussis* in nasopharyngeal swabs by PCR amplification of a region of the adenylate cyclase gene

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Summary. The polymerase chain reaction (PCR) was used to amplify a 522-bp region of the adenylate cyclase toxin (*cyaA*) gene of *Bordetella pertussis*. As few as 100 cfu from a suspension of *B. pertussis* could be detected by this procedure when the amplified PCR product was detected by ethidium bromide staining of agarose gels. However, simulated clinical specimens, prepared from swabs impregnated with known numbers of *B. pertussis* cells, only yielded a positive reaction with $\geq 10^4$ cfu. Hybridisation of a Southern blot of the PCR products from the swab samples with a *cya*-specific probe gave a positive reaction with as few as 8 cfu, but the hybridisation signal was uniformly weak with fewer than 10^4 cfu. Nevertheless, three of 13 nasopharyngeal swabs, taken from suspected clinically defined cases of whooping cough and stored frozen for up to 18 months, gave a positive PCR reaction.

Introduction

At present, the bacteriological confirmation of *Bordetella pertussis* in cases of whooping cough is often unsuccessful for a variety of reasons. These include the slow growth and fastidious nature of the organism, variability in swabbing techniques, time taken in transport of specimens to the laboratory, and the fact that the infection is being cleared by host defences as the typical disease symptoms appear. Isolation rates may be as low as 20% from suspected cases.¹ The alternative method of serological diagnosis can also be unreliable, with an efficacy ranging from 21 to 67% in one report.²

DNA probe technology is particularly suited to the detection of an organism like *B. pertussis*, which grows poorly on laboratory media. There is a further advantage in that, because growth is not required, it should be possible to collect specimens and store them frozen for long periods. *B. pertussis* produces, as one of its virulence factors, an adenylate cyclase toxin which is able to penetrate mammalian cells and raise the internal cyclic AMP concentration.³ The genetic determinant for the toxin has been cloned and sequenced.^{4,5} We report here on the use of primer-directed enzymic amplification of DNA (polymerase chain reaction or PCR⁶) to amplify a region of the

adenylate cyclase gene, and on the suitability of the technique for detection of bordetellae in clinical swabs.

Materials and methods

Bacterial strains

The following strains of *Bordetella* spp. were used and have been described.^{7,8} *B. pertussis* 18-323 (NCTC 10739); *B. parapertussis* NCTC 10520; *B. bronchiseptica* 276; and *B. avium* P-4084. *Bacillus subtilis* NCTC 3610, *Neisseria meningitidis* 4076 and *Streptococcus pneumoniae* (NIBSC laboratory stock strains) were also used.

Media and culture conditions

Strains of *Bordetella* spp. were grown at 37°C in a moist atmosphere on Bordet-Gengou (BG) medium (Gibco-BRL) containing defibrinated horse blood (Gibco-BRL) 20% v/v. Other organisms were grown at 37°C on Nutrient Agar (Oxoid), Tryptone Soya Agar (Oxoid) or Brain Heart Infusion Agar (Oxoid).

Sample preparation

Bacteria were washed from agar plates with sterile distilled water and routinely adjusted to 10 optical units with a standard opacity rod.⁹ This opacity is equivalent to $c. 2 \times 10^9$ cfu/ml in the case of *B. pertussis*, and this was confirmed by viable counting. Portions (100 µl) of serial dilutions were heated for

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10 min at 100°C; 5 µl of each boiled sample was then used in the PCR assay. Fine tip cotton-wool or alginate swabs (Medical Wire and Equipment, Corsham) for use in the simulation experiments were impregnated with 10 µl of a cell suspension of *B. pertussis*. Each swab was then immersed in 50 µl of distilled water, agitated thoroughly, and centrifuged for 10 min at 12000 rpm in a Heraeus microfuge. The swab was then removed to a small microfuge tube with a hole in the bottom, and then re-centrifuged inside the larger tube containing the first wash. A further 50 µl of distilled water was then added to the swab and the centrifugation step repeated. The combined eluate (c. 100 µl) was then heated to 100°C, as above, and 20 µl was used in the PCR assay. Nasopharyngeal cotton-wool swab specimens (Transwab; Medical Wire and Equipment) were collected from patients presenting with suspected, clinically-defined whooping cough (paroxysmal cough lasting for at least 2 weeks that was associated with a typical whoop, vomiting or apnoeic episodes). The initial, direct, hybridisation experiments were performed on samples supplied by Dr M. Thomas (St George's Hospital Medical School, London) that were culture-positive for *B. pertussis*, confirmed by agglutination with *B. pertussis*-specific antisera. Later samples for PCR analysis were supplied from various sources within the UK between February and August 1989. Such samples had been used for culture and were sent in the original charcoal or blood agar transport medium; they were then stored frozen for up to 18 months until processed as above.

PCR amplification

This was performed over 25 cycles, involving denaturation at 94°C (1 min), primer annealing at 55°C (2 min), and primer extension at 72°C (3 min), with a Techne PHC-1 automatic thermal cycler. Each 50-µl reaction mix contained: sample; 10 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; gelatin 100 µg/ml; 200 µM of each deoxyribonucleotide; 1 µM of each primer; and 2.5 U of *Taq* polymerase (Perkin-Elmer-Cetus). At the end of the reaction, 20 µl of the mixture was analysed by electrophoresis on an agarose (type II-A; Sigma) 1.5% gel.⁴ The 20-bp primers spanned bases 1–20 and 503–522, starting from the ATG translational start codon, of the *cyaA* structural gene.⁵ The primers were synthesised by the phosphite triester method with an ABI 381A automatic DNA synthesiser (Applied Biosystems) by Dr V. Math, Biochemistry Department, Glasgow University.

DNA techniques

Preparation of plasmid DNA, restriction digests, ligations, transformations, agarose gel electrophoresis, Southern blotting on to Hybond-N nylon membranes (Amersham), and autoradiography were as described previously.^{4,8} To obtain radiolabelled molecular size standards, λ DNA was digested with *Hind*III and labelled with ³²P-dATP as described by Downing *et*

*al.*¹⁰ For probe preparation, a 200-bp *Apa*LI/*Bst*EII fragment internal to the DNA fragment amplified by PCR (spanning bases 185–385 of the *cyaA* gene) was cloned into pSP64.¹¹ The purified plasmid DNA was digested with *Bam*HI and *Eco*RI, and a resulting 211-bp fragment was excised from an agarose gel and radiolabelled to high specific activity by the method of Feinberg and Vogelstein.¹² Hybridisations were performed at 42°C in the presence of formamide 50%. After 24 h, filters were washed twice in 1 × SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0), SDS 0.1% at 68°C for 2 h before autoradiography. For dot-blot analysis, 100-µl portions of bacterial cell suspensions were denatured by heating at 50°C for 10 min in 100 µl 0.4 N NaOH. The samples were then loaded into the wells of a multi-well manifold apparatus (Schleicher and Schuell) fitted with nylon membrane. Each well was washed with 0.5 M Tris, pH 7.4, 1.5 M NaCl, and hybridisation was performed as above.

Results

Direct hybridisation

Preliminary work assessed the efficacy of direct hybridisation to *B. pertussis* DNA immobilised on nylon membranes. A 211-bp DNA fragment, internal to the *cyaA* fragment amplified in later experiments by PCR, was radiolabelled and used as a probe against DNA passed through a dot-blot apparatus following release by boiling from dilutions of a *B. pertussis* cell suspension. The detection limit of this procedure, by visual inspection of the autoradiograph, was estimated to be 5×10^4 cfu. Attempts to recover sufficient cell material from six clinical swabs (taken from culture-confirmed cases of whooping cough) to give a positive reaction by the dot-blot assay were unsuccessful.

Fig. 1. Sensitivity of detection of *B. pertussis* by PCR. Dilutions of a standard cell suspension were assayed, subjected to agarose gel electrophoresis, and DNA was visualised by staining with ethidium bromide. Results are from two separate experiments. Numbers of cfu used for assay were: lane 1, 10^7 ; 2, 10^6 ; 3, 10^5 ; 4, 10^4 ; 5, 10^3 ; 6, 10^2 ; 7, 10^1 ; 8, negative control. M, fragment sizes (kb) of *Hind*III-digested λ DNA.

Fig. 2. Specificity of detection of *Bordetella* spp. by PCR. DNA from 10^8 cfu of each organism was processed as in fig. 1. The 522-bp *cyaA* gene fragment is indicated. Lane 1, *B. pertussis*; 2, *B. bronchiseptica*; 3, *B. avium*; 4, *B. parapertussis*; 5, 1-kb DNA ladder (Gibco-BRL) with relevant fragment size (bp) shown on right; 6, *Bac. subtilis*; 7, *N. meningitidis*; 8, *S. pneumoniae*.

PCR amplification

The problem of low numbers of bacteria on nasopharyngeal swabs, and hence small amounts of target DNA, was addressed by means of PCR amplification. The sensitivity of the procedure was assessed by PCR assays on boiled cell suspensions. When a *B. pertussis* cell suspension containing 2×10^9 cfu/ml was serially diluted and subjected to PCR, amplification of a 522-bp fragment, visible on ethidium bromide-stained agarose gels, was obtained from ≥ 100 cfu (fig. 1). The cloned *cyaA* gene has been shown previously to

Fig. 4. PCR assay of nasopharyngeal swab samples taken from patients with suspected, clinically defined, whooping cough. Lanes 1-8 are clinical specimens and lane 9 is a negative control. Lane M contains a 1-kb DNA ladder (Gibco-BRL), with the relevant sizes (bp) shown on the right.

hybridise in Southern blots to DNA from *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, but not to DNA from *B. avium*.⁴ This pattern was also exhibited with the PCR assay. The *cyaA* primers amplified a 522-bp fragment from all the bordetellae except *B. avium*, but no such amplification product was obtained with DNA from *Bac. subtilis*, *N. meningitidis* or *S. pneumoniae* (fig. 2). High cell numbers were deliberately used in each assay, which may be the reason for the presence of several low molecular size, non-specific amplification products visible in each lane.

Samples for routine diagnosis of *B. pertussis* in the UK are taken by nasopharyngeal swabs, so it was important to assess the sensitivity of detection of bacteria eluted from such swabs. Simulated swab specimens were prepared by applying known numbers

Fig. 3. PCR assay of simulated swab specimens. Dilutions of a standard *B. pertussis* cell suspension were applied to swabs, material eluted and assayed. The numbers of cfu used for each assay were: lane 1, 8×10^5 ; 2, 8×10^4 ; 3, 8×10^3 ; 4, 8×10^2 ; 5, 80; 6, 8; 7, negative control. Lane M contains radiolabelled fragments (sizes shown in kb) of *Hind*III-digested λ DNA. (a) Amplified DNA visualised by ethidium bromide staining. (b) Autoradiograph of DNA transferred to a nylon membrane and hybridised with a radiolabelled probe internal to the amplified fragment.

of *B. pertussis* cells to either cotton-wool or alginate swabs. Cells were then eluted and the eluates were used for PCR assays. No amplified product was detected when alginate swabs were used, but the 522-bp fragment was amplified and visible on agarose gels from cotton-wool swabs loaded with 8×10^4 cfu (fig. 3a), an appreciably lower level of detection than that obtained following simple dilution of a cell suspension when 100 cfu were detected. Hybridisation of a Southern blot of this gel, with the probe internal to the amplified fragment, revealed that as few as 8 cfu applied to the swab gave a positive hybridisation reaction following PCR assay (fig. 3b). However, the signal was uniformly weak from those cell dilutions which did not give a positive reaction by ethidium bromide staining. This pattern of a non-linear response of the PCR reaction with respect to cell numbers applied to the swabs was detected reproducibly, and indicated that the PCR reaction was unable to proceed to completion in simulated samples prepared from low numbers of bacteria.

PCR amplification of the 522-bp *cyaA* gene fragment was also used in an attempt to detect *Bordetella* spp. from swabs taken from suspected, clinically defined, cases of whooping cough. In an analysis of eight samples (fig. 4), one sample gave a positive reaction (lane 1), while in an analysis of five further samples, two were positive (data not shown).

Discussion

Previous reports^{13,14} have shown that the PCR can detect low numbers of *B. pertussis* cells in nasopharyngeal aspirates with primers that amplify regions of the pertussis toxin S1 subunit gene or a repetitive DNA sequence, both of which are species-specific. Houard *et al.*¹³ were able to detect *B. pertussis* in five of 10 nasal aspirates taken from suspected cases of whooping cough, while Glare *et al.*¹⁴ reported that, from a total of 332 samples, 63 of 66 culture-confirmed aspirates were positive by the PCR, as were 33 culture-negative specimens. In the present study, with nasopharyngeal swabs taken from patients with suspected whooping cough, three of 13 swabs gave a positive PCR reaction. This was encouraging since the swabs had been frozen and stored for a long period before testing. The swabs had been supplied after subculture

for *B. pertussis* diagnosis, but the culture response was not reported to us, except in three instances which were reported as culture-negative. One of the three latter swabs gave a positive PCR reaction, which emphasised the value of the PCR reaction for detection of *B. pertussis* in samples which present as culture-negative. However, the work with simulated swab specimens suggested that it was difficult to obtain a positive PCR reaction with low numbers of bacteria, either because of inhibitory material eluted from the swabs or because of a poor yield of bacteria in the eluates, a feature which may have been exacerbated by prolonged storage. As the work coincided with an inter-epidemic period, it was not possible to repeat the PCR assays on fresh specimens to assess the effect of prolonged storage on the efficacy of the PCR method.

This work extends previous reports by showing that PCR can be used to detect *B. pertussis* on clinical swabs as well as in aspirates. However, it was clear that insufficient cells, and hence DNA, were available on a clinical swab to ensure the detection of *B. pertussis* directly by hybridisation, at least with the *cya*-specific probe. This may be caused by inefficient removal of cells from the swab or sequestering of DNA by the swab material. Amplification of target DNA by PCR increased the sensitivity of detection, but more work is needed to demonstrate that the procedure used in conjunction with clinical swab specimens offers greater reliability than the standard culture and serological tests. The PCR reaction may, however, be unsuitable for routine use in a diagnostic laboratory, since it is very sensitive to cross-contamination resulting in false-positive results.^{15,16} The method may be more useful in vaccine efficacy trials where specimens can be collected and stored before assay. The alternative to use of the PCR for increasing the sensitivity of a gene probe assay is to amplify the signal-generating capacity of the system rather than the target DNA.¹⁷ The use of probes specific for *B. pertussis*, such as those used by Houard *et al.*¹³ and Glare *et al.*,¹⁴ in combination with a probe specific to other human pathogenic *Bordetella* spp., such as that used in this report, will be useful for identifying the presence of *B. parapertussis* or *B. bronchiseptica* in clinical specimens.

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