Journal of Medical Microbiology (2006), **55**, 331–334 **DOI** 10.1099/jmm.0.46373-0

Random amplified polymorphic DNA analysis of *Campylobacter jejuni* and *Campylobacter coli* isolated from healthy cattle and sheep

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Received 14 October 2005
Accepted 16 November 2005

The genetic heterogeneity among *Campylobacter jejuni* and *Campylobacter coli* isolates obtained from apparently healthy cattle and sheep was investigated by random amplified polymorphic DNA (RAPD) analysis. A total of 348 *Campylobacter* isolates, consisting of *C. jejuni* ($n=218$) and *C. coli* ($n=130$), were analysed. All these isolates were successfully typed by RAPD analysis. The total numbers of band patterns defined by RAPD in cattle and sheep were 42 and 45, respectively. Of the 42 distinct types obtained from cattle, 37 types were observed in *C. jejuni* isolates ($n=115$), and the remaining 5 were in *C. coli* isolates ($n=30$). Of 45 distinct types obtained from sheep, 21 types were observed in *C. jejuni* isolates ($n=103$), and 24 were in *C. coli* isolates ($n=100$). It was concluded that a high degree of heterogeneity existed among the *C. jejuni* and *C. coli* isolates of healthy cattle and sheep.

INTRODUCTION

Campylobacter jejuni and *Campylobacter coli* are aetiological agents that cause the highest frequency of acute bacterial diarrhoea worldwide, with an estimated 2·5 million people per year affected in the USA (Mead *et al.*, 1999). As thermophilic campylobacters are unable to grow in the environment, their reservoirs are the intestines of warm-blooded mammals and birds (Park *et al.*, 1991). The most important route of human *Campylobacter* infection is considered to be the consumption of contaminated poultry and poultry products (Nadeau *et al.*, 2002). However, the existence of *Campylobacter* species is well documented among many other animal species, including cattle and sheep (Stanley & Jones, 2003). The importance of cattle and sheep in campylobacteriosis is not just restricted to the contamination of milk at the farm and the carcass at slaughter, but also involves environmental and water contamination owing to the disposal of abattoir effluents and slurries to the land (Stanley & Jones, 2003).

The applicability of phenotypic methods for typing *Campylobacter* species is limited by the difficulty of obtaining standard antisera and phage reagents, and the lack of standardization of protocols between laboratories. In recent years, several genotypic methods have been described. One of the simplest and most cost-effective methods for the investigation of large numbers of isolates is the random amplified polymorphic DNA (RAPD) assay. This method is well recognized as a highly discriminatory tool for the molecular typing of a wide range of bacteria, including campylobacters, owing to the ability to determine polymorphisms in the entire bacterial genome. An RAPD assay that is based on the amplification of random DNA fragments using a single primer of arbitrary sequence was developed for *Campylobacter* spp. (Welsh & McClelland, 1990; Hernandez *et al.*, 1995), and has been reported to be of great asset in establishing genetic diversity among *C. jejuni* and *C. coli* isolates from different sources (Hilton *et al.*, 1997). RAPD analysis of *C. jejuni* and *C. coli* has frequently used 10-mer primers (Madden *et al.*, 1996; Ertas *et al.*, 2004).

While there are some reports about the genetic relationships among *C. jejuni* and *C. coli* strains of healthy cattle origin, information about the isolates of healthy sheep origin is limited. The objective of the present study was to investigate genetic heterogeneity among *C. jejuni* and *C. coli* isolates of healthy bovine and sheep origin, using an RAPD method.

Abbreviation: RAPD, random amplified polymorphic DNA.

METHODS

Bacterial isolates. A total of 348 *Campylobacter* isolates, consisting of 218 *C. jejuni* and 130 *C. coli*, were used in this study. All the isolates were grown on Preston *Campylobacter* broth and agar, and were identified by species-specific PCR (Açik & Çetinkaya, 2005). Of the 348 isolates, 145 were obtained from various samples from healthy cattle and the remaining 203 isolates originated from healthy sheep. Of the cattle isolates, 116 (115 *C. jejuni* and 1 *C. coli*) were obtained from gall bladders, 24 *C. coli* were obtained from faecal samples and 5 *C. coli* were from intestinal contents. Of the sheep isolates, 73 (42 *C. jejuni* and 31 *C. coli*) were obtained from gall bladders, 88 (53 *C. jejuni* and 35 *C. coli*) were from intestinal contents and 42 (8 *C. jejuni* and 34 *C. coli*) were from faecal samples. While gall bladder

and intestinal-content samples were collected from healthy cattle and sheep slaughtered at a local abattoir in the east of Turkey between July and September 2003, and between March and May 2004, faecal samples were collected from cattle at a local farm in the southeast of Turkey in October 2003, and from sheep of three different flocks in the east of the country in May 2004. Each individual animal was represented by only one sample; in other words, no more than one sample could be collected from the same cow or sheep. The geographical location of both cattle and sheep from which faecal samples were collected was different from those sampled for internal organs.

DNA extraction. A few representative colonies from pure cultures, which were identified as *C. jejuni* or *C. coli* by species-specific multiplex PCR, following inoculation of samples onto *Campylobacter* selective agar containing 7 % laked horse blood (SR0048C; Oxoid) and Preston *Campylobacter* selective supplement (SR117E; Oxoid) (Açik & Çetinkaya, 2005), were transferred into an Eppendorf tube containing 300 µl distilled water. The bacterial suspension was treated with 300 µl TNES buffer (20 mM Tris pH 8·0, 150 mM NaCl, 10 mM EDTA, 0·2 % SDS) and proteinase K (200 µg ml^{-1}), and was kept at 37 °C for 2 h. Following 10 min of boiling, an equal amount of phenol (saturated with Tris/HCl) was added to the suspension. The suspension was shaken vigorously by hand for 5 min and then centrifuged at 11 600 ***g*** for 10 min. The upper phase was carefully transferred into another Eppendorf tube and 3 M sodium acetate (0·1 volumes) and 95 % ethanol (2·5 volumes) were added to the suspension, which was left at −20 °C overnight to precipitate the DNA. The pellet, obtained following the centrifugation at high speed for 10 min, was washed twice with 90 and 70 % ethanol, respectively, each step was followed by 5 min centrifugation. Finally, the pellet was dried and resuspended in 50 µl distilled water.

RAPD analysis of *C. jejuni* and *C. coli* isolates. For the RAPD analysis of *C. jejuni* and *C. coli* isolates, the reaction mixture was prepared in a total volume of 50 µl, consisting of 5 µl DNA, 10 × PCR buffer (750 mM Tris/HCl, 200 mM $(NH_4)_2SO_4$, 0·1 % Tween 20), 3·5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 1·25 U *Taq* DNA polymerase (MBI Fermentas) and 1 µM OPA-11 primer (5′-CAA TCG CCG T-3′) (Hernandez *et al.*, 1995). The RAPD assay was performed in a thermal cycler with an initial denaturation step at 94 °C for 1 min, followed by 45 cycles at 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min, then, a last step of extension at 72 °C for 5 min. PCR products were separated by electrophoresis in 1·5 % (w/v) agarose gels and visualized by ethidium bromide staining. A 100 bp DNA ladder (MBI Fermentas; SM0321) molecular mass marker was used to evaluate the size of bands.

RESULTS AND DISCUSSION

This study was conducted to investigate strain variations among *C. jejuni* and *C. coli* isolates of healthy bovine and ovine origin, and for this purpose an RAPD assay, which is generally considered as the most advantageous assay in terms of discrimination ability, cost and ease, was used. In total, 87 different types (42 from cattle and 45 from sheep) were defined in this analysis of 348 *Campylobacter* isolates of various origins. In a previous study that employed the same primer, the number of RAPD types obtained in the examination of 208 samples was reported to be even higher (n=118) (Hernandez *et al.*, 1995). This might be expected as they were testing isolates from various sources, and in the current paper the isolates came from multiple sites in two ruminant species. Additionally, Nielsen *et al.* (2000) used a different primer and reported 56 RAPD types among the 80 strains examined. Subjective interpretation of the data, the choice of primers and type of samples, in addition to the variation in geographical locations, may play a role in these differences.

In the RAPD analysis of 115 *C. jejuni* isolates originating from the gall bladders of healthy cattle, 37 different types were obtained. Some of the RAPD types were represented by remarkably high percentages of isolates. For example, the most common types of *C. jejuni* were observed in 30 and 16 % of gall bladder isolates (Fig. 1). The other types were represented by less than 6 % of the isolates (Table 1).

In the RAPD analysis of 30 *C. coli* isolates originating from various samples from cattle, 5 different types were obtained. The most common type was represented by 40 % of intestinal-content samples and 33 % of faecal samples. All five different types were observed in faecal isolates. Four distinct types were obtained from intestinal isolates. The type observed from the gall bladder isolate was similar to one of the types obtained from faecal and intestinal-content isolates (Table 1). In the analysis, identical types were obtained from samples from different locations. The overall results suggest that the degree of heterogeneity among *Campylobacter* isolates from healthy cattle is relatively high, although some of the types of *Campylobacter* isolates were represented by a high percentage, and identical types of *C. coli* isolates were obtained from different locations. The fact that identical types were obtained from field and abattoir isolates suggests that there was no influence of location on the diversity in cattle.

Similar findings were also made for *C. jejuni* and *C. coli* isolates originating from intestinal contents, gall bladders

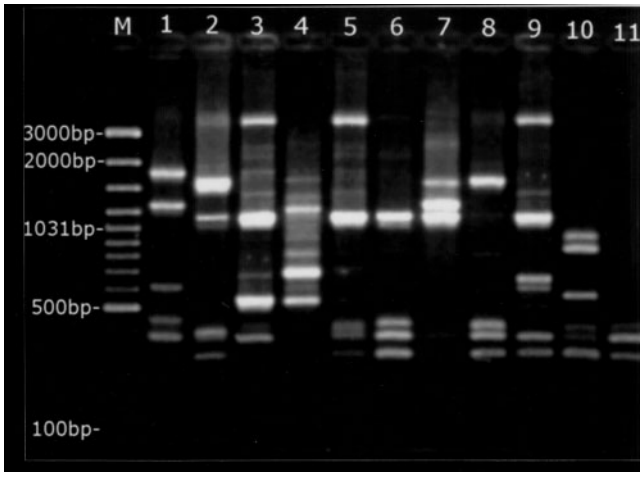

Fig. 1. RAPD types of *C. jejuni* isolates obtained from the gall bladder samples from cattle, using the OPA-11 primer. M, 100 bp molecular mass marker (MBI Fermentas; SM0321); lanes 1–11, different types (lane 6 represents the most frequent type).

Table 1. RAPD results of *C. jejuni* and *C. coli* isolates obtained from various specimens from cattle

Profile no.	Species	No. of isolates (%)	Source
1	*C. jejuni*	6 (5·2)	Gall bladder
2	*C. jejuni*	6 (5·2)	Gall bladder
3	*C. jejuni*	18 (15·7)	Gall bladder
4	*C. jejuni*	35 (30·4)	Gall bladder
5	*C. jejuni*	6 (5·2)	Gall bladder
6	*C. jejuni*	4 (3·5)	Gall bladder
7–8*	*C. jejuni*	6 (2·6)	Gall bladder
9	*C. jejuni*	4 (3·5)	Gall bladder
10–11†	*C. jejuni*	4 (1·7)	Gall bladder
12–37‡	*C. jejuni*	26 (0·9)	Gall bladder
38	*C. coli*	1 (100)	Gall bladder
		1 (20)	Intestinal content
		2 (8·3)	Faeces
39	*C. coli*	1 (20)	Intestinal content
		4 (16·7)	Faeces
40	*C. coli*	1 (20)	Intestinal content
		4 (16·7)	Faeces
41	*C. coli*	2 (40)	Intestinal content
		8 (33·3)	Faeces
42	*C. coli*	6 (25)	Faeces

*Each profile was represented by three isolates.
†Each profile was represented by two isolates.
‡Each profile was represented by one isolate.

and faecal samples of healthy sheep. In the RAPD analysis of 103 *C. jejuni* isolates originating from various samples from sheep, 21 different types were obtained (Table 2). Of these, nine types were obtained from gall bladders, ten from intestinal contents and two from faecal samples. The most common types were represented by 63 % of faecal samples, 26 % of gall bladders and 19 % of intestinal contents. Other types were detected at much lower percentages. In the analysis of 100 *C. coli* isolates originated from sheep, 24

Table 2. RAPD results of *C. jejuni* and *C. coli* isolates obtained from various specimens from sheep

Profile no.	Species	No. of isolates (%)	Source
1	*C. jejuni*	11 (26·2)	Gall bladder
2	*C. jejuni*	6 (14·3)	Gall bladder
3–4*	*C. jejuni*	6 (7·1)	Gall bladder
5	*C. jejuni*	6 (14·3)	Gall bladder
6–7†	*C. jejuni*	2 (2·4)	Gall bladder
8	*C. jejuni*	9 (21·4)	Gall bladder
9	*C. jejuni*	2 (4·8)	Gall bladder
10	*C. jejuni*	6 (11·3)	Intestinal content
11–12†	*C. jejuni*	6 (5·7)	Intestinal content
13	*C. jejuni*	4 (7·5)	Intestinal content
14–15‡	*C. jejuni*	4 (3·8)	Intestinal content
16	*C. jejuni*	5 (9·4)	Intestinal content
17	*C. jejuni*	8 (15·1)	Intestinal content
18	*C. jejuni*	10 (18·9)	Intestinal content
19	*C. jejuni*	10 (18·9)	Intestinal content
20	*C. jejuni*	5 (62·5)	Faeces
21	*C. jejuni*	3 (37·5)	Faeces
22	*C. coli*	8 (25·8)	Gall bladder
23	*C. coli*	15 (48·4)	Gall bladder
24–26‡	*C. coli*	6 (6·5)	Gall bladder
27–28†	*C. coli*	2 (3·2)	Gall bladder
29	*C. coli*	8 (22·9)	Intestinal content
30	*C. coli*	12 (34·3)	Intestinal content
31–33*	*C. coli*	9 (8·6)	Intestinal content
34–36‡	*C. coli*	6 (5·7)	Intestinal content
37–39‡	*C. coli*	6 (5·9)	Faeces
40	*C. coli*	10 (29·4)	Faeces
41	*C. coli*	3 (8·8)	Faeces
42	*C. coli*	12 (35·3)	Faeces
43–45†	*C. coli*	3 (2·9)	Faeces

*Each profile was represented by three isolates.
†Each profile was represented by one isolate.
‡Each profile was represented by two isolates.

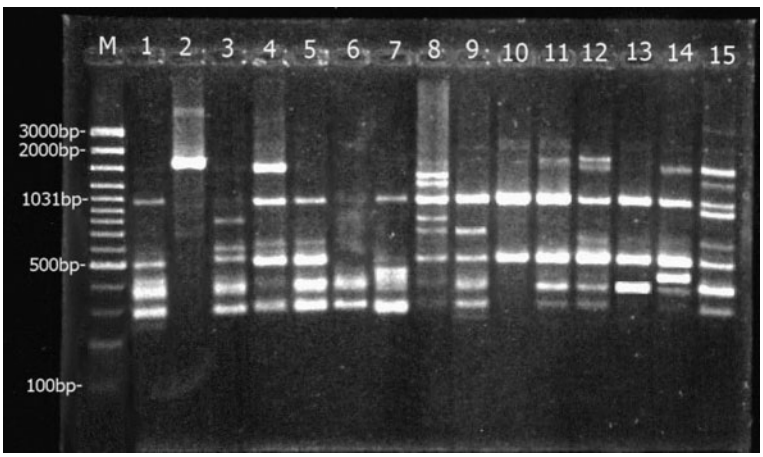

Fig. 2. RAPD types of *C. coli* isolates obtained from the intestinal contents and gall bladders of healthy sheep, using the OPA-11 primer. M, 100 bp molecular mass marker (MBI Fermentas; SM0321); lanes 1–8, types obtained from intestinal-content samples (lane 2 represents the most frequent type); lanes 9–15, types obtained from gall bladder samples (lane 10 represents the most frequent type).

different types were obtained (Table 2). Of these, seven types were obtained from gall bladder samples, eight from intestinal contents and nine from faecal samples. The most common types were represented by 48 % of gall bladder samples, 35 % of faecal samples and 34 % of intestinal-content samples (Fig. 2). Other types were detected at much lower percentages. Two of the types obtained from faecal isolates were observed in all three flocks, but the remaining seven distinct types were obtained in only one flock. The absence of identical types from field and abattoir isolates indicates that the geographical location might play a role in strain variation in sheep.

Information about the genetic relationships among *C. jejuni* and *C. coli* strains of healthy sheep origin is scarce. Scates *et al.* (2003) reported 8 distinct band types in the examination of 30 liver samples in sheep. To our knowledge, no studies investigating genetic heterogeneity among *Campylobacter* strains isolated from other types of sample from sheep have been conducted hitherto. The results of the present study showed that the number of RAPD profiles among *C. coli* isolates from sheep was higher than from cattle. On the contrary, the number of band types of *C. jejuni* isolates was higher in cattle.

The RAPD assay has been proved to have excellent discrimination ability due to the fact that the entire genome is the target in genotyping. A common opinion exists among researchers that the discrimination of an RAPD assay is higher than that of PFGE (Nielsen *et al.*, 2000). However, the major disadvantage of the RAPD assay appears to be its poor reproducibility and repeatability. Hernandez *et al.* (1996) noted that 17 % of their *Campylobacter* isolates could not be typed, due to DNase activity, using the arbitrarily primed-PCR fingerprint method . In this study, RAPD analysis was successfully applied in typing 100 % of *C. jejuni* and *C. coli* isolates. Moreover, the assay was repeated at least twice and identical results were obtained. The findings reported by Hilton *et al.* (1997) also supported the idea that RAPD may successfully be used to type *Campylobacter* species originating from various samples.

In conclusion, it was observed that a high degree of heterogeneity existed among *C. jejuni* and *C. coli* isolates of healthy cattle and sheep origin. RAPD analysis appears to be a valuable tool in epidemiological surveillance, and for investigating the distribution of types in animals, the environment and humans.

ACKNOWLEDGEMENTS

The technical support and help in sample collection of Dr H. Ongor, Dr M. Karahan and Mr M. Bazna is gratefully acknowledged. This work was supported by the Scientific Research Council of Firat University (FUBAP 800) and was part of a PhD thesis produced by M. N. Açik.

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