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RESEARCH ARTICLE

Emerging dynamics of human campylobacteriosis in Southern Ireland

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Abstract

Infections with *Campylobacter* spp. pose a significant health burden worldwide. The significance of *Campylobacter jejuni*/*Campylobacter coli* infection is well appreciated but the contribution of non-*C. jejuni*/*C. coli* spp. to human gastroenteritis is largely unknown. In this study, we employed a two-tiered molecular study on 7194 patient faecal samples received by the Microbiology Department in Cork University Hospital during 2009. The first step, using EntericBio® (Serosep), a multiplex PCR system, detected *Campylobacter* to the genus level. The second step, utilizing *Campylobacter* species-specific PCR identified to the species level. A total of 340 samples were confirmed as *Campylobacter* genus positive, 329 of which were identified to species level with 33 samples containing mixed *Campylobacter* infections. *Campylobacter jejuni*, present in 72.4% of samples, was the most common species detected, however, 27.4% of patient samples contained non-*C. jejuni*/*C. coli* spp.; *Campylobacter fetus* (2.4%), *Campylobacter upsaliensis* (1.2%), *Campylobacter hyointestinalis* (1.5%), *Campylobacter lari* (0.6%) and an emerging species, *Campylobacter ureolyticus* (24.4%). We report a prominent seasonal distribution for campylobacteriosis (Spring), with *C. ureolyticus* (March) preceeding slightly *C. jejuni*/*C. coli* (April/May).

Introduction

Campylobacter-related gastroenteritis is one of the most common causes of acute bacterial diarrhoeal illness in both developed and developing countries (Coker *et al.*, 2002). There is a persistently high incidence of campylobacteriosis in European countries, and the 2007; crude incidence rate in Ireland was a direct reflection of the European average of 45.2/100 000 (HSPC, 2007; EFSA, 2009). National surveillance programmes which have been employed by many industrialized countries have contributed to improved reporting of *Campylobacter* infections (HSPC, 2007). Yet despite the high reported incidence it is estimated that the true rate of infection may be between seven and thirty times higher (Crushell *et al.*, 2004; Moore *et al.*, 2005; Janssen *et al.*, 2008).

Campylobacteriosis is primarily regarded as a zoonosis and the reservoirs for infection vary depending on the species, but encompass a wide range of birds, livestock and domestic animals (McClurg *et al.*, 2002; Moore *et al.*, 2005). To date, 26 species are assigned to the *Campylobacter* genus (Euzeby, 1997), however, only some of these have been linked with human gastroenteritis (Mandrell *et al.*, 2005; Moore *et al.*, 2005). *Campylobacter jejuni* and *Campylobacter coli* combined have been reported as accounting for at least 95% of *Campylobacter* isolations in humans with gastroenteritis (Kulkarni *et al.*, 2002; Moore *et al.*, 2005), when using traditional culture methods for diagnosis. Advances in molecular diagnostics facilitate detection of non-*C. jejuni*/*C. coli* spp. from clinical samples (Duffy *et al.*, 2007; Bullman *et al.*, 2011). However, to date, the true contribution of non-*C. jejuni*/*C. coli* species to human gastroenteritis remains unknown, and the aims of our study were to determine the proportion of non-*C. jejuni*/*C. coli Campylobacter* in faeces from patients with acute gastroenteritis, and to analyse the complete collection of results with regard to seasonality and age distribution.

In this study we employed a two-tiered molecular-based analysis to examine the distribution of *Campylobacter* spp. in patients presenting with diarrhoeal illness

for the calendar year 2009. The first stage of the investigation was to detect a genus-specific marker for *Campylobacter* spp., and the second stage sought to determine identity to species level.

Materials and methods

Patient samples

A total of 7194 faecal samples collected over a 1-year period, from patients presenting with diarrhoea were screened for *Campylobacter* spp. using EntericBio® (Serosep Ltd, Limerick, Ireland). Of these, a total of 349 DNA samples were included in the study. These samples, stored at −20 °C, were extracted from anonymized faecal samples that tested positive for *Campylobacter* genus using the EntericBio® system. The samples were received by the Department of Medical Microbiology at Cork University Hospital, Ireland, between January 2009 and December 2009.

Control strains

The following control isolates were used in the study: *Campylobacter jejuni* ssp. *jejuni* DSM 4688, *Campylobacter coli* DSM 4689, *Campylobacter lari* ssp. *lari* DSM 11375, *Campylobacter fetus* ssp. *fetus* DSM 5361, *Campylobacter upsaliensis* DSM 5365, *Campylobacter hyointestinalis* ssp. *hyointestinalis* DSM 19053 and *Campylobacter ureolyticus* DSM 20703. All control strains were obtained from DSMZ, Braunschweig, Germany.

PCR template preparation

DNA was extracted from the faecal samples using the EntericBio® system in accordance with the manufacturer's instructions. The extracted DNA samples were either used immediately in the EntericBio® system or stored at −20 °C for subsequent use in *Campylobacter* species-specific and genus-specific PCR.

PCR assay

The 349 *Campylobacter* genus positive DNA samples were investigated using uniplex species-specific PCR assays for *C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, *C. hyointestinalis*, *C. upsaliensis* and *C. ureolyticus*, respectively, according to the methods used by Bullman *et al.* (2011).

16S rRNA gene sequencing

A *Campylobacter* genus-specific PCR targeting the 16S rRNA gene was conducted on DNA samples that were negative for species-specific PCR. Samples that produced a PCR amplicon were subject to 16S rRNA gene sequence analysis (Eurofins MWG Operon, Ebersberg, Germany). Returned sequences were subjected to BLAST analysis using the NCBI database and aligned by the CLUSTALW method in Megalign from the Lasergene suite of programs (DNAstar). Clinical strains were considered to be identified when sequence similarities with GenBank entries exceeded 99% identity over the entire length of the sequence.

Data collection

On receipt of the samples in the Microbiology Laboratory, each patient sample was assigned a unique specimen record number and the samples were anonymized for the purposes of this study. Details of the patients' age and gender along with sample details and collection information were provided on patients who were confirmed *Campylobacter*-positive, from which age and gender profiles were constructed.

Results

Confirmation of EntericBio® positive *Campylobacter* spp. results

Of 7194 patient faecal samples submitted to the Microbiology Laboratory from January to December of 2009, a total of 349 samples were determined to be *Campylobacter* genus-positive by the EntericBio® molecular method. *Campylobacter* species-specific PCR confirmed the presence of *Campylobacter* spp. in 329 (94.3%) of the 349 EntericBio®-positive samples. Of the 20 samples which were *Campylobacter*-positive by the EntericBio® but negative for the species-specific PCR, nine samples were positive for the *Campylobacter* genus-specific PCR (targeting the 16S rRNA gene); however, these samples failed to return results on sequencing. A further two samples were negative for the 16S rRNA gene PCR but were recorded as *Campylobacter* culture-positive by the clinical Microbiology Laboratory; thus, these 11 samples were reported as non-speciated *Campylobacter* positives. A total of nine samples were negative for both *Campylobacter* species-specific and genus-specific PCR and were reported as *Campylobacter* culture-negative by the clinical Microbiology Laboratory; thus, these nine samples were recorded as false positives. A total of 340 samples of the 349 patient samples which were positive by the EntericBio® molecular method were confirmed by alternative molecular methods (species-specific or genus-specific PCR) and/or routine culture to be true *Campylobacter* spp. positives.

Speciation of *Campylobacter*-positive samples

Campylobacter spp. were identified in 329 patient samples by species-specific PCR. There were 373 *Campylobacter* detections in all, as 33 samples contained mixed infections involving a total of 77 *Campylobacter* detections. Figure 1 shows the contribution of each species to the total number of organisms detected. The most common species, *C. jejuni*, was detected in 246 (72.4%) of patient samples and accounted for 66% of the total detections. *Campylobacter ureolyticus* was detected in 83 (24.4%) patient samples, accounting for 22.3% of the total detections. *Campylobacter ureolyticus* was the sole *Campylobacter* species detected in 55 (16.7%) patient samples (95% confidence interval: 12.8–21.2%). *Campylobacter coli* accounted for 6.7%; present in 25 (7.1%) of patient samples. Collectively *C. fetus*, *C. hyointestinalis*, *C. upsaliensis* and *C. lari* amounted to 5% of the total detections.

Age and gender distribution of *Campylobacter*-positive patients

Age and gender profiles were constructed (Fig. 2) for the 340 patients confirmed as *Campylobacter* positive; 52.4% were men. A bimodal age distribution was noted in both genders (Fig. 2); 51.8% of all *Campylobacter* detections were distributed between two age groups; children under 5 years (31.1%) and adults over 70 years (20.7%). In male patients, *Campylobacter* infection was commonest in those $\leq$ 5 years (37%), whereas in female patients, it was most frequent in those $\geq$ 70 years (29%) (Fig. 2). Infection remained at lower levels between late childhood and the 66–70 age group with the exception of a peak in male patients aged 41–45 (10.8%).

The age and gender profiles for *C. ureolyticus* exhibited a similar bimodal profile to those observed for the combined species, presenting peaks at extremes of age. This species was more commonly detected in female patients (62.7%) and more than 50% of detections in female patients were in the $\geq$ 70 age group (Fig. 2).

Seasonal prevalence of *Campylobacter* 2009

The hypotheses that the incidence of *C. jejuni*/*C. coli* and of *C. ureolyticus* is uniformly distributed across the 12 months were conclusively rejected by the chi-square goodness-of-fit test, showing *P*-values of 0.000, and 0.003, respectively).

As shown in Fig. 3, C. *jejuni*/*C. coli* infection peaked in April and May (12.9% and 12.6% of the total, respectively), declined from September onwards and remained low during the winter months.

An abrupt peak was observed for *C. ureolyticus* infections in early spring; 18.1% of the total number of detections occurred during March. The fewest detections of *C. ureolyticus* infections (2.4% of the total) were observed in December.

Discussion

Campylobacter infections remain a significant public health issue, not only in terms of morbidity but also

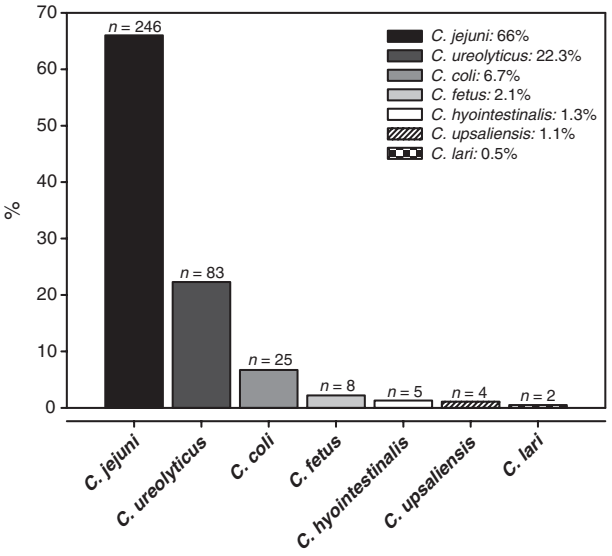

Fig. 1. The speciation of *Campylobacter* by PCR: percentage contribution of each species to the total number identified (n = 373).

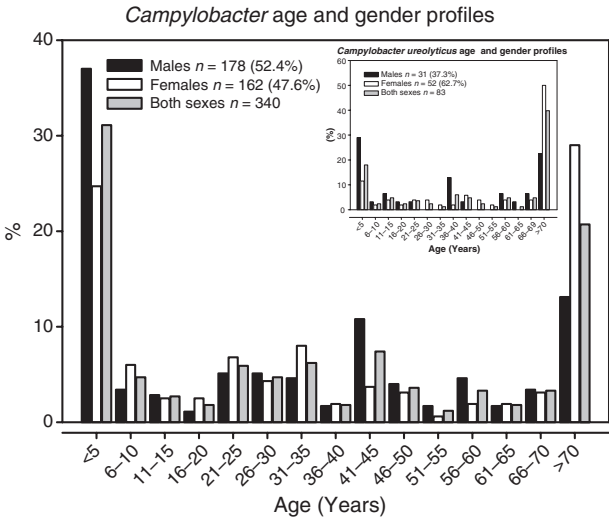

Fig. 2. Age and gender profiles of all *Campylobacter*-positive patients (n = 340); and age and gender profile of patients (n = 83) who tested positive for *Campylobacter ureolyticus*.

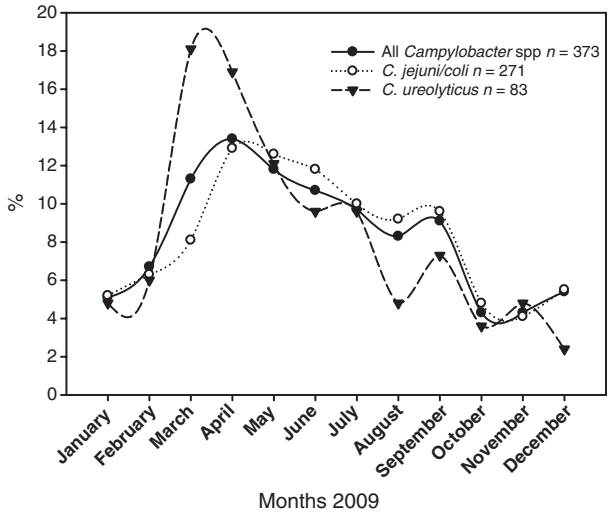

Fig. 3. Seasonal distribution of combined *Campylobacter* spp., *Campylobacter jejuni* and *Campylobacter ureolyticus* detected in 2009.

economic cost. In 2006, a total of 175 561 confirmed cases of campylobacteriosis were reported from 21 European countries (EFSA, 2009), a figure which is thought to be an underestimate (Moore *et al.*, 2005; Janssen *et al.*, 2008). Furthermore, the annual cost of *Campylobacter* infection in the US is estimated to be at least $8 billion; a large economic burden (Crushell *et al.*, 2004). Based on routine culture detection in diagnostic laboratories, *C. jejuni* and *C. coli* have been reported as being the most prevalent *Campylobacter* spp. causing human disease and, as a result, the majority of *Campylobacter* research has focused on these two species. With recent advances in diagnostic methods, there has been an increasing realization of the limitations of routine culture for the detection of non-*C. jejuni*/*C. coli* spp. (Lastovica & le Roux, 2000; Maher *et al.*, 2003; O'Leary *et al.*, 2009; Bullman *et al.*, 2011). Thus, traditional culture underestimates the true occurrence of both *Campylobacter*-related gastroenteritis and also the emerging campylobacteria that are detectable by molecular methods in cases of gastroenteritis (Lastovica & le Roux, 2000; Maher *et al.*, 2003; Bullman *et al.*, 2011). Herein, we employed a two-tiered molecular detection method, independent of culture, for the direct detection of *Campylobacter* spp. from the faeces of 7194 patients presenting with diarrhoeal illness.

Of 340 *Campylobacter*-positive samples, *C. jejuni* represented the largest proportion of PCR-positives, present in 72.4% of patients; although it has previously been reported that this species accounts for at least 80–90% of *Campylobacter*-related infections (Kovats *et al.*, 2005; Moore *et al.*, 2005). *Campylobacter coli*, traditionally ranked as the second most common *Campylobacter* species found in *Campylobacter* enteritis (Coker *et al.*, 2002; Moore *et al.*, 2005; Ivanova *et al.*, 2010); was identified in 7.4% of samples. An emerging species, *C. ureolyticus*, was detected in 24.4% of cases, and was detected solely (without other bacteria or viruses) in the samples of 55 symptomatic patients. There are a number of reasons why *C. ureolyticus* fails to be identified by routine culture: it is incapable of growth under the microaerobic atmosphere conditions used (5% O_2, 10% CO_2 and 85% N_2) unless hydrogen is supplied. In addition, certain strains appear to be sensitive to the antibiotic used in the *Campylobacter* selective agars. Finally, most clinical laboratories isolate *Campylobacter* from stools at incubation temperatures >40 °C; however, *C. ureolyticus* fails to grow above 37 °C (Vandamme *et al.*, 2010). To our knowledge, this is the first report of *C. ureolyticus* replacing *C. coli* as the second most common *Campylobacter* species in patients presenting with diarrhoeal illness. Furthermore, as previously reported (Bullman *et al.*, 2011), given that only 1.15% of the 7194 samples processed contained this organism, it is unlikely that *C. ureolyticus* exists as a common commensal in the gastrointestinal tract.

In support of the findings of Maher *et al.* (2003), we determined that non-*C. jejuni*/*C. coli* spp. accounted for 27.4% of the total *Campylobacter* organisms detected; a much greater percentage than the previously reported 1–5% (Galanis, 2007; Ivanova *et al.*, 2010). The large proportion of non-*C. jejuni*/*C. coli* species in this study is primarily due to the novel identification of *C. ureolyticus*, which as mentioned previously, is not normally isolated by routine *Campylobacter* culturing techniques. This finding highlights the advantages of molecular diagnostics for the detection of such fastidious organisms. Recent observations by O'Leary *et al.* (2009), when comparing a multiplex PCR assay to routine culture indicated that approximately 30% of samples positive for *Campylobacter* by the molecular method failed to grow on culture. Similar results were reported by Bessède *et al.* (2011), who reported considerably lower sensitivity for culture-based techniques compared to molecular methods. The hypothesis that the true proportion of *Campylobacter* detections due to *C. jejuni*/*C. coli* is 95% or more in the current study is conclusively rejected by the appropriate statistical test (P-value = 0.000).

The reported incidence of the less commonly grown campylobacteria varies in different regions of the world (Moore *et al.*, 2005), and interestingly, we have detected *C. fetus* in 2.4% of the *Campylobacter*-positive samples (0.11% of the 7194 samples analysed). *Campylobacter fetus* has been considered to be a very rare cause of gastroenteritis in humans and acts most often as an opportunistic pathogen in immunocompromised individuals (Umehara *et al.*, 2009); our findings may suggest the need for further investigation of this species as a gastrointestinal pathogen.

Human *Campylobacter* infection in temperate countries has been acknowledged as having a seasonal occurrence. Reports demonstrate a trough during winter months followed by a two- to threefold higher peak in late spring or early summer (Kovats *et al.*, 2005). Our findings reflect this for the combined *Campylobacter* spp. as well as for *C. jejuni*/*C. coli* and *C. ureolyticus* individually. *Campylobacter jejuni*/*C. coli* and *C. ureolyticus* infections peak in spring. Figure 3 suggests a slightly earlier peak (March) for *C. ureolyticus* in comparison to *C. jejuni* (April and May).

A second epidemiological feature of *Campylobacter* infection is that incidence shows associations with patient age and gender (Crushell *et al.*, 2004). European studies report (HSPC, 2007, EFSA, 2009) that in developed countries *Campylobacter* infection is most common in children under 5 years; however, a second peak is noted in the young adult, the 15–24 year age group, which is not observed in developing countries. In our study, *Campylobacter* was most frequently detected in the extremes of age, with more than half of all detections in children ≤ 5 years and adults ≥ 70 years. *Campylobacter ureolyticus* was shown in this study to be more common in female patients. Previously, *Campylobacter* infections have been reported as more common in male patients (Moore *et al.*, 2005; EFSA, 2009). In addition, the peak which we report in the elderly (particularly for *C. ureolyticus*) accords with a recent study in the UK (Gillespie *et al.*, 2009), which found that the elderly are the population most at risk of *Campylobacter* infection.

In conclusion, the results of our investigation for the period of January–December inclusive, 2009, in Southern Ireland, suggest that non-*C. jejuni*/*C. coli* spp. contribute considerably to the proportion of *Campylobacter*-positive samples from patients presenting with diarrhoeal illness. This finding illustrates the power of molecular-based detection over less sensitive culture techniques (O'Leary *et al.*, 2009; Bullman *et al.*, 2011). The resulting positive impact of increased sensitivity of diagnostics on clinical care is likely to be significant. Although *Campylobacter*-related gastroenteritis is usually self-limiting in the immunocompetent host, it imposes a high economic burden primarily due to hospitalization and man hours lost to industry. As *Campylobacter* infections are notifiable in Ireland, increased sensitivity of detection might be expected to heighten infection control efforts, in particular for organisms such as *C. ureolyticus*, whose reservoir is currently unknown.

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