Plasmid profiles of “Campylobacter upsaliensis” isolated from blood cultures and stools of paediatric patients

FERNANDA M. DA SILVA TATLEY, A. J. LASTOVICA† and L. M. STEYN

Department of Medical Microbiology, Medical School, University of Cape Town, Observatory 7925, Cape Town, South Africa and *Department of Medical Microbiology, Red Cross War Memorial Children’s Hospital, Rondebosch 7700, Cape Town, South Africa

Summary. Seventy-three clinical isolates of “Campylobacter upsaliensis” were screened for the presence of plasmids. Plasmid bands were found in 68 (93%) isolates, from which 14 plasmid types were identified. Type 5 was found only in blood-culture isolates, whereas types 7–14 were found only in faecal isolates. Plasmid-free isolates and the other plasmid profiles were present in both faecal and blood isolates. The reproducibility of these profiles was largely dependent on the method of plasmid isolation. The chloroform-phenol lysis method was the most efficient and reliable method of preparing plasmid DNA for plasmid profile analysis. The success of this method may be largely attributable to two factors: (1) the efficacy of cell lysis was independent of either an alkaline agent or of heat; (2) the inactivation of nucleases by the utilisation of chloroform-phenol to lyse the cells. Furthermore, this method of plasmid DNA preparation is ideally suited for use on clinical isolates, especially when rapid plasmid profile analysis may be required.

Introduction

The genus Campylobacter includes the well-recognised thermophilic species Campylobacter jejuni, C. coli and C. lari as well as a more unusual group of campylobacters, the catalase-negative or catalase-weak strains (CNW strains).1-4 These strains have been assigned to a new species,2,5 and the name “C. upsaliensis” has been proposed for them. Recently, it has been suggested that “C. upsaliensis” is a human pathogen.6-8 This was not surprising since other campylobacters such as C. jejuni and C. coli have been implicated in diarrhoeal disease.10-13 C. jejuni and C. coli, can be typed by the serotyping scheme of Penner et al.,13 however, there is no method of typing “C. upsaliensis”. The use of plasmid-profile analysis to type “C. upsaliensis” was considered by Owen and Hernandez.14 These authors compared the plasmid profile patterns of “C. upsaliensis” strains from patients inhabiting geographically distinct regions. Their findings suggest that there is an association of specific plasmid profiles with defined geographical regions. Goosens et al.9 characterised clinical isolates of “C. upsaliensis” by combining data acquired from protein electrophoretogram analysis, plasmid-profile analysis and phenotypic traits. These authors identified a plasmid-profile pattern which was present in 60-7% of all plasmid-containing strains. Nevertheless, despite the high percentage of these strains (89%) which contained plasmids, Goosens et al.9 were unable to correlate antibiotic resistance or other phenotypic markers with any of the plasmids identified. Plasmid-profile analysis has been of little value as an epidemiological tool in most Campylobacter species,15 primarily due to the variable and predominantly low frequency with which plasmid bands have been found in these strains.16-18 Since “C. upsaliensis” appears to differ from other campylobacters in this regard, plasmid-profile analysis may be a useful typing tool.

“C. upsaliensis” strains are frequently isolated from diarrhoea stools and blood cultures of patients at Red Cross War Memorial Children’s Hospital. In 1990 alone, the clinical isolates of “C. upsaliensis” accounted for 22% (114/527) of all campylobacter isolates. “C. upsaliensis” isolates are collected routinely as part of a survey to monitor the distribution of campylobacters and their contribution to disease either as a causative agent or as opportunistic pathogens. Samples included in this study were accumulated over a period of 5 years (1985–1990) and were not associated with specific outbreaks of disease.

The aims of the study were: (1) to determine the percentage of “C. upsaliensis” clinical isolates that contained plasmids; (2) as plasmids were present in a significant percentage of these strains, to determine the frequency with which each plasmid profile occurred; and (3) to evaluate the available methods for plasmid DNA extraction to determine the most reliable and expedient for use with “C. upsaliensis”.

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† Correspondence should be sent to Dr A. J. Lastovica.
Materials and methods

Bacterial strains, isolation and culture

The automatic Bactec 460 System was used to isolate "C. upsaliensis" from blood; blood was cultured in 6B, 16B or 7C bottles and examined daily. The 16 blood-culture isolates investigated in this study were accumulated between 1985 and 1989. The established protocol in our laboratory for the isolation of Campylobacter spp. from faeces has involved the use of antibiotic-containing blood-agar plates. However, in 1989, a new antibiotic-free filtration method was adopted, and for the first time we were able to isolate "C. upsaliensis" from diarrhoea stools; 57 clinical isolates of "C. upsaliensis" from faeces were used in this investigation. All isolates were grown under microaerophilic conditions (CO₂ 12%, air 88%, humidity 95%) at 37°C, on Tryptose Blood agar (Oxoid, CM233) containing lysed horse blood 5% v/v, for up to 4 days.

Identification

Clinical isolates were designated "C. upsaliensis" on the basis of the following criteria. None of the strains grew at 25°C but all grew at 37°C and most grew at 42°C. All isolates were motile, gram-negative, spiral or curved bacteria. Four isolates were very weakly catalase-positive, the rest were catalase-negative. All isolates were oxidase- and nitrate reductase-positive. Each isolate was hippurate- and H₂S-negative, and sensitive to nalidixic acid and cephalothin.

Serotyping

Serotyping on the basis of thermostable somatic (O) lipopolysaccharide antigens was performed with antisera to the 65 reference strains of C. jejuni and C. coli by the scheme of Penner et al. Clinical isolates were also tested against an additional 11 antisera to new serotypes not included in the scheme.

Antimicrobial susceptibilities

Antibiotic sensitivities of "C. upsaliensis" isolates were determined by the antibiotic disk diffusion method of Barber and Stokes to gentamicin 10 μg, amoxycillin 10 μg and erythromycin 15 μg disks (Oxoid).

Plasmid DNA extraction

Specimens were inoculated onto two plates of tryptose agar containing lysed horse blood 5% v/v and incubated for 48 h as previously described. Colonies from the primary inoculum were not used for plasmid DNA isolation. The remaining colonies were scraped off the plates and were suspended in the buffer appropriate to each method of plasmid DNA extraction. Plasmid DNA was isolated by three methods: (i) the chloroform-phenol method of Alter and Subramanian; (ii) the rapid lysis method of Kado and Liu; (iii) the alkaline lysis method of Birnboim and Doly. Since the method of plasmid DNA extraction of Alter and Subramanian is relatively unknown, a brief description follows. Bacterial cells were suspended in 500 μl of 10 mM Tris-HCl, pH 8.0, buffer, containing 1 mM EDTA and 1-25 mM NaCl. To each sample, 400 μl of chloroform/isoamylalcohol/phenol (24:1:25) previously equilibrated with 1 mM Tris-HCl, pH 7.8, followed by 1 mM NaCl, was added. The tubes were inverted until the two phases were mixed and were left on ice for 15 min. The phases were separated by centrifugation in a Beckman Microfuge at 12000 g for 15 minutes at 4°C. The aqueous layer was recovered and the plasmid DNA was precipitated with isopropanol (0.7 volume) at room temperature. The DNA pellet was dissolved in water and re-precipitated with 0.1 volume of 4 M LiCl and 2-5 volumes of absolute alcohol, either at -70°C for 30 min or at -20°C overnight.

Gel electrophoresis

Before electrophoresis, the DNA samples were incubated at room temperature in the presence of RNase (50 μg/ml; Boehringer Mannheim, Germany) in water for 30 min. The DNA was electrophoresed overnight at 1 V/cm through horizontal agarose gels (0.7%) in a 40 mM Tris-Acetate buffer, pH 8.0, containing 1 mM EDTA. At the end of the run, the gels were stained with ethidium bromide (50 pg/ml in water for 30 min) and were transilluminated with ultraviolet light and photographed (Ilford FP4 film).

Results

Serotyping

Serotyping results were not useful; most of the isolates did not react with any of the 76 antisera to C. jejuni or C. coli. Three isolates reacted strongly with the antiserum to serotype 028, a C. coli strain, indicating that antigenic determinants were shared by C. coli and "C. upsaliensis".

Antimicrobial susceptibilities

All specimens tested were sensitive to gentamicin and ampicillin. However, three faecal isolates were resistant to erythromycin.

Plasmid profiles of "C. upsaliensis" obtained by the method of Alter and Subramanian

Plasmid bands were present in 68 (93%) of the "C. upsaliensis" strains examined (figure A; table).
Plasmid-containing strains, included 15 of the 16 blood-culture isolates and 53 of the 57 faecal isolates.

Fourteen plasmid profiles (types 1–14) were identified in the plasmid-containing strains of C. upsaliensis. The most common plasmid profile identified among the blood-culture isolates was type 1 (five isolates); type 5 was represented by a single blood culture isolate. In contrast, types 7–14 were found only amongst faecal isolates; of these, type 9 occurred most frequently (17 isolates). Plasmid-free strains, as well as types 1–4 and 6 were found in both blood-culture and faecal isolates (table).

The plasmids identified ranged in size from 1.8 to >98 Mda (figure A; table). A 2.4-Mda plasmid band was common to 10 of the 14 plasmid-profile types (table). This was confirmed by Southern hybridisation (results not shown). Since this plasmid band occurred in 58 (85%) of the 68 plasmid-containing strains, it
Figure. Agarose gel electrophoresis of plasmid DNA from a representative sample of "C. upsaliensis" strains. Panel A: plasmid DNA isolated by the method of Alter and Subramanian. Panel B: plasmid DNA isolated by the method of Kado and Liu. Panel C: plasmid DNA isolated following the method of Birnboim and Doly. The numbers above each lane indicate the plasmid-profile type. M1 and M2 denote reference strains with covalently closed circular plasmids of known mol. wt: M1, E. coli NCTC 50192 (98.0, 42.0, 23.0 and 46 Mda) and M2, E. coli NCTC 50192 (32.0, 5.2, 3.5, 3.0, 2.2, 1.7, 1.5 and 1.2 Mda); M3 consists of a mixture of plasmids which have been characterised in our laboratory (46.0, 18.0, 15.0, 6.6, 5.5, 5.0, 2.7 and 1.8 Mda).

Table. Plasmid-profile types and sizes of "C. upsaliensis" strains

<table>
<thead>
<tr>
<th>Plasmid-profile types</th>
<th>Plasmid sizes (Mda)</th>
<th>Number of strains</th>
<th>Source of isolates</th>
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<tr>
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<td>24, 24, 0, 18, &gt; 98</td>
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<td>B/F</td>
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<td>14</td>
<td>18, 24, 0, 28, &gt; 98</td>
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Po, plasmid-free strains; B, blood-culture isolate; F, faecal isolate.

may be a useful plasmid marker. Other plasmid bands also observed in the 68 plasmid-containing strains were: a 1.8-Mda plasmid in 16 (23.5%), a 3.4-Mda plasmid in 2 (3%), a 4.0 Mda plasmid in 3 (4%), and a 8.0-Mda plasmid in 2 (3%), a 24.0-Mda plasmid in 41 (60.3%), a 28.0 Mda plasmid in 12 (17.6%), an ≈ 98.0-Mda plasmid in 19 (28%) and two plasmids > 98.0 Mda in 54 (79%). No association was found between plasmid profiles and antimicrobial resistance patterns. Two of the isolates which were resistant to erythromycin were plasmid free.

Plasmid profiles of "C. upsaliensis" obtained with other plasmid isolation methods

The figure (B) shows the plasmid profiles of some "C. upsaliensis" clinical isolates after plasmid DNA isolation by the method of Kado and Liu. The plasmids migrate as two groups, one fast and one slow. The faster migrating plasmid bands (1-4-8.0 Mda) were easily identifiable, though a significant proportion of each plasmid appears to be present in the corresponding relaxed (open) form. However, the slower migrating plasmid bands equivalent to those in panel A of the figure (24.0-98.0 Mda) were indistinct and were not resolved from the contaminating chromosomal DNA background.

A similar picture is seen with the plasmid profiles of "C. upsaliensis" obtained with the method of Birnboim and Doly (figure, C). In some clinical isolates (e.g., figure, C, lane 1) the slower migrating plasmid bands (> 24.0 Mda) were distinct, but generally these plasmid bands were obscured by the contaminating
fragmented DNA background. Furthermore, the faster migrating plasmid bands were more diffuse than the corresponding bands obtained by either of the other methods and appeared to be present in multiple conformational forms. Consequently, the plasmid profile types described in the table, were no longer recognisable.

Discussion

Plasmid bands were observed in 68 (93%) of the 73 clinical isolates of “C. upsaliensis” examined in this study, regardless of the method of plasmid DNA isolation. The figure is similar to the 87% reported by Owen and Hernandez14 for the prevalence of plasmids in isolates from geographically different regions. Goosens et al.6 also reported a similarly high percentage (90%) of plasmid-containing strains.

Fourteen plasmid profile types were identified among the plasmid-containing strains. Types 7-14 were present in 34 (64%) of the 53 faecal isolates but not in any of the blood-culture isolates. Except for type 5, which was obtained from a single blood-culture isolate, all other profile types were identified in both blood-culture and faecal isolates. Initially, when “C. upsaliensis” was isolated from blood cultures, the method of isolation of this species from faeces was not established in our laboratory. Thus, between 1985 and 1989 only blood-culture isolates were obtained for analysis. Furthermore, since no blood-culture and faecal isolates were obtained from the same patient during 1989 and 1990 we have not been able to compare isolates from different sources in the same patient. However, as suggested by Lastovica et al.,7 the finding of “C. upsaliensis” in blood cultures may reflect the opportunistic nature of this pathogen in man. Thus the bacteriæmias are most probably secondary to intestinal infection with the same organism.

The fact that the same plasmid profile was observed in isolates collected as much as 5 years apart suggests plasmid stability, though this supposition needs confirmation by analysis of sequential isolates from the same patient or during outbreaks. One of the blood-culture isolates of plasmid type 4 was obtained from a patient who absconded following hospitalisation. On re-admission of the patient, 4 days later, a second isolate of “C. upsaliensis” was obtained and shown to be plasmid free. Since only rehydration therapy had been administered to the patient in hospital, it was not possible to ascribe the change in plasmid profile to the effects of therapy. This case may indicate that the original infection was with multiple strains or that re-infection with another strain occurred.

Owen and Hernandez14 and Goosens et al.6 identified a small plasmid (2-6 Mda) that appears to be equivalent to the 2-4 Mda plasmid described in this study. This plasmid was present in 58 (85%) of the plasmid-containing isolates and may be a useful plasmid marker. As yet, no genetic markers, including antibiotic-resistance genes, have been assigned to any of the plasmids, i.e., the plasmids remain cryptic. This finding is in agreement with those of Taylor et al.23 and Pang et al.24 with other Campylobacter species. The size estimation of the slower migrating plasmid bands (> 240 Mda) from blood-culture and faecal isolates was ambiguous and inaccurate. Therefore, in the table, the sizes of the slower migrating plasmid bands are given as approximate values, because it was difficult to assess which, if any, of these plasmid bands were present in other conformations. The inability to give precise mol.-wt estimations of these plasmids is largely due to the inadequate resolving capacity of the horizontal agarose gel electrophoresis system, as small differences in electrophoretic mobility correspond to marked differences in size. Also, the absence of covalently closed circular markers > 98 Mda make it impossible to give accurate estimations. Vertical gel or pulse field electrophoresis may be useful in resolving large plasmids; however, our aim was to assess a simple method of plasmid-profile analysis. The plasmid isolation method of Alter and Subramanian20 and horizontal gel electrophoresis fulfils these criteria.

Differences in plasmid mol.-wt determinations became particularly apparent when we compared our profile types obtained with the method of Alter and Subramanian20 with those of Owen and Hernandez14 who used the method of Kado and Liu21 to isolate plasmid DNA from “C. upsaliensis”. Owen and Hernandez14 had included in their survey some of our blood-culture isolates (provided by Dr A. Lastovica), and although some of the plasmid profiles types were similar to the types that we determined, our plasmid mol.-wt estimations differed from theirs, e.g., a plasmid band which we identified to be of 2-4 Mda corresponded to the 2-6-Mda band of Owen and Hernandez.14 Although this is a relatively insignificant size difference (< 10%), sometimes the difference was as much as 40%. To test the possibility that these differences may have arisen from comparison of plasmids isolated by different methods, we re-isolated the plasmid DNA from some of our strains by the method described by Owen and Hernandez14 (see figure, B). The resolution of the higher mol.-wt bands remained poor. Also, the fast migrating group of plasmids was not as sharply defined, and a significant proportion of the plasmid bands were present in a nicked or linear form rather than in the covalently closed circular conformation. To determine if similar differences apply to other plasmid isolation methods, we re-isolated the plasmid DNA from the same strains by the method of Birnboim and Doly.22 The plasmid profiles obtained with this method are shown in the figure C. An inspection of these plasmid profiles reveals two major features: (1) that except for one isolate (figure, C, lane 1) the slower migrating plasmid bands were not visible; and (2) that in place of the 2-4-Mda plasmid band (figure, A), multiple unfocused plasmid bands were present. These bands may represent differing degrees of denaturation of the 2-4-Mda
plasmid. Hybridisation studies with the linear form of this plasmid confirms this possibility (data not shown).

The isolation and conversion of the covalently closed circular DNA to nicked open circular and linear conformation can complicate the profile analysis. The correct identification of these forms is especially important when more than one plasmid band is present as shown in panel A of the figure, lanes 6, 10 and 11; e.g., in lane 6 there are five plasmid bands of 1.8-5.5 Mda, yet only two of these bands are in the covalently closed circular form, one corresponding to 1.8 Mda and another to 2.4 Mda. The other bands are the linear or nicked forms of these plasmids. Repeated agarose gel electrophoresis on the stored DNA preparations confirmed the conversion to these forms. In our hands, the most reliable and suitable method of plasmid isolation from “C. upsaliensis” was that of Alter and Subramanian30 (figure, A). Though this procedure is not without limitations, there may be two reasons for its success: (1) the lysis procedure is far more rapid than with the other methods, and obviated exposure of the plasmids to the denaturing effect of an extreme alkaline environment or to heat; and (2) the use of phenol-chloroform in the lysis step may reduce nuclease attack and remove a significant proportion of proteins which may interfere with gel running conditions. Consistently, the faster migrating plasmid bands (1.8-8.0 Mda) were isolated predominantly in the covalently closed circular form. The slower migrating plasmid group (> 24.0 Mda) was readily identified and, when several high-mol.-wt species were present, each was resolved, despite significant chromosomal contamination.

It has been suggested that plasmid-profile analysis is of little value in the genus Campylobacter, mostly because of low plasmid carriage rates, especially in the clinically important organisms such as C. jejuni,14 but a recent report by Simor et al.25 on C. (Helicobacter) pylori indicates that the technique may be more useful than previously thought, if used in conjunction with other data. Owen and Hernandez14 also suggest that the technique may have some potential as a typing method for “C. upsaliensis”. The high prevalence of plasmids in this organism make it attractive for plasmid-profile analysis. Data presented here show that the plasmid DNA extraction procedure may influence plasmid-profile analysis.

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