Epidem Inf. (1987), 99, 361–372
Printed in Great Britain

The isolation of fusobacteria from tropical ulcers

By B. ADRIAANS and B. S. DRASAR

London School of Hygiene and Tropical Medicine, University of London, Keppel Street, London WC1E 7HT

(*Accepted 11 June 1987*)

SUMMARY

Tropical ulcer is a disease found most commonly in the tropics, although the disease is not confined to those areas. Young children are affected most frequently and the disease is thought to be a polymicrobial infection with fusobacteria, aerobic microorganisms and spirochaetes each playing a role. The fusobacteria have been reported on smears but have not been cultured thus far. We report the isolation and culture of these organisms from 46 ulcers and have also demonstrated the same organisms in skin biopsies from these lesions.

INTRODUCTION

Tropical ulcer is a form of skin ulceration of unknown aetiology predominantly affecting children. However, in many cases the disease seems to follow trauma at the site of the lesion followed by the development of dermal necrosis. Clinically the lesions are quite distinct, with ulceration usually occurring through the epidermis and dermis, very rarely extending to the subcutaneous tissues. Ulcers are found most commonly on the lower leg, although they may occasionally be found on the upper leg and the arm. The ulcers have a well-defined edge with some surrounding hyperpigmentation. The subsequent progress of the ulcers is rapid, with lesions a few centimetres in diameter developing within a few days. The clinical manifestations have been reviewed by Robinson & Hay (1985). Fusiform bacilli and spirochaetes have been observed in smears from these ulcers (Lowenthal, 1963; Lindner, 1968) but have been neither cultured nor characterized. They are thought to play a role in the pathogenesis of the disease (Lowenthal, 1963). We have investigated the bacteriology of tropical ulcers in 120 patients, with particular reference to the anaerobic flora.

MATERIALS AND METHODS

Patients and clinical findings

Patients with tropical ulcers were examined in areas where such ulcers are prevalent, namely Papua New Guinea, southern India, Zambia and Gambia. All patients were examined to see whether there was any underlying disease or medical abnormality predisposing them to the development of a tropical ulcer.

Table 1. *Media used in the isolation procedures*

1 Brain Heart Infusion agar (BHI) plates (Difco Laboratories, East Molesey, UK) supplemented with cysteine, haemin and vitamin K as described in the VPI manual (Holdeman, Moore & Cato, 1977). and also 10% horse blood. The medium (supplemented BHI agar) served as a general-purpose medium and for comparison of growth from the antibiotic-containing media.

2 Supplemented BHI agar with 10% horse blood and crystal violet 1:1000 for the isolation of fusobacteria (Ninomiya, 1972).

3 Supplemented BHI with rifampicin 7·5 μg/ml for isolating fusobacteria.

4 Supplemented BHI with kanamycin 1000 μg/ml and vancomycin 7·5 μg/ml for isolating bacteroides.

5 Supplemented BHI with 100 μg/ml nalidixic acid for anaerobic Gram-positive rods.

6 Columbia blood agar plates (Oxoid, Basingstoke, UK) with 10% horse blood for the isolation of aerobes.

7 MacConkey agar (Oxoid) for differentiating aerobic Gram-positive cocci and Gram-negative rods.

Bacteriological studies

Swabs taken from the edge and the base of the active lesions were placed in sterile ¼-strength Ringer's solution with 0·25% peptone. Each swab was thoroughly mixed in the solution, which was then aspirated with a needle and syringe and the air expelled. The mixture was inoculated into a pre-prepared anaerobically sterilized Hungate tube containing peptone–yeast–glucose broth (VPI manual, 1977). Details of the method have been reported previously (Adriaans *et al.* 1986). The sample was inoculated into the Hungate tube through the self-sealing butyl rubber stopper, thus maintaining the anaerobic conditions. Samples were maintained at room temperature for up to 6 weeks due to lack of storage facilities in the villages. Samples from the Hungate tubes were inoculated on to a set of culture plates (Table 1) in an anaerobic cabinet (Don Whitley, Shipley, UK).

Each sample was inoculated on to a set of plates in an anaerobic cabinet using a spiral plater system (Spiral Systems, Cincinnati, USA). After the plates were inoculated with the stylus, a non-selective plate was streak-inoculated with transport fluid, using a sterile plastic disposable loop (Don Whitley, Shipley, UK). A 5 μg disk of metronidazole (May and Baker, Dagenham, UK) was placed on each of the streak-inoculated plates. The anaerobic plates were incubated at 37 °C in an anaerobic cabinet filled with 85% nitrogen, 5% hydrogen and 10% carbon dioxide. Plates were incubated for up to 5 days.

The plates for aerobic incubation were transferred from the anaerobic cabinet to an incubator at 37 °C and supplied with 5–7% carbon dioxide. These plates were examined after 24 h and at 48 h.

All colonies growing in the anaerobic environment, sensitive to metronidazole or with no comparable growth on Columbia blood agar under aerobic conditions, were regarded as obligate anaerobes. These were picked and streaked for single colonies on to a BHI non-selective plate. Growth from these plates was sufficient for biochemical and chromatographic testing. Colonial morphology and Gram

staining characteristics were examined after 48 h growth and after 5 days growth on the non-selective BHI plate. All the anaerobic Gram-negative non-sporing rods were further tested with various antibiotics.

Isolation of spirochaetes

The media used in an attempt to isolate spirochaetes from the samples include those described by Kelly (1971), Leschine & Canale-Parola (1980) and Moore, Holdeman & Smibert (1984). All media were prepared anaerobically in Hungate tubes and autoclaved. Filter-sterilized supplements were added through the self-sealing rubber stoppers once the agar cooled. Rifampicin 7·5 μg/ml and polymixin B 8000 units per ml were initially added to all tubes. Tubes were incubated for up to 2 weeks, and individual colonies which had grown were picked out and subcultured in freshly prepared medium incorporating the same antibiotics.

Antibiotic resistance testing

This was carried out using the method previously described by Bennett & Duerden (1985). Antibiotic disks used included kanamycin 1000 μg per disk, vancomycin 5 μg/disk, rifampicin 7·5 μg/disk, penicillin G 2 U/disk, neomycin 1 mg/disk and phosphamycin 300 μg/disk (Duerden *et al.* 1976). The antibiotic concentrations used provided taxonomic identification and were not used for therapeutic evaluation.

Gas chromatography (GLC)

GLC was carried out using the method previously described by Holdeman & Moore (1977), using a Pye Unicam chromatograph series 204 (Philips, Cambridge, UK) equipped with a flame ionizer and 1·5 × 4 mm glass column packed with Chromosorb W (80–100 mesh). The temperature settings for the injection port, column and detector were 250 °C, 150 °C and 250 °C respectively. The flow rates of hydrogen, air and nitrogen were 44 ml/min, 400 ml/min and 40 ml/min respectively. The area under each peak produced on the graph was calculated by a computer (PU 4180 computing integrator, Philips) programmed to the chromatograph. Those organisms producing large amounts of butyric acid without any isobutyric or isovaleric acids were identified as fusobacteria and submitted for further studies.

Biochemical tests

The fusobacteria were tested for oxidase, catalase, gas and extracellular DNAase production, as well as aesculin hydrolysis, casein digestion and nitrate and nitrite reduction using the methods described by Bennett & Duerden (1985). Carbohydrate fermentation and hydrogen sulphide production were tested using the API 20A kit (API Laboratories, Montalieu, Vercieu, France). A BHI purity plate was inoculated at the same time.

Cell-wall electrophoresis

The fusobacteria were grown for 48 h in a Brain Heart Infusion Broth supplemented with 0·01% calcium carbonate. A strain of *Bacteroides fragilis* isolated from an ulcer and a reference strain of *Fusobacterium varium* (VPI 0499)

and *F. rusii* (VPI 0307) were similarly prepared. A strain of *Streptococcus faecalis* grown in the same medium but with added 0·08% Tween 80 served as the internal reference strain. Cell-wall electrophoresis was performed as described by Moore *et al.* (1980) using an 8·5% resolving gel.

Electron microscopy studies

The individual fusobacterial colonies were subcultured on three different media, namely Supplemented Brain Heart Infusion agar with 10% horse blood, Fastidious Anaerobe agar (Lab M, Salford, UK) with 10% horse blood and Wilkins Chalgren agar (Oxoid) with 10% horse blood. The colonies were collected from the plates with a sterile loop, fixed in 3% glutaraldehyde for 60 min, washed thrice over 24 h in 1 M cacodylate buffer and then fixed in 2% molten agar. Sections were post-fixed in 2% osmium tetroxide before dehydration and embedding. Biopsies of the ulcers carried out under local anaesthetic (1% lignocaine) were also prepared in the same way for electron microscopy.

RESULTS

We isolated a variety of anaerobes from these ulcers (Adriaans *et al.* 1987). All the anaerobes were isolated from ulcers of less than 6 weeks duration. The anaerobic non-sporing Gram-negative rods which produced large amounts of butyric acid but no isobutyric or isovaleric acid on GLC were regarded tentatively as fusobacteria and further characterized. The 46 fusobacteria isolated from the ulcers were all obligate anaerobes. They were all Gram-negative rods, although there was considerable morphological variation.

Morphology

There were two distinct morphological patterns. Group 1 fusobacteria formed large dome-shaped colonies which were cream-coloured initially but became more yellow in colour with age. No haemolysis was observed. The edge of the colony was smooth and the entire colony lifted easily off the plate. Group 2 fusobacteria formed smaller colonies which were more translucent. The colonies were flatter, and non-haemolytic.

Gram stain characteristics

All strains were Gram-negative rods. Group 1 were short, almost coccal in appearance, with slightly pointed ends (Fig. 1). The second group stained more irregularly and were very variable in shape and size. They often displayed a bulbous swelling in the centre of the rod and were much longer than the rods in the first group (Fig. 2).

Biochemical profiles of the fusobacteria isolated

These are presented in Table 2.

Electron microscopy

The morphology of the same organisms varied on different media, as is shown in the electron microscopic pictures (Figs. 3–5). Figure 3 shows the group 1

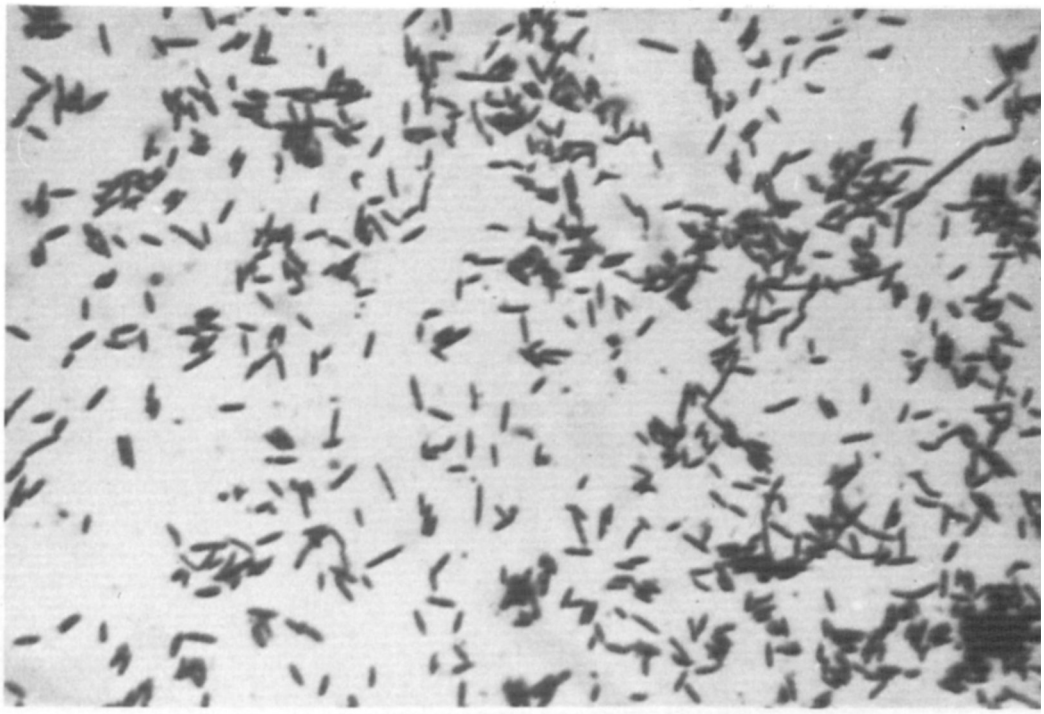

Fig. 1. Gram stain of group 1 fusobacteria showing long thin Gram-negative rods. Carbol fuchsin stain. × 1000.

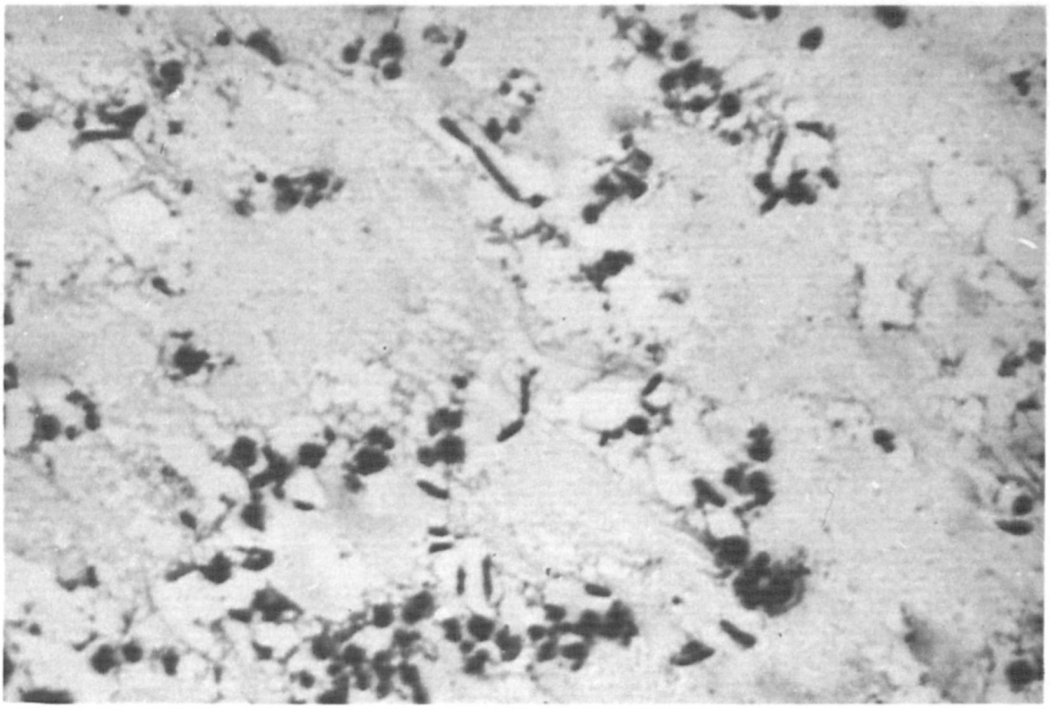

Fig. 2. Gram stain of group 2 fusobacteria showing much more irregular Gram-negative rods with bulbous swellings. Carbol fuchsin stain. × 1000.

https://doi.org/10.1017/S0950268800067844 Published online by Cambridge University Press

Table 2. *Some characteristics of the fusobacteria isolates*

	Group 1	Group 2
Oxidase test	—	—
Nitrate/nitrite test	—	—
DNAase production	—	—
Sugar fermentation	Glucose	Glucose and mannose
Aesculin hydrolysis	—	—
Catalase production	—	—
Casein hydrolysis	—	—
End products of metabolism	Acetic, propionic, butyric	Acetic, propionic, butyric
Indole	—	—
H_2S production	—	—
Growth on bile	++	—

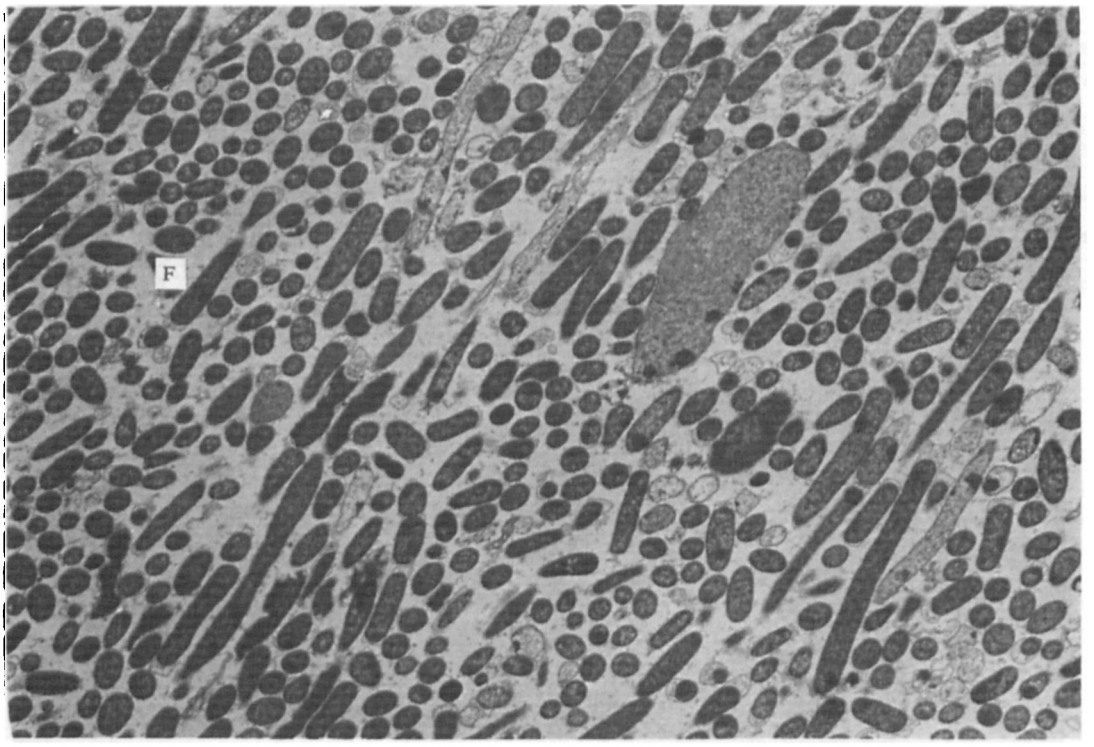

Fig. 3. Electron microscopy of group 1 fusobacteria on BHI medium. ×3200.

organisms on supplemented Brain Heart Infusion agar. The organisms are pleomorphic with rather pointed ends. The cell walls are intact and the cytoplasmic membrane is represented by a dark line surrounding the organism. Figure 4 shows the organisms grown on fastidious anaerobe agar, and Fig. 5 shows the same organisms grown on Wilkins Chalgren agar. In Fig. 5 they appear much more pleomorphic, with considerable variation in shape and also diameter. Group 2 fusobacteria showed similar variation on the different media. We saw identical pleomorphic bacteria in the dermis of skin biopsies from the ulcers (Fig. 6). These bacteria therefore are present not only on the surface of the ulcers but also in the deeper tissues.

https://doi.org/10.1017/S0950268800067844 Published online by Cambridge University Press

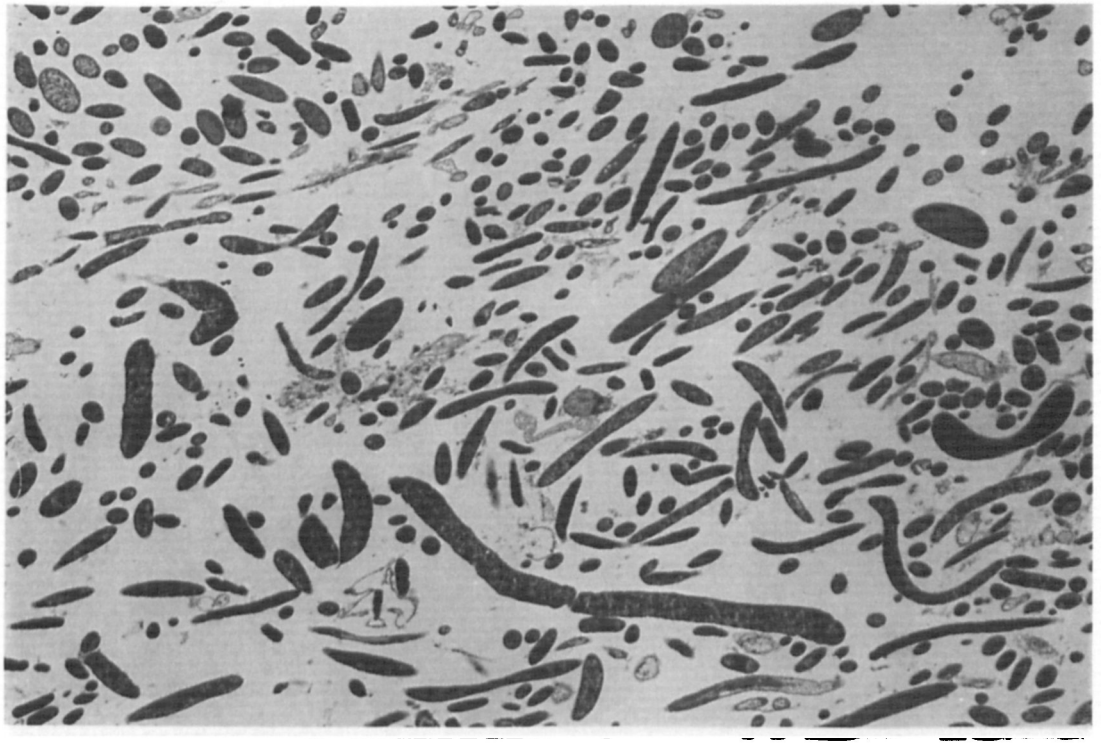

Fig. 4. Electron microscopy of group 1 fusobacteria on FABA medium. ×5000.

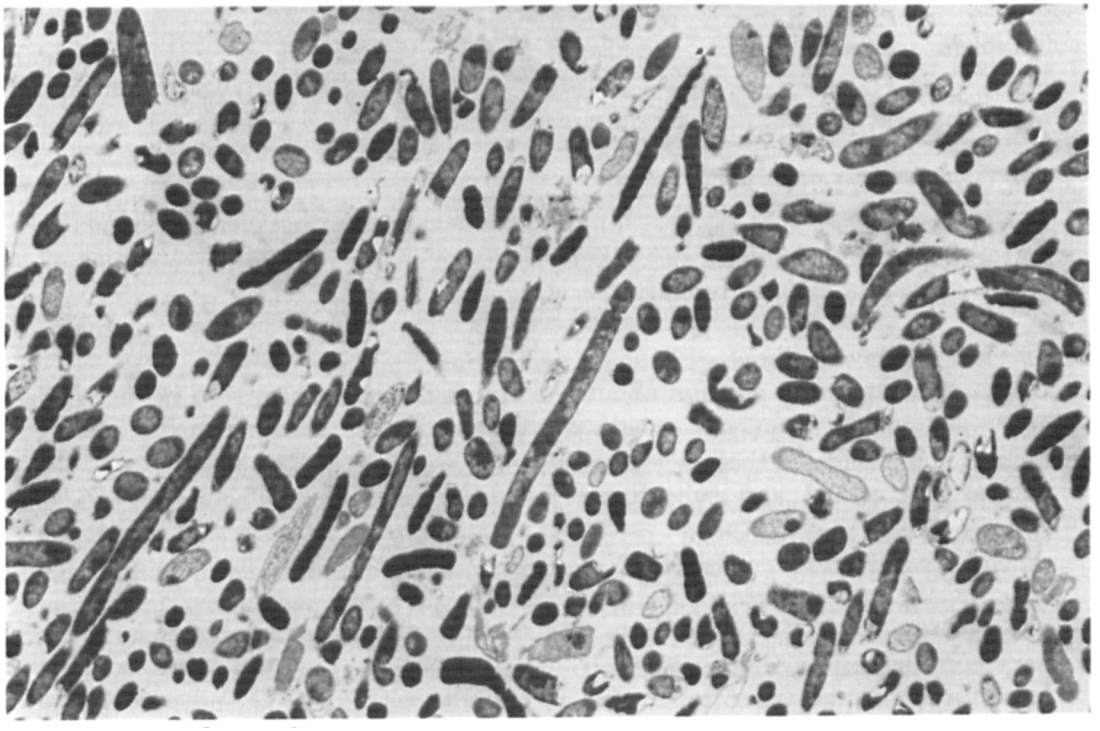

Fig. 5. Electron microscopy of group 1 fusobacteria on Wilkins Chalgren agar. ×5000.

https://doi.org/10.1017/S0950268800067844 Published online by Cambridge University Press

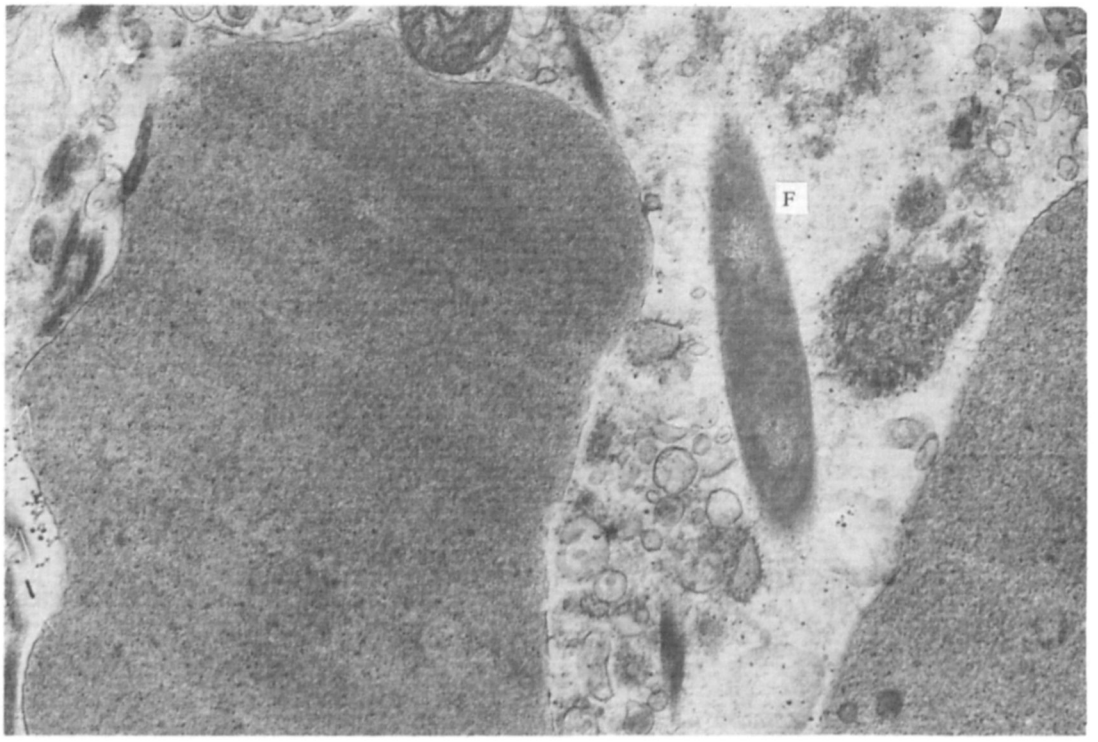

Fig. 6. Fusobacteria (F) in the dermis of a skin biopsy taken from a tropical ulcer. × 5000.

Antibiotic sensitivities

All the strains of fusobacteria isolated were sensitive to penicillin and phosphomycin and resistant to rifampicin.

Cell-wall electrophoresis

All strains belonging to group 1 and group 2 isolates showed identical bands on PAGE (Fig. 7). The patterns differ from those of the reference strains of fusobacteria and the bacteroides.

Spirochaete isolation

Several white fluffy colonies about 3–5 mm in diameter were seen in the NOS agar medium. No significant growth was obtained from the other media. Carbol fuchsin stains showed that they were indeed spirochaetes (Fig. 8). However, the culture was mixed and subcultures failed to yield a significant growth of spirochaetes in pure culture.

DISCUSSION

Fusobacteria are Gram-negative non-sporing obligate anaerobic rods which vary considerably in shape and size. They are normally found in the faeces, but have been cultured from the gingiva in patients with gingival and periodontal disease (Moore *et al.* 1984). The organisms have also been thought to play a role in cancrum oris, a mixed infection seen in malnourished children (Finegold, 1977).

https://doi.org/10.1017/S0950268800067844 Published online by Cambridge University Press

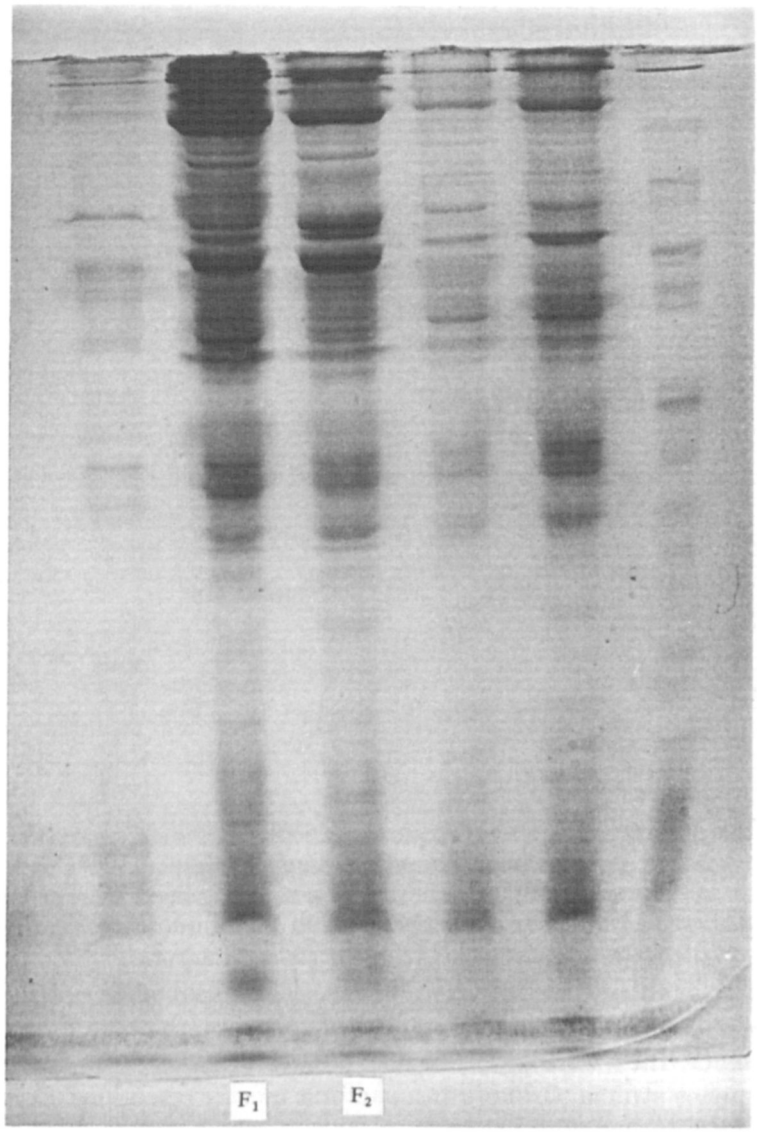

Fig. 7. PAGE of cell walls of both groups of fusobacteria.

To date there are several species recorded, classified mainly on morphology, biochemical tests, chromatography of end products of metabolism and sensitivity to antibiotics (Holdeman, Moore & Cato, 1977). The characteristic finding on gas–liquid chromatography is the production of *n*-butyric acid as their major end product of metabolism. More recently their cell walls have been characterized by electrophoresis (Moore *et al.* 1980).

Although reports on the bacteriology of tropical ulcers date back to the early part of this century (Clements, 1936), the fusiform bacilli seen on microscopy had not been cultured. Despite this, many authors have alluded to their role in the aetiology and pathogenesis of these ulcers (Loewenthal, 1963). Improved methods

https://doi.org/10.1017/S0950268800067844 Published online by Cambridge University Press

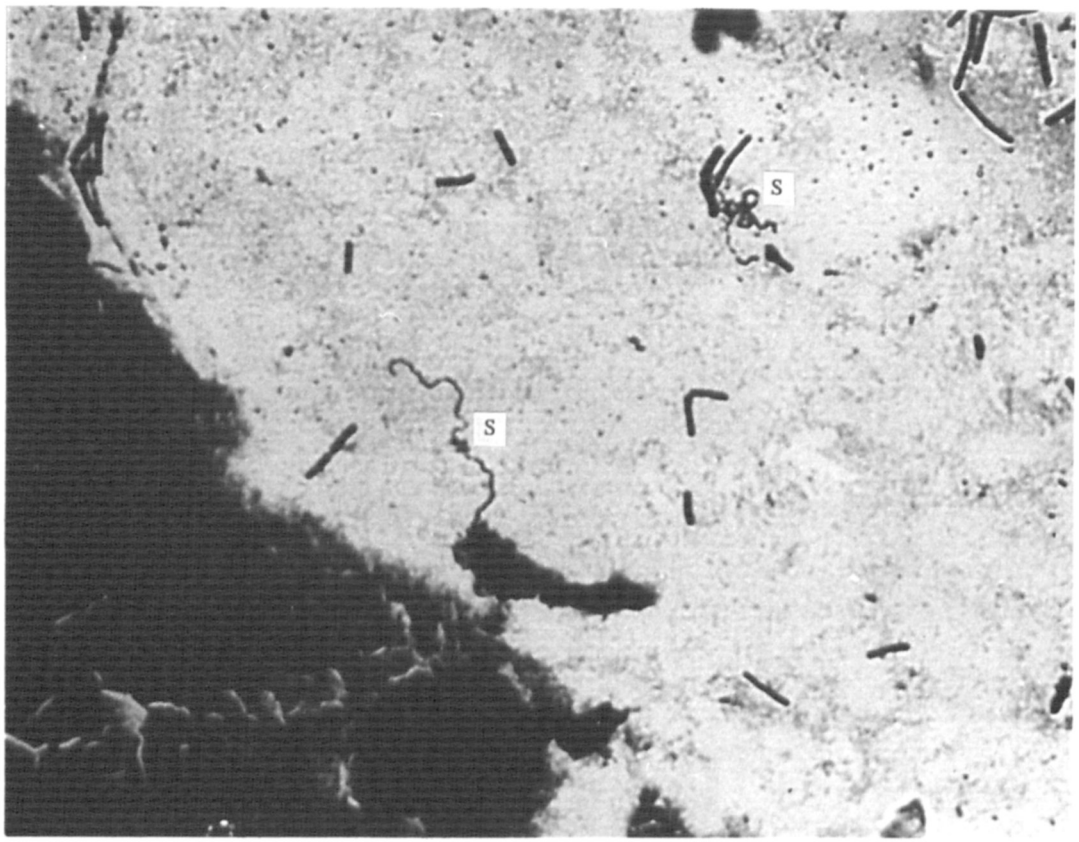

Fig. 8. Spirochaetes (S) in culture. Carbol fuchsin. × 1000.

of transportation of samples and better isolation techniques have contributed to the successful culture and characterization of these anaerobes.

We isolated fusobacteria from 46 ulcers. The fusobacteria fell into two categories. Morphologically the fusobacteria of group 1 resembled *F. varium* most closely. However, they were indole-negative, thus differing from the previously recorded reference strains. Group 2 fusobacteria mostly resembled *F. moriferum*. However, they were aesculin-negative, a feature which differs from previously recorded results of similar known strains of this species.

Cell-wall analysis by PAGE shows identical bands from all the strains isolated. Thus despite the variation in morphology, Gram-staining characteristics and biochemical tests, these are probably all the same species with individual variation. The cell-wall patterns do not correspond to any of the previous strains evaluated by this method (W. E. C. Moore, personal communication). Though these strains have been isolated from patients in various parts of the tropics, they are all very similar. That these bacteria have been isolated from the surface and have been identified in the dermis of the lesions strongly suggests tfhat they are not just commensals. Further, the repeated isolation of these organisms from several patients in the early phase of tropical ulcers also suggests that they are likely to be involved in the pathogenesis of the disease. The method of entry of the bacteria into the skin is not entirely clear, but it seems probable that organisms

enter after some form of trauma to the skin. The isolates are now being examined for virulence factors such as toxins that may explain their role in the aetiology and pathogenesis of tropical ulcer.

Tropical ulcer is probably a polymicrobial disease with fusobacteria and possibly other aerobic or facultative anaerobic organisms contributing to the infection. Similar fuso-spirochaetal infections have been described with footrot in sheep (Roberts & Egerton, 1977) and Vincent's infection of the mouth (Finegold, 1977). In our patients spirochaetes were also identified on dark-field examination of the pus from the ulcers (25%) and were also cultured in three patients, although not in pure culture as yet as further subculture was not successful. Electron microscopy studies morphologically identified them as treponemes (Adriaans *et al.* 1987). The relationship of spirochaetes to the infection is currently under investigation.

We have isolated fusobacteria which differ morphologically, biochemically and on PAGE examination of their cell walls from previously recorded species of this genus. We consider these organisms to be representative of a previously undescribed species of the genus *Fusobacterium*. It seems likely that these bacteria play an essential part in the causation of tropical ulcers.

The authors would like to acknowledge the support of the Wellcome Trust for Dr Adriaans' work. Technical help and advice was kindly provided by Miss Z. Ahmet.

REFERENCES

Adriaans, B., Hay, R. J., Drasar, B. S. & Robinson, D. C. (1986). Anaerobic bacteria in tropical ulcer – the application of a new transport system for their isolation. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 793–794.

Adriaans, B., Hay, R. J., Lucas, S. & Robinson, D. C. (1987). The light and electron microscopic findings of tropical ulcers. *Journal of Clinical Pathology* (In the Press.)

Adriaans, B., Hay, R. J., Drasar, B. S. & Robinson, D. C. (1987). The infectious aetiology of tropical ulcer – a study of the role of anaerobic bacteria. *British Journal of Dermatology* **116**, 31–37.

Bennett, K. & Duerden, B. I. (1985). The identification of Fusobacteria in a routine diagnostic laboratory. *Journal of Applied Bacteriology* **59**, 171–181.

Clements, F. N. (1936). Tropical ulcer with special reference to its aetiology. *Medical Journal of Australia* **2**, 615–644.

Cowan, S. T. (1974). *Cowan & Steel's Manual for the Identification of Medical Bacteria*, 2nd edn, p. 167. London: Cambridge University Press.

Duerden, B., Holbrook, W. P., Collee, J. G. & Watt, B. (1976). The characterisation of clinically important gram-negative anaerobic bacilli by conventional bacteriological tests. *Journal of Applied Bacteriology* **40**, 163–188.

Finegold, S. M. (1977). *Anaerobic Bacteria in Human Disease*, p. 394. London and New York: Academic Press.

Holdeman, L. V., Moore, W. E. C. & Cato, E. (1977). *Anaerobe Laboratory Manual*, 4th edn. Blacksburg, VA: Virginia Polytechnic Institute and State University.

Kelly, R. (1971). Cultivation of *Borrelia hermsi*. *Science* **173**, 443–444.

Leschine, S. B. & Canale-Parola, E. (1980). Rifampicin as a selective agent for isolation of oral spirochaetes. *Journal of Clinical Microbiology* **12**, 792–795.

Lindner, R. R. & Adeniyi-Jones, C. (1952). The effect of metronidazole on tropical ulcers. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **62**, 712–715.

Lowenthal, L. J. (1963). Tropical phagedenic ulcer; a review. *International Review of Tropical Medicine* **2**, 267–291.

MOORE, W. E. C., HASH, D. E., HOLDEMAN, L. V. & CATO, E. P. (1980). Polyacrylamide slab gel electrophoresis of soluble proteins for studies of bacterial floras. *Applied and Environmental Microbiology* **39**, 900–907.

MOORE, W. E. C., HOLDEMAN, L. V., SMIBERT, R. M., CATO, E. P., BURMEISTER, J. A., PALCANIS, K. G. & RANNEY, R. R. (1984). Bacteriology of experimental gingivitis in children. *Infection and Immunity* **46**, 1–6.

NINOMIYA, K., OHTANI, F. & KOOSAKA, S. (1972). Simple and expedient methods of differentiation among Bacteroides, Sphaerophorus and Fusobacterium. *Japanese Journal of Medical Science and Biology* **25**, 63–73.

ROBERTS, D. S. & EGERTON, J. R. (1969). The aetiology and pathogenesis of ovine footrot. II. The pathogenic association of *Fusiformis nodosus* and *F. necrophorus*. *Journal of Comparative Pathology* **79**, 217–227.

ROBINSON, D. A. & HAY, R. J. (1985). Tropical ulcer in Zambia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **80**, 132–137.