RESTRICTION ENDONUCLEASE ANALYSIS OF CAMPYLOBACTER STRAINS WITH PARTICULAR REFERENCE TO *CAMPYLOBACTER FETUS* SS. *FETUS*

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SUMMARY. Forty-three strains of *Campylobacter fetus* ss. *fetus* isolated from sheep abortions in New Zealand, and reference strains of *C. fetus* ss. *fetus* (four), *C. fetus* ss. *venerealis* (two), *C. jejuni* (one) and *C. coli* (one) were examined by restriction endonuclease analysis with the enzymes *Bst*E II and *Xho* I. DNA fragment patterns of *C. fetus*, *C. jejuni* and *C. coli* differed strikingly from each other, but there were many similarities in the patterns obtained for all strains of *C. fetus* ss. *fetus* (47) and *C. fetus* ss. *venerealis* (three). The 43 local strains of *C. fetus* ss. *fetus* were clearly divided into four types. Two of these types produced patterns nearly identical with those of overseas reference strains.

INTRODUCTION

The genus *Campylobacter* has undergone several major changes of classification in the last 20 years but is at present strongly influenced by the following “approved names” (Skerman, McGowan and Sneath, 1980): *C. fetus*, with its subspecies *fetus* and *venerealis* (the subspecies *intestinalis* and *fetus*, respectively, of Smibert, 1978); *C. jejuni* and *C. coli*, which together represent Smibert’s (1978) *C. fetus* subspecies *jejuni*; and *C. sputorum*, with its subspecies *sputorum* and *bubulus*. However, doubts have been expressed about the present scheme (Karmali *et al.*, 1983) and a new species has recently been proposed (Gebhart *et al.*, 1983). Campylobacters are important in human and veterinary medicine. *C. jejuni* causes enteritis in man and animals (Prescott and Munroe, 1982) and abortion in sheep. *C. fetus* is associated with abortion and other reproductive disorders in cattle and sheep (Smibert, 1978) and also occasionally with human infections (Righter *et al.*, 1983). In New Zealand, *C. fetus* ss. *fetus* is an important cause of abortion in sheep (Gumbrell and Christiansen, 1982; Quinlivan and Jopp, 1982). Although the annual incidence of abortions associated with *C. fetus* ss. *fetus* varies, in every year some farms experience major outbreaks, sometimes affecting over one third of the animals in a flock. The reasons for the variation in annual incidence and for the sporadic occurrence of major outbreaks are not clear. Attempts to study the problem are hampered by the lack of an adequate typing system. Serological typing has been attempted, but only two broad serogroups could be confidently identified (Bird *et al.*, 1984).

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Restriction endonuclease analysis, a technique used widely for comparing and typing viruses (Robinson, Ellis and Balassu, 1982; McIntosh and Ignoffo, 1983), has recently been applied successfully to the typing of bacteria (Marshall, Wilton and Robinson, 1981; Kaper *et al.*, 1982). The technique consists of extraction of DNA from the organism, digestion of the DNA with a restriction endonuclease, and separation of the DNA fragments by agarose-gel electrophoresis. Restriction endonucleases recognise and cleave specific base-pair sequences and so digest a particular DNA strand into a unique set of fragments. Electrophoretic migration of the fragments produces a pattern of bands that may be used as a "fingerprint" to characterise the organism from which the DNA was extracted.

This study investigated the potential of restriction endonuclease analysis for distinguishing between different species of *Campylobacter* and for differentiating between strains of *C. fetus* ss. *fetus*.

MATERIALS AND METHODS

Bacteria. The reference strains of *Campylobacter* examined in this study are listed in the table. Wild strains of *C. fetus* ss. *fetus* were isolated from 43 sheep abortions occurring on 10 different farms over a period of 3 years. Strain C30 was an isolate of *C. fetus* ss. *venerealis* from a local bovine-infertility study. The identity of strain C30 and of the wild *C. fetus* ss. *fetus* strains was confirmed by standard biochemical tests (Véron and Chatelain, 1973).

Preparation of DNA. Strains were cultured on Columbia Agar (Oxoid Ltd, Basingstoke, Hants) containing 7% defibrinated sheep blood (Gibco, Penrose, Auckland, New Zealand) and Skirrow antibiotic supplement for *C. fetus* (Oxoid). Cultures were grown for 4–5 days at 37°C in a microaerophilic atmosphere produced by a gas generating kit (Oxoid) in an anaerobic jar without a catalyst. Approximately 0.2 g wet weight of cells was washed in 0.15 M phosphate-buffered saline, pH 7.3, and centrifuged at 3000 *g* for 20 min. This procedure was repeated twice to remove traces of culture medium. The final pellet was suspended in 1 ml of buffer consisting of 100 mM ethylenediamine tetra-acetic acid disodium salt (EDTA) and 100 mM Tris-HCl, pH 8.5. DNA was extracted by the method of Marshall *et al.* (1981). This consists of treating the cells with lysozyme, lysing them with sodium dodecyl sulphate in the presence of non-specific proteases, and extracting DNA from the lysis mixture with phenol:chloroform:isoamyl alcohol (25:24:1); the DNA extract is then subjected to exhaustive dialysis. The concentration of DNA in each extract was measured by fluorimetry (le Pecq and Paoletti, 1966) after adding RNAase (Type X-A, Sigma Chemical Company, Saint Louis, MO 63178, USA). The purity of each extract was assessed by measuring the ratio of UV absorbance at 260 nm to that at 280 nm.

Restriction endonuclease analysis. *Campylobacter* DNA (0.6 µg) was digested to completion with either of the two restriction enzymes *Xho* I or *Bst* E II (New England Biolabs, Beverly, MA 01915, USA) at the digestion temperature and with the buffer specified by the supplier. Gel electrophoresis was performed in horizontal slabs of 0.5% agarose (standard low *M_r*; Bio-Rad, Richmond, CA 94804, USA) immersed in a buffer consisting of 40 mM Tris-acetate, 5 mM sodium acetate, and 1 mM EDTA, pH 7.8. After electrophoresis at 1 V/cm for 16 h the gels were stained for 30 min in ethidium bromide 0.5 µg/ml. Stained gels were placed on an ultraviolet transilluminator (Ultraviolet Products, San Gabriel, CA 91778, USA) and the fragment patterns were photographed on Kodak Tri-X film by a 120 format plate camera with a Wratten 23A gelatin filter.

RESULTS

The mean and standard deviation of the 260 nm to 280 nm absorbance ratios of the DNA extracts was 1.94 ± 0.10 . The amount of DNA obtained from each extract was in the range 30–120 µg in a total volume of *c.* 2 ml.

FIG. 1.—Fragment patterns after *Bst*E II digestion of DNA from (1) *C. coli* (NH12607), (2) *C. jejuni* (NH12397), (3–5) *C. fetus* ss. *venerealis* (NH12399, NH11095, C30), (6–8) *C. fetus* ss. *fetus* (NH12398, NH11084, NH11214). Lane 9 contained uncut λ phage plus a *Hind* III digest of λ phage; the mol. wts of the fragments are indicated ($\times 10^6$).

The results of agarose-gel electrophoresis of restriction endonuclease digests are shown in figs. 1, 2 and 3. DNA extracts were always completely digested into discrete lines in the conditions used, and doubling the digestion time or the amount of restriction enzyme did not change any of the patterns. The diffuse spot at the low-mol.-wt end of each lane is due to the presence of RNA. To facilitate comparisons, strains whose patterns are most similar have been placed in adjacent

FIG. 2.—Fragment patterns after *Bst*E II digestion of DNA from strains of *C. fetus* ss. *fetus*; local (New Zealand) strains are designated types A-D: (1) NHI2398, (2) NCTC5850, (3) type A strain, (4) NHI1084, (5) type D strain, (6) type B strain, (7) type C strain, (8) NHI1214. Lane 9 contained uncut λ phage plus a *Hind* III digest of λ phage; the mol. wts of the fragments are indicated ($\times 10^6$).

lanes. Uncut λ phage together with a *Hind* III digest of λ phage have been placed in one lane of each gel to indicate the mol. wt of the campylobacter DNA fragments.

Fig. 1 shows a comparison of *Bst*E II digests of DNA from reference strains of *C. coli*, *C. jejuni*, *C. fetus* ss. *venerealis* and *C. fetus* ss. *fetus*. The patterns produced by the *C. coli* and *C. jejuni* strains were strikingly different from each other and from those of the *C. fetus* strains. In contrast, nearly half of the DNA bands were shared by all strains of the two subspecies of *C. fetus* (ss. *venerealis* and ss. *fetus*).

FIG. 3.—Fragment patterns after *Xho* I digestion of DNA from strains of *C. fetus* ss. *fetus*; local strains are designated types A-D: (1) NHI2398, (2) NCTC5850, (3) type A strain, (4) NHI1084, (5) type D strain, (6) type B strain, (7) type C strain, (8) NHI1214. Lane 9 contained uncut λ phage plus a *Hind* III digest of λ phage; the mol. wts of the fragments are indicated ($\times 10^6$).

Figs. 2 and 3 show *Bst*E II and *Xho* I digests of DNA from reference and local strains of *C. fetus* ss. *fetus*. Four restriction patterns were distinguished in the local strains of *C. fetus* ss. *fetus*. These have been designated types A-D and are shown in lanes 3, 5, 6 and 7 (figs. 2 and 3). All 43 local strains gave patterns identical to one or other of these four types and those that gave a particular pattern with *Bst*E II invariably produced a single pattern when digested with *Xho* I. There were 13 type-A strains, 24 type B, 5 type C, and 1 type D. No more than two different types were

isolated from any farm. In the single instance in which the organism was isolated in each of three successive years from the same farm, all isolates were of the same type. The four patterns produced by *Bst*E II DNA-digests from the local strains were easily distinguished from each other by the absence or the position of bands with mol. wts of $(10-11.5) \times 10^6$ (fig. 2). In the *Xho* I digests the distinctive differences were in the regions immediately above and below the two common bands centred on a mol. wt of 10×10^6 (fig. 3).

DNA patterns from the reference strains NHI2398 and NCTC5850 appeared identical (lanes 1 and 2, figs. 2 and 3) and were also closely similar to the patterns produced by the type-A local strains, although a slight difference was evident in the thickness of the band of mol. wt 15×10^6 in the *Bst*E II digests (lane 3, fig. 2). This slight difference was reproducible and was not an artefact. Reference strain NHI1084 produced a pattern nearly identical to that of the local type-D strain (lanes 4 and 5, figs. 2 and 3).

DISCUSSION

The 260 nm to 280 nm absorption ratios of the DNA extracts indicated that the DNA in this study was substantially free of protein contamination. The DNA concentrations were measured fluorimetrically because experience showed this to be more reliable than basing them on the absorption at 260 nm.

No difficulty was experienced in digesting these DNA extracts with the enzymes used. Digestion was invariably complete, though this is not apparent in the high mol.-wt regions of the illustrations because the films were given a generous exposure to reveal the low-mol.-wt fragments. After digestion with either of the two enzymes, the largest DNA fragments had mol. wts greater than that of λ phage (31.5×10^6). For such large fragments to be obtained it is important that the DNA has an even higher mol. wt before it is digested. Common techniques of DNA preparation such as ultrasonic disintegration, ethanol precipitation, spooling, and vigorous extraction were avoided as these generally cause extensive shearing of very high-mol.-wt DNA. No attempt was made to remove RNA from the extracts as it did not interfere with the technique.

The recognition of four *Campylobacter* spp., implicit in acceptance of the "approved names" (Skerman *et al.*, 1980), is based on phenotypic tests and supported by studies of the guanine plus cytosine content of the DNA and of DNA homology (Véron and Chatelain, 1973; Owen and Leaper, 1981; Belland and Trust, 1982). *C. fetus* ss. *fetus* and *C. fetus* ss. *venerealis* are distinguished by their different biochemical and ecological characters (Véron and Chatelain, 1973). It is informative to compare the DNA-fragment patterns of the various strains analysed in this study in the light of this classification. The definite differences in pattern produced by *Bst*E II digestion of DNA from the separate species *C. coli*, *C. jejuni* and *C. fetus* and the many regions of similarity in the patterns produced by all strains of the two subspecies *C. fetus* ss. *fetus* and *C. fetus* ss. *venerealis* agree well with the current classification system, although it must be admitted that *C. jejuni* and *C. coli* were represented by only one strain each (table). Digestion by the restriction enzyme *Xho* I (gel not shown) produced similar results. Restriction endonuclease analysis clearly has a useful role to play in classification of the genus *Campylobacter*.

TABLE
Reference strains of *Campylobacter* used in this study*

Organism	Strain number	Synonymous designation
<i>C. fetus</i> ss. <i>fetus</i>	NHI1214	
	NHI1084	CDCD1278
	NHI2398	NCTC10842, ATCC27374, CIP5396
	NCTC5850	
<i>C. fetus</i> ss. <i>venerealis</i>	NHI1095	
	NHI2399	NCTC10354, ATCC19438, CIP6829
<i>C. jejuni</i>	NHI2397	NCTC11351, CIP702
<i>C. coli</i>	NHI2607	NCTC11366, CIP7080

* Abbreviations: NHI, National Health Institute, Wellington, New Zealand; NCTC, National Collection of Type Cultures, London, England; ATCC, American Type Culture Collection, MD, USA; CDC, Centers for Disease Control, Atlanta, GA, USA; CIP, Collection of the Institute Pasteur, Paris, France.

It is difficult to compare quantitatively the results obtained by restriction endonuclease analysis with those obtained by DNA homology studies, because the two approaches are so different. However, it is interesting to note that although a number of differences are apparent between the patterns produced by the two reference strains *C. fetus* ss. *venerealis* NHI2399 and *C. fetus* ss. *fetus* NHI2398 (lanes 3 and 6, fig. 1), these two strains could not be distinguished by DNA homology under stringent conditions (Belland and Trust, 1982). Restriction endonuclease analysis thus appears to be a more sensitive technique than DNA homology for detecting differences in the genome of *C. fetus* strains. However, it is not possible on the basis of this present work to assess the biological significance of these differences.

Restriction endonuclease analysis revealed four different types of *C. fetus* ss. *fetus*. It also showed that reference strains isolated many years ago in other countries were very similar to two of the types associated with sheep abortion in New Zealand. The finding of a single restriction type in isolates obtained from one farm in three successive years indicated the stability of the restriction patterns through many generations. Typing by restriction endonuclease analysis is currently being used to investigate and improve serological typing systems of *C. fetus* ss. *fetus* and to study the epidemiology of sheep abortion in New Zealand.

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