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Characterisation of *Campylobacter concisus* strains from South Africa using Amplified Fragment Length Polymorphism (AFLP) profiling and a Genomospecies-specific Polymerase Chain Reaction (PCR) assay: Identification of novel genomospecies and correlation with clinical data

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Amplified Fragment Length Polymorphism (AFLP) profiling was used to evaluate the distribution of phenotypically indistinguishable, but genetically distinct, among *Campylobacter concisus* strains from South Africa. A Polymerase Chain Reaction (PCR) assay described for identifying strains belonging to Genomospecies 1 and 2 was applied in this study. Forty-seven *C. concisus* strains were studied in total, of which 42 were of South African origin. Forty of the South African isolates were assigned to the major existing genomospecies typified by the type strain of oral origin (GS1), and reference strains from bloody diarrhoea (GS2). Eighteen South African isolates were distributed in the GS1 cluster including two oral strains. Twenty-two faecal South African isolates clustered with reference GS2 strains. Two novel genomospecies (GS 5 and 6) were inferred by their AFLP profile characteristics. Use of an existing PCR assay first described for identification of GS1 and GS2 strains generally indicated that the tool was accurate, although the novel genomospecies described here yielded an amplicon in the GS2 assay. No consistent clinical pattern among the diarrhoea South African strains could be discerned. The study extends the known genetic diversity among *C. concisus,* elucidates the presence of multiple genomospecies in South Africa, and confirms for the first time an association of GS1 with diarrhoea as well as the utility (with caveats) of a PCR assay for identifying GS1 and GS2 strains.

Key words: AFLP, *Campylobacter concisus* strains, bloody diarrhoea, genomospecies, PCR.

INTRODUCTION

Campylobacter concisus was described in 1981 having been recovered from oral specimens of patients with periodontal disease (Tanner et al., 1981). Since this initial description, various studies have demonstrated it to be associated with cases of human gastroenteritis (Vandamme et al., 1989; Lastovica, 2006; Cornelius et

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al., 2012; Nielsen et al., 2012). In most of these investigations, the prevalence of *C. concisus* rivals that of the entero-pathogenic species *C. jejuni*, now well established as the most frequent bacterial cause of gastroenteritis worldwide (Olson et al., 2008). However, studies of *C. concisus* have been hampered since most culture methods used routinely are poorly suited for its recovery (Lastovica, 2006).

Although the use of more suitable methods (principally "the Cape Town protocol", or variants thereof) have frequently shown *C. concisus* to be prevalent in diarrhoea (Lastovica, 2006; Nielsen et al., 2012), its clinical significance is as yet unproven, since it has been shown to be as frequently detected in healthy stools as diarrhoea ones (Van Etterijck et al., 1996; Cornelius et al., 2012). One explanation for this apparent conundrum has been the characterisation of *C. concisus* as a highly diverse species-complex that comprises several genomospecies (GS): phenotypically indistinguishable, yet genetically distinct, species that may differ in their pathogenic potential (Vandamme et al., 1989; Aabenhus et al., 2005; Kalischuk and Inglis, 2011). The application of a high-resolution genotyping method, Amplified Fragment Length Polymorphism (AFLP) profiling (Aabenhus et al., 2005) identified four genomospecies. A subsequent study of some pathogenic characteristics of GS1 and GS2 strains revealed that GS1 strains (that include the type strain of oral origin) exhibited lower levels of epithelial invasion and translocation than GS2 isolates (Kalischuk and Inglis, 2011), an observation consistent with known roles for these factors in diarrhoeal disease.

At the Red Cross Children's hospital, the long-standing use of "The Cape Town Protocol" (Lastovica, 2006), which is well suited to the recovery of a wider range of Epsilonproteo-bacterial species (including the genera *Campylobacter, Arcobacter*and *Helicobacter*) has resulted in consistent reports of high prevalence of *C. concisus* in, particularly, paediatric diarrhoea cases (Lastovica, 2006). The genetic diversity of South African *C. concisus* strains has been demonstrated in a study using *Not*I– based macro-restriction profiling (Matsheka et al., 2002), but this method does not allow for the classification of strains into genomospecies. In this study, we examine a range of *C. concisus* isolates of South African origin using AFLP profiling, to obtain a snapshot of the distribution of GS in this locality. These results are also used as the basis for a study of the specificity of PCR assays originally designed to identify strains of GS1 and GS2 (Bastyns et al., 1995), but developed prior to the identification of additional genomospecies (Aabenhus et al., 2005).

MATERIALS AND METHODS

Bacterial strains

A total of 47 *C. concisus* isolates were studied (Table 1). Forty-two were field isolates from the Red Cross Children's Hospital in Cape Town that had been characterised previously by macro-restriction profiling (Matsheka et al., 2002). Clinical details, including stool white blood cell count and co-infections known, are also given in Table 1. Representatives of the four genomospecies, identified previously by AFLP (Aabenhus et al., 2005), included the type strain of oral origin (CCUG 13144^T: GS1), CCUG 19995 and 13826 (GS2), 7656 (GS3) and 13570 (GS4).

AFLP profiling

DNA was extracted from three-day old bacterial cultures, and subjected to AFLP profiling, as described previously (Aabenhus et al., 2005). In brief, ca. 625 ng of genomic DNA from each isolate was digested simultaneously with 1 U of MfeI and 1 U of BSpDI in NEB4 buffer (New England Biolabs Ltd.), in a total reaction volume of 20 µl. Ligation was performed directly in the restriction digestion by adding 1 U of T4 DNA ligase, 2 µl of 10X T4 DNA ligase buffer (USB Corporation, Cleveland, Ohio), 2 µM MfeI (5' AATTCCAAGAGCTCTCCAGTAC 3' and 5′ TAGTACTGGAGAGCTCTTGG 3) and 20 µM BSpDI adapters (5′-CAG GTA TCG CCG AGT TTA GAC A-3' and 5′-CGT GTC TAA ACT CGG CGA TAC-3') complementary to the respective restriction sites. The final volume was adjusted to 40 µl, and ligation was performed at 37°C for 3 h. The digestion-ligation was subsequently diluted by the addition of 960 µl of Milli-Q H2O. PCR was performed with 5 µl of the diluted digestion-ligation mixture. The PCR primer sequences were MfeI-F, synonymous with BGL2F-0 (5' GAG AGC TCT TGG AAT TG 3'; 6-carboxylfluorescein labeled at the 5' end); and BspDI, (5' GTG TAC TCT AGT CCG AT 3') (DNA Technology, Århus, Denmark). The number of cycles used was 25. AFLP fragments were detected with an ABI 377 automated sequencing machine (Amersham Biosciences AB, Uppsala, Sweden) as previously described (26) and processed with GeneScan, version 3.1 (Applied Biosystems, Foster City, Calif.). AFLP profiles were then exported, assimilated into a database and compared using the program Bionumerics 5.1 (Applied Maths, Kortrijk, Belgium). A dendrogram of inter-strain relationships was produced using the Dice coefficient and UPGMA clustering as described before (Aabenhus et al., 2005).

Genomospecies-specific PCR analysis

A subset of South African isolates, and type and reference strains of GS1 and GS2, was examined using the assay described by Bastyns et al. (1995). PCR amplification of the specific *C. concisus* 23S rRNA gene regions was performed as described by these authors, except that the two reverse primers were used independently (Kalischuk and Inglis, 2011). Briefly, genomic DNA was prepared from each isolate using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) as per manufacturer's instructions for Gram-negative bacteria. PCR reactions were prepared containing the following: 100 ng genomic DNA, 1.5 mM $MgCl_2$, 1 X PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 0.2 mg/ml BSA, 250 uM each dNTP, 200 µM MUC1 forward primer, 200 µM CON1 reverse primer or 200 µM CON2 reverse primer and 1.25 U AmplitaqTaq DNA polymerase (Applied Biosystems). PCR amplification was performed using a GeneAmp® PCR System 9600 thermo-cylcer with the following cycling conditions: 94°C for five minutes, followed by 30 cycles of 94°C for 60 s, 60°C for 60 s, 72°C for 60 s, followed by 72°C for 5 min. PCR amplicons were analyzed by a MCE-202 Multi-NA Microchip Electrophoresis System (Shimadzu Corporation, Kyoto, Japan) using standard operating procedures for on-chip mixing with the DNA 500 Reagent Kit (Shimadzu); detection was performed using SYBR Gold (Invitrogen Corporation, Carlsbad, CA, USA) and

Table 1. Strain details. All strains from South Africa unless otherwise stated. CCUG, Culture Collection of the University of Gothernburg, Sweden.

Strain no.	Clinical details	Patient sex	Patient age (months)	Co-isolations	Stool WBC	Stool RBC	AFLP Cluster / Genomospecies assignation	PCR – based identification
45.99	Persistent loose stools	M	12	None	0	0	1	1
15.99	HIV + diarrhoea	M	7	*Ascaris* spp.	2	0	1	1
38.99	Protein-losing enteropathy	M	13	None	ND	ND	1	
205.94	Bloody diarrhoea	F	21	None	1	2	1	
220.96	Dysentery	M	9	None	1	0	1	1
D9	Adult, dental	F	384	N/A	NA	NA	1	
61.99	Dysentery	M	12	*Shigella dysenteriae*	3	2	1	1
64.99	Dysentery	F	27	None	1	0	1	1
219.96	Chronic diarrhoea	F	11	None	0	0	1	
CCUG 13144[T]	Adult, gingivitis, USA	NA	NA	NA	NA	NA	1	1
115.99	Aplastic anaemia	M	3	None	0	ND	1	1
24.99	Fever, ventilated	F	40	None	0	0	1	1
389.96	Chronic diarrhoea	F	20	None	0	0	1	1
28.99	Dysentery	F	14	None	0	0	1	1
12.99	Recent history of worms	F	30	Not at time of examination	0	0	1	
204.93	Chronic diarrhoea	M	9	None	0	0	1	
26A	Adult, dental	M	336	NA	NA	NA	1	
396.96	Pneumonia, diarrhoea + vomiting	F	6	None	0	0	1	
312.98	Chronic diarrhoea	M	6	None	1	0	2	2
47.99	Gastroenteritis	M	15	None	0	0	2	
331.98	Bloody diarrhoea	F	18	None	1	1	2	
207.94	Chronic gastritis	M	3	None	0	0	2	
25.99	Chronic diarrhoea	M	19	*Ascaris* spp.	0	3	2	
20.93	Biliary atresia	M	6	None	1	0	2	
59.99	Chronic diarrhoea	F	9	None	1	0	2	
62.99	Chronic diarrhoea	F	13	None	0	0	2	
CCUG 19995	Faeces, patient with pyrexia & exanthema, Sweden	NA	NA	NA	NA	NA	2	2
318.98	HIV + bloody stools	M	21	None	1	2	2	
113.99	Prolonged diarrhoea	F	15	*Cryptosporidium* spp.	1	0	2	2
25.94	Microcytic anaemia	F	24	*Ascaris* spp.	1	0	2	
135.99	Malabsorption	F	6	*Shigella dysenteriae*	1	3	2	2
122.99	Dysentery	F	9	None	0	1	2	
130.99	Diarrhoea	M	9	None	2	0	2	
377.96	Dysentery	F	5	None	2	2	2	2
131.99	Dysentery	F	25	*Shigella flexneri*	3	3	2	2
140.99	Dysentery	F	17	None	ND	ND	2	2
35.99	Diarrhoea	F	11	*Cryptosporidium* spp.	0	0	2	

Table 1. Continued**.**

275.95	Rectal prolapse, diarrhoea	F	39	*Trichuris* spp.	0	0	2	2
104.93	Loose stools	F	29	None	1	0	2	2
316.98	Bloody diarrhoea	M	4	*Shigella flexneri*	3	1	2	2
305.98	Dysentery	F	17	None	1	0	2	
306.98	Hirschprung´s disease	M	19	None	1	0	2	
RH 13826	Bloody stool, Denmark	UN	324	UN	ND	ND	2	
RH 7656	Diarrhoea, insulin-dependant diabetes mellitus patient, Denmark	UN	540	UN	ND	ND	3	
RH 13570	Diarrhoea, patient with liver abscess, Denmark	UN	552	UN	ND	ND	4	
127.99	Chronic diarrhoea	M	7	None	0	0	5	2
393.96	Loose mucoid stools	F	5	None	1	0	6	2

RH, Rigshospitalet, Denmark; M, male; F, female; ND, not done; NA, not applicable; T, type strain; UN, unknown.

amplicon sizing was determined by comparison to a 25 bp DNA ladder (Invitrogen). Analysis of Multi-NA data was performed using the Multi-NA Viewer Software (Shimadzu).

RESULTS

AFLP typing of *C. concisus*

The AFLP profiles of the 47 isolates examined contained 2 – ca. 50 fluorescently-labelled fragments in the 50 – 500 bp detection range (Figure 1). Reproducibility was evaluated by examining 19 strains on different occasions and subsequent cluster analysis of the AFLP profiles (data not shown). The mean similarity (S-) values obtained between duplicates was 92.0%.

AFLP analysis revealed a high level of genetic diversity among the strains, with no two profiles alike. Cluster analysis of the profiles delineated six clusters at the 18.0% S-level (Figure 1). Clusters 1, 2, 4 and 5 contained the type and reference strains designated previously as Genomospecies 1-4, respectively. Clusters 3 and 6 were represented by single South African isolates (Lasto 127.99 and Lasto 393.96, respectively) with AFLP patterns distinct from those studied previously. South African strains representing GS 3 or 4 were not identified in this study. However, 17 isolates from the region were identified as *C. concisus* GS1, and 23 as GS2, on the basis of the AFLP cluster analysis.

Clinical data

With the exception of one (D9), all South African isolates were from faecal samples from children, 15 of which suffered from co-infections and/or other underlying conditions (Table 1). The adult dental isolate was confirmed as *C. concisus* GS1. We believe our study is the first to identify a GS1 strain (205.94) associated with bloody diarrhoea, a condition hitherto only associated with GS2 infection. However, no consistent clinical pattern among the diarrhoeal South African isolates of the four *C. concisus* genomospecies could be discerned (Table 1).

Genomospecies-specific PCR assay results

Twenty South African isolates and type and reference strains representing *C. concisus* GS1 (CCUG 13144^T) and GS2 (CCUG 19995) were examined with the genomospecies-specific assay described previously (Bastyns et al., 1995). Strains assigned to GS1 and GS2 on the basis of the cluster analysis of their AFLP profiles were similarly identified as GS1 and GS2 in the PCR assay (Table 1). However, two South African strains assigned to novel genomo-species 5 and 6 on the basis of their AFLP profiles (Lasto 127.99 and Lasto 393.96: Figure 1) yielded an amplicon in the GS2 PCR assay (Bastyns et al.,1995) (Table 1).

DISCUSSION

As with previous studies using AFLP (Aabenhus et al., 2005; Kalischuk and Inglis, 2011), PFGE (Matsheka et al., 2002) and Multi-Locus Sequence Typing (Miller et al.,

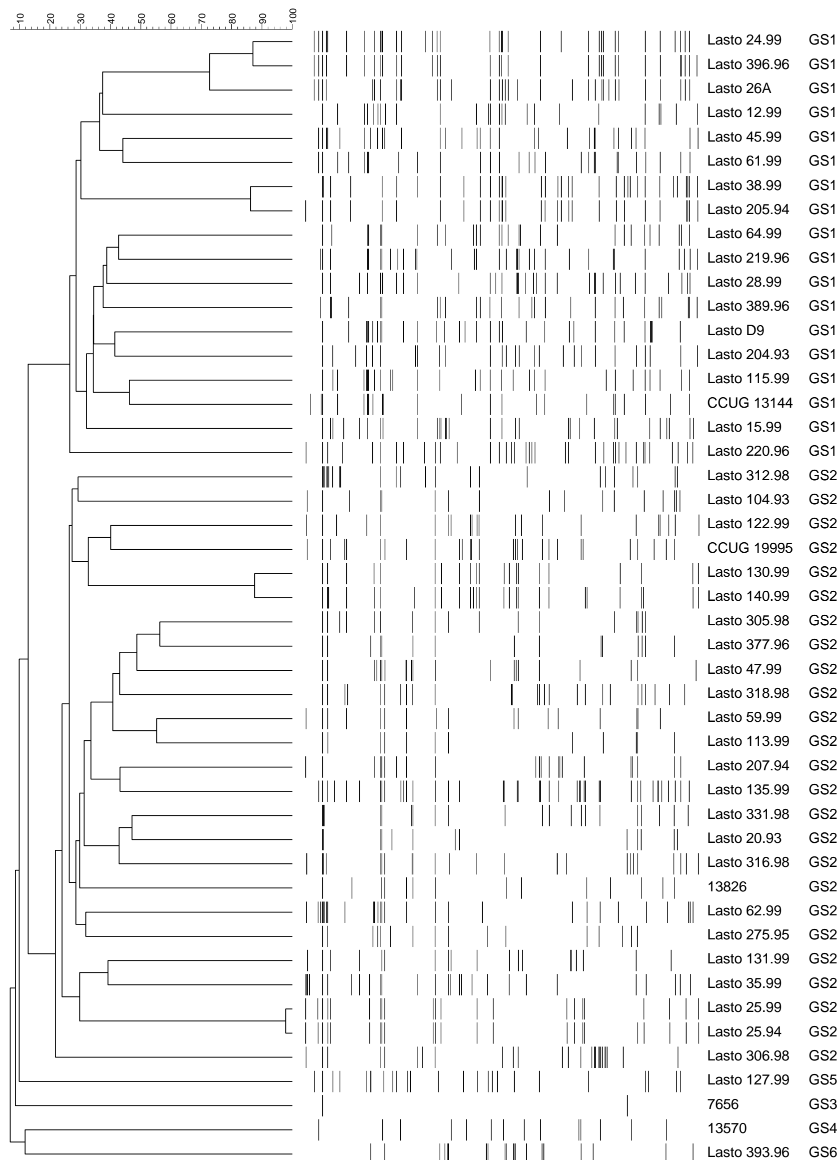

Figure 1. AFLP profile analysis of *C. concisus* strains studied. Six clusters are defined at the 18.0% S-level that correspond to known and novel genomospecies.

2012), the *C. concisus* strains examined in this study displayed a high genetic diversity, with each strain yielding a unique AFLP profile. Of the *Campylobacter* species for which DNA-based subtyping has been undertaken on appreciable numbers of strains, *C. concisus* appears to be the most polymorphic. Interestingly, despite the apparent lack of clonality as inferred in AFLP (Aabenhus et al., 2005; Kalischuk and Inglis, 2011; this study) or MLST analysis (Miller et al., 2012), the latter has not yet identified substantive recombination events between strains. Additional work is needed to resolve the population genetics of this intriguing species.

Despite the apparent lack of clonality between strains, groupings of patterns based around established genomospecies could be seen. As in previous studies (Aabenhus et al., 2005; Kalischuk and Inglis, 2011), two major groups were revealed, that corresponded to GS1 (that includes CCUG 13144, the type strain of oral origin) and GS2 (that includes reference strains CCUG 19995 and RH 13826 from human gastroenteritis). No appreciable similarity between South African isolates was obtained with reference profiles of GS3 (RH 7656) and GS4 (RH 13570; which was indicated by MLST to be *C. curvus:* Miller et al. (2012). On the other hand, two profiles were observed, that were highly distinctive and occupied well separated positions in the dendrogram, and potentially representing new *C. concisus* genomo-

species (GS 5 and 6 respectively). Additional work is, however, required to substantiate this suggestion.

Aabenhus and colleagues (2005) observed a statistically significant correlation between the prevalence of GS2 strains in immune-competent patients not infected with other gastrointestinal pathogens that, together with the finding that bloody stools were only found in cases of GS2 infection and the tendency for GS2 strains to be more frequently associated with fever exceeding 38°C, led the authors to propose that GS2 strains were more invasive and aggressive than GS1 isolates. Similar results were described in a Canadian study (Kalischuk and Inglis, 2011) in which AFLP cluster 2 strains (equivalent to our GS2 designation) demonstrated increased invasion and translocation abilities in *in vitro* assays, and were more frequently associated with diarrhoea, than AFLP cluster 1 (that included the oral type strain and equivalent to our GS1 designation) isolates. However, our results differ somewhat from the Danish and Canadian studies (Aabenhus et al., 2005; Kalischuk and Inglis, 2011), in both the study population demographic (where our focus was on paediatric cases) and the genomospecies associations. Although our oral isolates were identified as GS1, and three of four strains associated with bloody stools identified as GS2, we believe our study is the first to demonstrate *C. concisus* GS1 can be associated with bloody diarrhoea. Another important distinction between our study and that of Aabenhus and colleagues (2005) is the prevalence of co-infections among gastroenteritis cases containing GS1 or GS2. In the current study, other enteric pathogens (including *Shigella, Cryptosporidium* and *Ascaris* spp.) were detected in only 2 of 15 GS1 (13%) diarrhoea cases, but the corresponding data for GS2 diarrhoea cases was 8 of 23 (35%). Using a Fishers Exact Test, there is no significant difference between these groups (p=0.26). Elevated stool WBC counts are indicative of active infections, while those with raised RBC are used to diagnose conditions such as Crohn's Disease, ulcerative colitis and cancer. Again, the Fishers Exact Test revealed no statistically significant differences in our observations of elevated levels of either marker between GS1and GS2 infections. It should also be noted that technical and fiscal limitations in the present study precluded stool analysis for norovirus and rotavirus; however even with that limitation, our study indicates that the pathogenic potential of the major *C. concisus* genome-species remains complex. This is ratified by a recent study (Kaakoush et al., 2011), in which *C. concisus* strains containing a plasmid or the exotoxin 9 gene, demonstrated a higher invasive potential than those lacking this characteristic and were recovered only from patients with chronic gastrointestinal illness (including Crohn's Disease).

In addition, pathogenic characteristics including haemolytic activity, induction of DNA apoptosis, and toxin gene carriage have been determined in GS1 strains (Kalischuk and Inglis, 2011).

The fastidious nature, taxonomic complexity and lack of validated tools for identification of *C. concisus* genomospecies have significantly limited studies of this organism, despite various investigations showing it to be frequently associated with gastrointestinal illness. Recent developments including AFLP and MLST analyses have proven to be effective discriminatory tools, but are complex for routine use. For that reason, we performed a limited comparison of our AFLP results with those of a simple PCR described in 1995 to distinguish GS1 and GS2 isolates (Bastyns et al., 1995). We found an excellent correlation with *C. concisus* genomospecies identification between these two approaches, although the novel genome-species (GS5 and GS6) we describe would be identified as GS2 according to the PCR assay (Table 1). However, MLST analysis of the GS5 strain places it in a cluster dominated by known GS2 strains (Miller et al., 2012). This may indicate that the PCR assay correlates more accurately with more distant strain phylogenetic relationships than recent evolutionary events detected by those more finely resolved by AFLP. This is not entirely surprising, given that the PCR design was from 23S rRNA gene sequence data, an important phylogenetic marker. More work is needed to clarify this, but we believe our data validates the use of the simple PCR in further investigations by clinical laboratories aimed at clarifying the role and significance of the major genomospecies of this ubiquitous, yet poorly understood *Campylobacter*.

In conclusion, this study extends the known genetic diversity among *C. concisus,* elucidates the presence of multiple genomospecies in South Africa, and confirms for the first time an association of GS1 with diarrhoea as well as the utility (with caveats) of a PCR assay for identifying GS1 and GS2 strains. Given recent studies demonstrating *C. concisus* is present in well established vectors of disease, namely domestic pets (Petersen et al., 2007) and poultry and beef samples (Lynch et al., 2011), further studies of this organism are certainly warranted.

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