Epidemiol. Infect. (1993), **110**, 289–295

Non-radioactive ribotyping of *Haemophilus ducreyi* using a digoxigenin labelled cDNA probe

T. J. BROWN AND C. A. ISON

Department of Medical Microbiology, St Mary's Hospital Medical School, Paddington, London W2 1PG

(*Accepted 17 October 1992*)

SUMMARY

Haemophilus ducreyi, the causal organism of chancroid, has increased in significance recently due to its association with HIV transmission. Most previous typing systems have exploited phenotypic characteristics. Detection of ribosomal RNA cistrons, ribotyping, was successfully developed to examine *H. ducreyi*, but required the use of ^{32}P.

We have used digoxigenin to define ribotypes from 30 strains of *H. ducreyi* from diverse geographical locations. This was achieved by agarose gel electrophoresis of restriction enzyme (RE) digested DNA extracts. These extracts were vacublotted onto nylon membrane and probed using digoxigenin labelled complementary DNA probe, prepared from *Escherichia coli* 16S and 23S ribosomal RNA. From 19 REs tested, *Ava* II, *Hinc* II, *Bgl* II and *Bst*E II gave clear ribotypes. The ribotypes of *Bst*E II and *Bgl* II used together gave the highest index of discrimination ($D = 0{\cdot}95$), 16 types, and showed good reproducibility. This non-radioactive method demonstrates the three important features of a typing system: discrimination, typability and reproducibility.

INTRODUCTION

Haemophilus ducreyi, the causal organism of chancroid, is a fastidious gram-negative rod, that can be cultured on complex media. Although described first in 1889 [1] it has remained a little studied organism as chancroid was predominantly a disease of the tropics and was not life-threatening. Recently interest in this organism has increased with the rise in the incidence in the western world [2]. Further the presence of chancroid ulcers is thought to be a risk factor in HIV transmission [3].

There is no established system for typing *H. ducreyi* although several methods have been examined including protein profiles [4], enzymic activity [5], lectin typing [6], indirect immunofluorescence [7] and plasmid analysis [8].

Digestion of genomic DNA by restriction-enzymes (REs) produces banding patterns on agarose gel, as fragments will vary in length. This restriction fragment length polymorphism may be used as a typing system. A problem with the technique is that patterns are often complex. By examining a specific sequence

that may be repeated throughout the genome the patterns may be simplified. Such a sequence is the ribosomal RNA (rRNA) cistron. This sequence in bacteria is evolutionarily conserved and is often present in multiple copy numbers [9]. rRNA sequences can be detected by hybridization between the genomic DNA and a labelled complementary DNA (cDNA) probe. A problem with ribotyping, as this technique has become known, is that ^{32}P is often used as the label. Sarafian and colleagues [10] identified clear types amongst strains of *H. ducreyi* using a ^{32}P end-labelled rRNA probe. The technique however would be more useful if a non-radioactive label was incorporated into the probe. The synthesis of such a probe has been described by Pitcher and colleagues [11]. Biotin or digoxigenin (DIG) are two non-radioactive labels that have become well established in detection techniques.

The purpose of this study was to develop a typing system for *H. ducreyi* that does not use radioactive markers but in which ribotypes can be visually distinguished from one another. The typability, reproducibility and discrimination of the system using DIG labelled cDNA was also assessed.

MATERIALS AND METHODS

Bacteria

We selected a panel of 30 strains of *H. ducreyi*, 23 from 14 different geographical origins (South Africa, USA, Kenya, Gambia, Thailand, France, Vietnam, Belgium, Bangladesh, Denmark and four cities in UK) and 7 strains from unknown origin, from a collection stored in liquid nitrogen at St Mary's Hospital Medical School (SMHMS). Strains were cultured on solid medium (Mueller–Hinton agar with chocolatized horse blood (50 ml/l), 1 × IsoVitaleX, 5% foetal calf serum), incubated for 48 h aerobically at 33 °C with 5% CO_2. Identity [12] was confirmed as *H. ducreyi* if the colonies could be pushed intact across the medium, and if they were Gram-negative rods which were oxidase positive, catalase negative, nitrate reductase positive, protoporphyrin negative and alkaline phosphatase positive.

Digestion of bacterial DNA and separation of fragments

Washed bacterial cells suspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) were lysed using GES (60% guanidinium thiocyanate w/v, 3·7% EDTA w/v, 0·5% Na lauroylsarcosine w/v) and DNA was recovered by phenol:chloroform extraction and ammonium acetate precipitation in ethanol [13]. 5–10 μg of DNA were digested according to the RE manufacturer's instructions. A range of 19 restriction enzymes were evaluated (see Table 1). Electrophoresis of digests was carried out overnight at 25 volts on a 0·9% agarose gel.

Preparation of cDNA probe

The reverse transcriptase reaction mixture (200 μl) based on that of Pitcher and colleagues [11], contained: 0·1 mM each of dATP, dCTP and dGPT (Boehringer–Mannheim (BM); 18·2 units/ml random $p(dN)_6$ primers (BM); 80 μg/ml denatured *E. coli* 16S and 23S ribosomal RNA (BM); 80 units/ml human placental RNase inhibitor (BM); 5 mM dithiothreitol (Gibco–BRL); 50 μg/ml BSA (Gibco–BRL);

Table 1. *Discrimination index* (D) *of ribotype banding patterns*

Restriction enzyme	Number of strains examined (*N*)	Index of discrimination (*D*)	Number of bands	Number of ribotypes
*Bst*E II	29	0·754	10–11	7
Bgl II	28	0·823	10–11	10
*Hin*c II	29	0·416	9–10	3
Ava II	30	0·186	4	2
*Hin*d III	26	0·748	7–14	10
*Bst*E II and *Bgl* II	28	0·950	–	16
*Hin*d III and *Hin*c II	26	0·818	–	12
*Bst*E II and *Bgl* II and *Hin*d III	26	0·975	–	18

0·1 mM DIG–dUTP (BM); 8000 units/ml murine leukaemia virus reverse transcriptase (Gibco–BRL); 1 × reaction buffer (50 mM Tris–HCl (pH 8·3), 75 mM KCl and 3 mM MgCl). After incubation at 37 °C for 1 h 4 μl 5 mM dTTP (BM) was added. The reaction was stopped after 30 min by adding 4 μl 0·5 M EDTA. The probe was purified by phenol:chloroform extraction followed by sodium acetate precipitation in ethanol, and finally by ammonium acetate precipitation in ethanol [13]. The cDNA was dissolved in 200 μl TE buffer.

Southern blot hybridization

The DNA separated in the gel was transferred onto a nylon membrane (Hybond-N, Amersham) using Vacugene XL (Pharmacia) according to the manufacturer's protocol. Briefly, the gel was treated with depurination solution (0·2 M HCl), denaturation solution (0·5 M NaOH, 1·5 NaCl) and neutralization solution (1 M Tris pH 7·5, 1·5 NaCl). Once complete the DNA was fixed using a transilluminator for 6 min.

Using a hybridization oven system (Hybaid), membranes were incubated for 1 h in 20 ml pre-hybridization solution (750 mM NaCl, 75 mM Na citrate, 0·1% Na lauroylsarcosine, 0·02% SDS, 1·5% Boehringer–Mannheim blocking reagent) and 15–18 h in 2·6 ml hybridization solution (2·5 mls pre-hybridization solution, 100 μl DIG–cDNA probe denatured by boiling for 5 min). Probe cDNA was detected according to the protocol given in the Boehringer–Mannheim application manual. Briefly DIG was detected using an anti-DIG alkaline phosphatase conjugate and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and Nitroblue tetrazolium salt (NBT) was used for colour development.

Analysis of ribotypes

Arrays of DNA fragments hybridized with the probe were compared and judged as to whether they had common banding patterns. Two strains were placed in the same ribotype if all the bands from one strain visually corresponded with all the bands from the second. Strains were placed in separate ribotypes if one or more bands varied.

The discriminating power of different REs was examined by comparing values of Simpson's Index of Diversity, D, as suggested by Hunter and Gaston [14] calculated according to the following equation:

$$D = \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j-1),$$

where s is the number of types, n_j is the number of strains falling into the jth type and N is the size of the population. This statistic gives the probability that two randomly selected strains will belong to different types. A value of $D = 1$ would indicate that all the population members differ from one another, and $D = 0$ would indicate that all the population members were identical.

RESULTS

Evaluation of restriction enzymes

Nineteen REs were evaluated as to their suitability for the production of ribotypes. Ideally the ribotype patterns will contain approximately 10 discrete bands that are spread across the gel. *Hae* III, *Cla* I, *Sau* 3A and *Hpa* II gave banding patterns made of fragments that were too small to be separated into discrete bands on the electrophoresis system used in this study. *Pst* I, *Hin*f I, *Eco*R I, *Sma* I, *Bam* HI, *Kpn* I, *Not* I, *Dra* I, *Sal* I and *Bgl* I either showed no digestion, incomplete digestion or diffuse bands. *Ava* II, *Hin*c II, *Bst*E II, *Bgl* II and *Hin*d III gave banding patterns that were suitable for typing.

Typability

Thirty strains were typed using *Ava* II, *Bgl* II, *Bst*E II, *Hin*c II and *Hin*d III giving 2, 10, 7, 3 and 10 types respectively. The 3 types seen with *Hin*c II resembled 3 out of the 4 types obtained by Sarafian and colleagues [10] although the general resemblance between the *Hin*d III types produced by the two methods was only revealed when molecular weights of the main bands were calculated. We found *Hin*d III ribotypes of little use because visual comparison was difficult due to low intensity of the bands and complex patterns of the fragments between 8 and 10 kilobases. Examples of all the *Bst*E II and *Bgl* II types can be seen in Figures 1*a* and 1*b*.

Discrimination

Values of D were calculated (Table 1). Adding *Hin*c II or *Ava* II or both did not improve on $D = 0{\cdot}95$ obtained with *Bgl* II and *Bst*E II. Adding *Hin*d III gave an improvement in discrimination to $D = 0{\cdot}975$, but this increase must be weighed against the difficulties of achieving it. Sixteen types were generated by the *Bst*E II/*Bgl* II combination from 7 and 10 patterns respectively (Fig. 1). A maximum of four strains were found in any one type.

Reproducibility

Twelve strains that included examples of all the patterns seen with REs *Bgl* II and *Bst*E II were typed on four independent occasions. All strains gave patterns that were visually identical (the same number and distribution of bands) on three

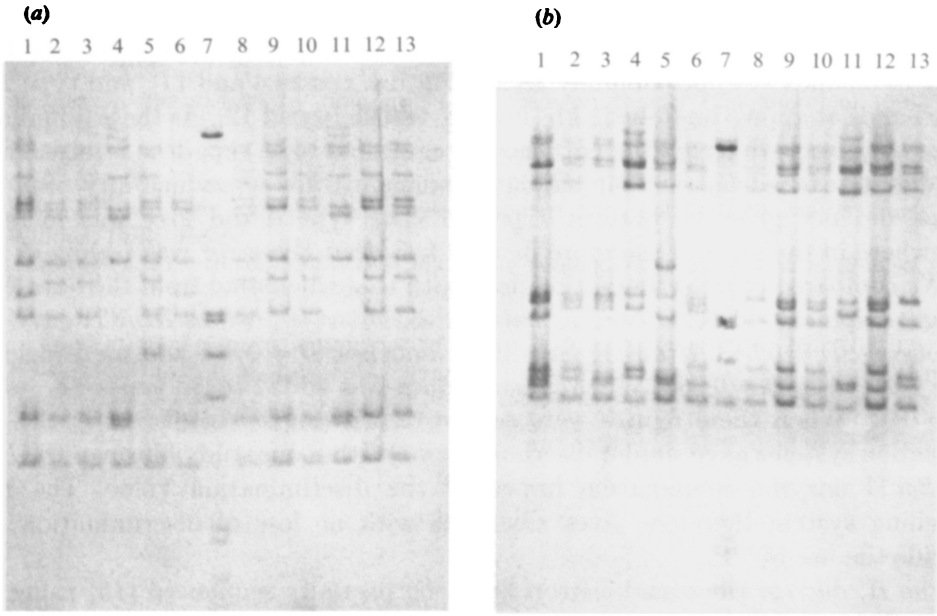

Fig. 1. Probed membrane containing all the patterns observed with (*a*) *Bst*E II and (*b*) *Bgl* II. *Bst*E II patterns designated 1–7. Tracks 1–6 and 8–13 on gel A are *Bst*E II types 3, 4, 4, 1, 7, 5, 4, 6, 2, 1, 2 and 4. *Bgl* II patterns designated 1–10. Tracks 1–6 and 8–13 on gel B are *Bgl* II types 3, 4, 5, 6, 7, 8, 10, 2, 2, 1, 2 and 9. Track 7 on both gels are DNA markers of 21226, 5148, 4973, 4268, 3530, 2027, 1904 and 1584 bps.

occasions. On the fourth occasion 11 strains gave visually identical patterns. The remaining strain gave the same *Bgl* II pattern but a different *Bst*E II pattern (type 5 rather than type 2) to that seen on the previous three occasion.

DISCUSSION

When examining typing systems for *H. ducreyi* an organism which will only grow on ill-defined media, variation in the expression of phenotypic characteristics may produce unstable systems. Systems that have been previously examined have made use of protein profiles [4] and enzymic activity [5] which are both dependent on modulation of protein expression. Indirect immunofluorescence [7] is dependent on expression of antigens, and lectin typing [6] on expression of cell wall carbohydrate moieties. Plasmids have been analysed [8], but their mobility between strains makes them an unstable characteristic. Ribotyping makes an ideal typing system based on chromosomal characteristics that are unaffected by growth conditions [9].

In this study strains of *H. ducreyi* were chosen from diverse geographical locations so as to challenge the system with strains that are unlikely to be related. We observed 16 different types using the *Bst*E II/*Bgl* II combination. The ribotypes were assigned according to the similarities or differences in the electrophoretic patterns (Fig. 1 *a*, *b*). For example in Figure 1 *a* tracks 2 and 3 have the same pattern and are assigned the same ribotype; tracks 1 and 4 are both different and are assigned to two further ribotypes. Types were designated *Bgl* II

https://doi.org/10.1017/S0950268800068229 Published online by Cambridge University Press

1–10 and *Bst*E II 1–7. *Bst*E II types 1 and 2 on some membranes could both be separated into two further types on the basis of the separation of two bands; type 1 by two bands at approximately 18 kb (Fig. 1*a*, tracks 4 and 11), and type 2 by two bands at approximately 21 kb (Fig. 1*a*, tracks 10 and 12). As these differences were not always detected the patterns were referred to as type 1 or 2 only. Of the 72 digests carried out in four batches to demonstrate reproducibility only one track did not give the pattern expected. The type it did give was included elsewhere in the gel and therefore it was likely that the error was technical.

We compared our discrimination data with that calculated from the data from 13 geographically diverse strains, given by Sarafian [10], where *Hin*c II gave two ribotypes, $D = 0{\cdot}513$, *Hin*d III gave five ribotypes, $D = 0{\cdot}808$ and used together five ribotypes, $D = 0{\cdot}808$. When plasmid data was added seven types were seen, $D = 0{\cdot}91$. When these figures were compared with our data (Table 1) the two detection systems gave similar discrimination. Within our study however the *Bst*E II/*Bgl* II enzyme combination improved the discrimination value. The DIG labelling system therefore gives ribotypes with no loss of discrimination and avoids the use of ^{32}P.

The *H. ducreyi* ribosomal cistron has been partially sequenced [15] using the type strain (NCTC 10945), 1498 bases on the 16S gene and 456 bases on 23S gene. This data indicates at least one cutting site for each of the restriction enzymes used to produce ribotypes in this study. Frequent cutters such as *Ava* II with five sites indicated yield low discrimination whereas less frequent cutters such as *Bgl* II with one site indicated give a higher discrimination.

This typing scheme should be capable of determining whether *H. ducreyi* strains in the western world have been imported or are indigenous. Other investigations would include the examination of strains for possible correlations between antibiotic susceptibility and virulence.

Using the two restriction enzymes *Bst*E II and *Bgl* II to produce ribotypes we have defined at least 16 distinct types of *H. ducreyi*, and have demonstrated that this system gives good discrimination ($D = 0{\cdot}95$) and good reproducibility. The system is non-radioactive, gives a higher discrimination index than the radioactive system previously described [10] and may therefore prove a more powerful epidemiological tool in investigations into *H. ducreyi* and chancroid.

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