MICROBIAL CHARACTERISATION

Heterogeneity of human intestinal spirochaetes demonstrated by one-dimensional polyacrylamide gel electrophoresis of proteins visualised by ³⁵S-methionine labelling and Coomassie Blue staining

S. P. BARRETT, J. J. M. HOLTON*, J. V. HOOKEY†, M. COSTAS‡, M. GANNER‡, R. MUNDY and D. J. M. WRIGHT§

Departments of Medical Microbiology, St Mary's Hospital Medical School, Norfolk Place, London W2 1PG, †University College London Medical School, 67–73 Riding House Street, London W1P 7PN, ‡Virus Reference Division and §National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT and §Department of Medical Microbiology, Charing Cross and Westminster Medical School, Charing Cross Hospital, St Dunstan's Road, London W6 8RP

The relatedness of strains of a human intestinal spirochaete was investigated by comparison of electrophoretic protein profiles produced by Coomassie Blue staining of proteins separated by polyacrylamide gel electrophoresis (PAGE) of lysed organisms and by examination of autoradiographs following PAGE of lysed ³⁵S-methionine-labelled organisms. A wide diversity of strains was revealed by both techniques but clustering of strains was different by the two methods. These findings support the view that the human intestinal spirochaetes comprise a group of bacteria of considerable heterogeneity.

Introduction

The human intestinal spirochaetes are a relatively poorly characterised group of bacteria. At least two distinct groups (taxa) exist but only one, *Brachyspira aalborgi* [1] which is generally accepted as a commensal, has been named. A second group of human intestinal spirochaetes, showing superficial similarities to the animal pathogen *Serpulina hyodysenteriae*, is found infrequently in the western hemisphere where reports have often associated it with intestinal disorders, particularly in male homosexuals and patients with HIV infection [2–5].

This second organism appears much more common in populations of the developing world where association with disease has been less frequently suggested [6, 7]. Informal names such as '*Serpulina jonesii*', '*Anguillina coli*', '*Anguillina jonesii*' [8] and HIS [9] have been applied to strains in this general group. Isolates of this human intestinal spirochaete have closely similar macroscopic and microscopic morphology, and give similar reactions in standard biochemical identification tests [4, 6, 10]. However, on the basis of multilocus enzyme electrophoresis (MEE), Lee and Hampson [8] demonstrated considerable diversity

among apparently similar spirochaetes. The relatedness of human intestinal spirochaetes was investigated by two related but independent methods: SDS-PAGE of lysed bacterial cells followed by comparison of total protein profiles, and ³⁵S-methionine labelling of organisms followed by SDS-PAGE and autoradiography.

Materials and methods

Isolates

Most strains were collected from inhabitants of the Muscat area of the Sultanate of Oman [6]. A few were obtained from homosexual men in the UK during 1994; others were isolated in 1987 from a mixed group of Asian subjects returning from the Indian subcontinent and from homosexuals in the UK. Two isolates from Australian Aborigines were kindly provided by Dr D. Hampson (Murdoch University, Australia) who also provided isolates of *S. hyodysenteriae* (strain B7B) and *S. innocens* (strain B256); the type strain of *B. aalborgi* (NCTC 11492) and a reference strain of *Treponema denticola* (ATCC 33520) were also included for comparison. Strains of human intestinal spirochaetes had the following characteristics: typical cultural morphology when grown anaerobically on blood agar, typical appearance on darkground or phase-contrast microscopy and a characteristic profile in the API-ZYM system (API-bioMérieux, 69280 Marcy-l'Etoile,

France): strongly positive in the test for production of β -galactosidase; weakly positive for α -galactosidase and esterases C4 and C8; negative in other enzyme tests. Thirty-one isolates were examined by both methods, 11 by Coomassie Blue-stained PAGE only and 45 by ^{35}S -methionine autoradiography only.

Coomassie Blue-stained one-dimensional SDS-PAGE examination

Isolates stored in glycerol broth 15% v/v at -70°C were plated on to Columbia base agar with horse blood 5% v/v and incubated anaerobically for 4 days at 37°C . Growth from a plate was used to inoculate Tryptone-soya broth (Oxoid), supplemented with horse serum 10% v/v, glucose 0.25% w/v and L-cysteine hydrochloride 0.05% w/v, that was incubated for 2 days at 37°C . The broth culture was centrifuged and the organisms were washed once by resuspension in 1 ml of de-ionised water. *B. aalborgi*, which grows only on solid medium, was suspended directly from blood agar in de-ionised water. About 20–40 μg wet wt of the resulting bacterial pellet was suspended in 50 μl of double-strength lysis buffer (glycerol 20% v/v, 2-mercaptoethanol 2% v/v, SDS 4% w/v stacking buffer 70%). Cell debris was removed by centrifugation, and 6 μl of tracking dye (bromophenol blue 0.001% w/v) was added to the supernate. Samples were stored at -70°C . Each strain was electrophoresed in duplicate on an SDS 10% polyacrylamide gel. Full details of the electrophoretic operating conditions have been described previously [11]. Gels were stained and fixed overnight in a solution containing Coomassie Brilliant Blue R-250 0.1% w/v, methanol 25% v/v and acetic acid 10% v/v. Destaining was continued in the same solution, but without Coomassie Blue, until the background was clear and the gels were dried between dialysis-membrane sheets.

Dried gels were scanned with an LKB Ultrosan XL laser densitometer (Pharmacia-LKB Biotechnology, Sweden). A programme package was used to calculate the similarities of all pairs of traces as the Pearson product moment correlation coefficient and these were clustered by the method of unweighted pair group average linkage (UPGMA) to provide a relationship dendrogram.

^{35}S -methionine autoradiography following one-dimensional SDS-PAGE

Details of the composition of reagents used for ^{35}S -methionine labelling have been given elsewhere [12]. Briefly, isolates were stored and cultured anaerobically as above. Growth from the agar surface was transferred into sterile saline and pelleted by centrifugation; bacteria were then labelled by resuspension in 50 μl of labelling medium containing 10 μCi ^{35}S -methionine (Amersham). After incubation for 2 h at 37°C , labelling was stopped by the addition of 50 μl of lysis buffer

containing SDS 20%. The lysate was boiled in a water bath for 2 min. Cell debris was removed by centrifugation for 1 min in a MicroCentaur MSE centrifuge at high speed and 6 μl of supernate was loaded into each well of the sample applicator. Each strain was electrophoresed in duplicate in the Ambis system [13] with SDS-polyacrylamide 12.5%. After drying in an Ambis vertical drier the gels were scanned by an Ambis Mk2 radioanalytic imaging system. Full details of the operating conditions have been given elsewhere [12]. Pearson product correlation coefficients were calculated and clustered by UPGMA as above.

Results

Dendograms of the similarity of protein profiles of isolates obtained by Coomassie Blue staining (CB) (Fig. 1) or by ^{35}S -methionine labelling (SM) (Fig. 2) are shown. Reproducibility was 95% within individual gels and 90% between gels. Similarity levels as low as 50% are apparent by Coomassie Blue staining and of 68% with ^{35}S -methionine labelling. A degree of similarity between certain geographically related isolates is evident; thus, strains from Australia and from the UK (designations beginning with A and U, respectively) group with similarity levels $\geq 70\%$ (Fig. 1); most of the UK isolates from 1987 (strain designations beginning FT) group with a similarity level $\geq 80\%$ (Fig. 2).

The independence of the two methods in distinguishing strains is apparent on inspection of the different degrees of relatedness shown by specific pairs of isolates. For example, strains 23 and 74 showed c. 90% similarity by ^{35}S -methionine labelling but $\leq 50\%$ by Coomassie Blue staining; similar values were obtained for strains 39 and 91; conversely, strains 15 and 74 showed $>80\%$ similarity in both methods, as did strains 25 and 47.

Both methods showed that the control spirochaetes examined—*B. aalborgi* and *S. hyodysenteriae*—were widely separated from the main group of human intestinal spirochaetes.

Discussion

Koopman *et al.* [14] drew attention to the diversity of intestinal spirochaetes when comparing DNA-digest fragment patterns of isolates from man and from various animals. Not only was there a marked diversity of strains from different animal species, but also among isolates from any one host species including man. Lee and Hampson [8] further demonstrated by MEE the heterogeneity of human intestinal spirochaetes and found similarity levels below 50% which is in agreement with the results of the present study; they suggested that this finding might indicate the presence

Fig. 1. Relatedness dendrogram of human intestinal spirochaetes indicated by SDS-PAGE electrophoresis and visualisation by Coomassie Blue staining.

Fig. 2. Relatedness dendrogram of ^{35}S -methionine labelled human intestinal spirochaetes indicated by SDS-PAGE electrophoresis and autoradiography.

of several species within the group. Our observations demonstrate that the heterogeneity of strains revealed by MEE is not peculiar to that method and we have also found that the relatedness of individual isolates differs according to the technique used. Like Lee and Hampson [8], we too have found the human intestinal spirochaetes to be more closely related to the strains of *S. hyodysenteriae* than to *B. aalborgi*; nevertheless, the work of Hookey *et al.* [9], in which 16S rDNA gene sequencing was used, indicated that the latter species were more closely related to each other than to human intestinal spirochaetes.

The use of CB and SM to compare different isolates of a species, including other gut-related organisms such as *Helicobacter pylori* and various coliforms, has not normally revealed diversity levels in excess of 25% [15, 16]. The heterogeneity of human intestinal spirochaetes in this and other studies seems unprecedented for strains which generally appear to be identical in routine laboratory tests; it suggests that intestinal spirochaetes may comprise several taxa.

Human intestinal spirochaetes do, in fact, exhibit a number of curious features, an explanation of which might be that they comprise several species. The major difference in geographic prevalence [6, 17, 18] is unparalleled in other commensal bacteria and the apparent decline in prevalence in western populations during the course of this century – whilst at the same time remaining common in the developing world – is unexplained [18, 19]. Furthermore, studies of the role of human intestinal spirochaetes in disease have produced conflicting evidence. Tompkins *et al.* [18] in the UK and Barrett [6] in Oman found no evidence of an association with gastrointestinal disease, whereas Lee and Hampson [7] found the organisms significantly more commonly in patients with abnormal stools. The clustering of isolates A, FT and U (Figs 1 and 2) suggests that related strains may be prevalent in different areas; thus, the disparate clinical findings may result from a diversity of phenotypes with some strains, yet to be clearly described, being more pathogenic than others. In this respect analogies may be drawn with strains of *H. pylori* which are of differing virulence and whose prevalence shows a variation linked to socioeconomic factors [20].

It is clear from this and other investigations that the superficial similarity of isolates of human intestinal spirochaetes conceals considerable diversity. An investigation of this diversity may lead to the recognition of virulent strains and other taxa.

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