Campylobacter concisus pathotypes are present at significant levels in patients with gastroenteritis

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Given that Campylobacter jejuni is recognized as the most common cause of bacterial gastroenteritis worldwide, recent findings showing comparable levels of Campylobacter concisus in patients with gastroenteritis would suggest that this bacterium is clinically important. The prevalence and abundance of Campylobacter concisus in stool samples collected from patients with acute gastroenteritis was examined using quantitative real-time PCR. The associated virulence determinants exotoxin 9 and zonula occludens toxin DNA were detected for Campylobacter concisus-infected samples using real-time PCR. Campylobacter concisus was detected at high prevalence in patients with gastroenteritis (49.7%), higher than that observed for Campylobacter jejuni (~5%). The levels of Campylobacter concisus were putatively classified into clinically relevant and potentially transient subgroups based on a threshold developed using Campylobacter jejuni levels, as the highly sensitive real-time PCR probably detected transient passage of the bacterium from the oral cavity. A total of 18% of patients were found to have clinically relevant levels of Campylobacter concisus, a significant number of which also had high levels of one of the virulence determinants. Of these patients, 78% were found to have no other gastrointestinal pathogen identified in the stool, which strongly suggests a role for Campylobacter concisus in the aetiology of gastroenteritis in these patients. These results emphasize the need for diagnostic laboratories to employ identification protocols for emerging Campylobacter species. Clinical follow-up in patients presenting with high levels of Campylobacter concisus in the intestinal tract is needed, given that it has been associated with more chronic sequelae.

INTRODUCTION

Despite substantial decreases in the incidence of gastroenteritis over recent decades, acute gastroenteritis is the second greatest burden of all infectious diseases worldwide (Murray & Lopez, 1996). It occurs when intestinal epithelial cells are breached by an enteric pathogen or toxin resulting in inflammation, diarrhoea and disrupted physiological functions. It is reported that there are up to 2 billion cases of gastroenteritis every year, with up to 5000 children dying every day as a result (Webb & Starr, 2005).

Despite employing current reference-standard laboratory diagnostics, in a substantial amount of cases (up to 40% in some studies), a pathogen is not identified.

Currently, Campylobacter jejuni is recognized as the most common bacterial cause of gastroenteritis worldwide, accounting for approximately 400 million cases every year (Allos, 2001; Kaakoush et al., 2015b). Other Campylobacter species are important, with Campylobacter coli considered a clinically important pathogen, accounting for around 5–19% of Campylobacter-associated gastroenteritis worldwide (Allos, 2001; Kaakoush et al., 2015b). Recently, Campylobacter species other than Campylobacter jejuni and Campylobacter coli have been suggested to play a role in human disease. However, these species are relatively fastidious and require an H2-enriched environment.
to grow (Lastovica, 2006; Kaakoush & Mitchell, 2012; Nielsen et al., 2013a). Such conditions are generally not employed by most clinical laboratories and it is therefore believed that the clinical relevance of these emerging *Campylobacter* species, including *Campylobacter concisus*, is highly under-reported (Lastovica, 2006; Kaakoush et al., 2015b). While initial studies focused on the role of *Campylobacter concisus* in oral diseases such as periodontitis, more recent studies have reported *Campylobacter concisus* to play a role in gastrointestinal diseases such as inflammatory bowel diseases, Barrett’s oesophagus and gastroenteritis (Lastovica, 2006; Macfarlane et al., 2007; Kaakoush & Mitchell, 2012; Blackett et al., 2013; Nielsen et al., 2013a; Kaakoush et al., 2014b, 2015b). Indeed, in more recent years, improved isolation and identification techniques have elucidated *Campylobacter concisus* to have a comparable prevalence to that of *Campylobacter jejuni* in cases of gastroenteritis (Lastovica, 2006; Collado et al., 2013; Nielsen et al., 2013a, 2015; Ferreira et al., 2014).

*Campylobacter concisus* is genetically and taxonomically diverse. For example, some *Campylobacter concisus* strains have been shown to invade intestinal epithelial cells and survive intracellularly, while other strains possess different virulence characteristics such as the ability to secrete a zonula occludens toxin (ZOT) (Kaakoush et al., 2010, 2011; Man et al., 2010a). These characteristics, along with the ability to induce an inflammatory response, suggest that certain *Campylobacter concisus* strains may be more associated with causing disease than others. Recently, based on these virulence characteristics, it has been proposed that *Campylobacter concisus* may be divided into two pathotypes: adherent and invasive *Campylobacter concisus* (AICC) and adherent and toxigenic *Campylobacter concisus* (AToCC), which are genetically different to commensal strains (Kaakoush et al., 2014b). Conserved amongst AICC pathotypes are extrachromosomal virulence determinants including a restriction-modification (R-M) system that comprises at least four genes: a restriction endonuclease, a DNA-methyltransferase, a site-specific recombination and exotoxin 9 (Deshpande et al., 2013). In contrast, AToCC pathotypes have the ability to produce a ZOT that can potentially target and disassemble intercellular tight junctions in a similar manner to ZOT found in *Vibrio cholerae* (Fasano et al., 1995; Fasano et al., 1997; Kaakoush et al., 2010). This may lead to the leakage of luminal contents that represents the clinical manifestation of diarrhoea.

While most cases of gastroenteritis are self-limiting, it has recently been reported that *Campylobacter concisus*-associated gastroenteritis is more prolonged in nature (Nielsen et al., 2012, 2013b). Furthermore, 25 and 12.5% of patients with gastroenteritis resulting from *Campylobacter concisus* infection go on to develop microscopic colitis and irritable bowel syndrome, respectively (Nielsen et al., 2012, 2013a, b, 2014). These findings highlight the need to further investigate the role of *Campylobacter concisus* in the aetiology of acute gastroenteritis. In this study, we employed real-time quantitative PCR (qPCR) on faecal samples of patients with gastroenteritis to determine the levels of *Campylobacter concisus*, as well as levels of exotoxin 9 and ZOT, to qualitatively classify *Campylobacter concisus*-positive samples into AICC and AToCC, respectively. Additionally, faecal samples were tested for the presence of commonly known gastrointestinal pathogens.

**METHODS**

**Faecal samples.** A total of 499 faecal samples obtained from 499 subjects (one per subject) presenting with clinical gastroenteritis (loose stools for >24 h with or without concomitant signs of fever, weight loss and upper gastrointestinal symptoms) were submitted to the South Eastern Area Laboratory Services (SEALS) Microbiology laboratory. Faecal samples were initially subjected to routine diagnostic testing in SEALS Microbiology to detect common gastrointestinal pathogens (Siah et al., 2014). The testing employed included bacterial multiplex PCR to detect *Salmonella* species, *Shigella* species, *Campylobacter jejuni/coli*, toxigenic *Clostridium difficile* and *Yersinia enterocolitica*. Any samples positive for *Campylobacter jejuni/coli* by PCR were subjected to cultivation to distinguish which *Campylobacter* species was present in the stool. If isolation of either species remained unsuccessful, the samples were reported as *Campylobacter jejuni/coli* positive. Testing also included viral multiplex PCR to detect rotavirus group A, norovirus genotype II, adenovirus serotype 40/41 and astrovirus. Finally, faecal samples were subjected to enzyme immunoassays (EIAs) to detect *Giardia lamblia* and *Cryptosporidium*. EIAs positive for either of these protozoans were subjected to microscopic examination to confirm their presence. Upon completion of these tests, faecal samples were stored at −4°C until use. To ensure no sampling bias occurred, the age, gender and results of these tests were blinded until completion of testing for *Campylobacter concisus*.

**Bacterial strains and DNA extraction.** *Campylobacter concisus* strains UNSWCD (AICC) and BAA-1457 (AToCC) and *Campylobacter jejuni* NCTC 11168 were used in this study. All bacteria were grown on horse blood agar plates [blood agar base no. 2 supplemented with 7% (v/v) defibrinated horse blood (Oxoid)], and incubated at 37°C under microaerobic conditions supplemented with H2 for 48 h. Bacterial DNA was extracted using the Puregene Core A kit (Qiagen) according to the manufacturer’s instructions.

**Detection and quantification of *Campylobacter concisus*, exotoxin 9 and ZOT DNA.** DNA was extracted from 300 mg of faeces using a phenol/chloroform/isooamyl alcohol DNA extraction method following the procedures outlined in Griffiths et al. (2000). Quantification of the levels of *Campylobacter concisus*, exotoxin 9 and ZOT was accomplished on a Rotor Gene 6000 real-time PCR cycler (Qiagen). *Campylobacter concisus* DNA was amplified using the primers Concisis-F and Concisis-R, which have been previously optimized (Man et al., 2010b; Kaakoush et al., 2014a). Cycling conditions were 95°C for 5 min, 40 cycles of 95°C for 10 s, 65°C for 10 s and 72°C for 30 s, followed by 72°C for 5 min. Exotoxin 9 DNA was amplified using the primers Exotox-F and Exotox-R, which have been previously optimized by Kaakoush et al. (2014a). Cycling conditions were 94°C for 5 min, 30 cycles of 94°C for 20 s, 53°C for 20 s and 72°C for 1 min, followed by 72°C for 5 min. ZOT DNA was amplified using the primers ZotF and Zot2, which have been previously optimized by Kaakoush et al. (2015a). Cycling conditions were 94°C for 5 min, 30 cycles of 94°C for 20 s, 53°C for 20 s and 72°C for 1 min, followed by 72°C for 5 min. Each 10 μl reaction mixture consisted of 3.5 μl Sensimix SYBR mastermix (Bioline), 10 pmol each primer, 1 μl extracted DNA and sterile water to complete the volume.
In order to quantify the levels of DNA in the faeces, standard curves were constructed for the *Campylobacter concisus*, exotoxin 9 and ZOT qPCRs employing a range of concentrations (0.25, 0.5, 1, 10, 25, 50, 100, 250, 500 and 1000 pg DNA) from *Campylobacter concisus* UNSWCD and BAA-1457 stocks to obtain a linear plot. A triplicate real-time PCR was run on 60 (12%) random samples to calculate the mean percentage standard error for the *Campylobacter concisus* (13.32%), exotoxin 9 (17.15%) and ZOT (13.22%) qPCRs.

**Putative identification of Campylobacter concisus pathotypes.**
Samples positive for *Campylobacter concisus* were putatively categorized into their respective pathotypes based on the detection and abundance of exotoxin 9 and ZOT. Those positive for exotoxin 9 only were considered AICC, while those positive for ZOT only were considered ATOCC. Those that were positive for both exotoxin 9 and ZOT were categorized into their pathotypes based on the ratio of abundance of these virulence determinants. Those significantly higher in exotoxin 9 were considered AICC while those significantly higher in ZOT were considered ATOCC. Where there was no significant difference between the abundance of exotoxin 9 and ZOT, the sample was considered to be ‘mixed’. Those negative for both exotoxin 9 and ZOT were classified into the ‘other’ subgroup.

**Clinical relevance of Campylobacter concisus-positive samples.** In order to assess the clinical relevance of *Campylobacter concisus*-positive samples, real-time qPCR was conducted on samples shown to be negative for *Campylobacter concisus* and *Campylobacter coli* but positive for *Campylobacter jejuni* (n=12). Amplification of *Campylobacter jejuni* DNA was accomplished on a Rotor Gene 6000 real-time PCR cycler (Qiagen). *Campylobacter jejuni* DNA was amplified using the primers C412F and C1288R, which have been previously optimized by Linton et al. (1996). Cycling conditions were 95°C for 5 min, 40 cycles of 95°C for 10 s, 55°C for 10 s and 72°C for 45 s, followed by 72°C for 5 min. To quantify levels of *Campylobacter jejuni* DNA in the faeces, a standard curve was constructed by employing a range of concentrations (1, 5, 100, 1000, 2500 and 5000 pg DNA) of *Campylobacter jejuni* NCTC 11168 DNA to obtain a linear plot. The lowest abundance of *Campylobacter jejuni* was used as a threshold to determine which *Campylobacter concisus* samples may be clinically relevant and which may be potentially transient. Those *Campylobacter concisus* samples above the minimum abundance of *Campylobacter jejuni* were considered to be clinically relevant, while those under the minimum abundance of *Campylobacter jejuni* were considered potentially transient.

**Statistical analysis.** All statistical tests were performed using Fisher’s exact test on GraphPad Prism 6 (GraphPad software).

**RESULTS**

**Prevalence and abundance of Campylobacter concisus**
Of the 499 faecal samples analysed, 248 (49.7%) were found to be positive for *Campylobacter concisus* DNA. Of the 248 samples in which *Campylobacter concisus* was detected, 113 had very low abundances of DNA ranging between 1 and 300 pg (g faeces)^{-1}, 35 had low abundances of DNA ranging between 301 and 3000 pg (g faeces)^{-1}, 32 had moderate abundances of DNA ranging between 3001 and 30 000 pg (g faeces)^{-1} and 58 had high abundances of DNA greater than 30 000 pg (g faeces)^{-1}.

**Putative identification of Campylobacter concisus pathotypes**
Of the 248 *Campylobacter concisus*-positive faecal samples, 20 were only positive for exotoxin 9, 27 were only positive for ZOT, 191 were positive for both exotoxin 9 and ZOT and 10 were negative for both exotoxin 9 and ZOT. Of the 191 samples positive for both exotoxin 9 and ZOT, 62 samples had significantly greater abundances of exotoxin 9, 83 samples had significantly greater abundances of ZOT and 46 samples had similar levels of exotoxin 9 and ZOT. Based on the presence and abundance of exotoxin 9 and ZOT in *Campylobacter concisus*-positive samples, 82 (33.1%) were classified as AICC, 110 (44.4%) were classified as ATOCC, 46 (18.5%) were classified as ‘mixed’ and 10 (4.0%) were classified as ‘other’ (Fig. 1a).

**Assessment of the clinical relevance of Campylobacter concisus pathotypes in gastroenteritis**
Of the 12 *Campylobacter jejuni* samples examined, five had abundances ranging between 3001 and 30 000 pg (g faeces)^{-1} and seven had abundances greater than 30 000 pg (g faeces)^{-1}. Based on the lowest abundance of *Campylobacter jejuni* as a threshold for clinical relevance, *Campylobacter concisus* was classified as clinically relevant in 90 samples and potentially transient in 158 samples (Fig. 1b). Within the clinically relevant subgroup, 37 samples (41.1%) were classified as AICC and 32 (35.6%) were ATOCC, while 45 (28.5%) were AICC and 76 (48.1%) were ATOCC within the potentially transient subgroup (Fig. 1b). AICC were borderline significantly more enriched in the clinically relevant subgroup as compared with AICC in the potentially transient subgroup (P=0.0497), and ATOCC were more enriched in the potentially transient subgroup as compared with ATOCC in the clinically relevant subgroup (P= 0.0628).

**Rate of co-infection of Campylobacter concisus and other gastrointestinal pathogens**
The rate of co-infection of *Campylobacter concisus* with other gastrointestinal pathogens was also examined (Table 1). Of the 499 faecal samples tested, 385 (77.1%) had no gastrointestinal pathogen detected. No significant differences were found in the prevalence rates of any pathogen in the *Campylobacter concisus*-positive and negative samples (Table 1). However, a trend was observed for a higher prevalence of *Campylobacter jejuni* in *Campylobacter concisus*-negative samples than in *Campylobacter concisus*-positive samples. Moreover, of the six *Campylobacter jejuni*-positive samples that were also positive for *Campylobacter concisus*, five had potentially transient levels of *Campylobacter concisus*, indicating low levels of *Campylobacter concisus* in *Campylobacter jejuni*-positive samples. Importantly, of the 385 samples where no gastrointestinal pathogen was detected, 192 (49.9%) were positive for *Campylobacter concisus*, of which 70 (36.5%) were classified in the clinically relevant subgroup.
Correlation of Campylobacter concisus infection with patient age and gender

A total of 248 males and 251 females were included in this study. Of the 248 Campylobacter concisus-positive samples, 124 were males and 124 females, suggesting no correlation existed between the presence of Campylobacter concisus and gender. Within the clinically relevant group, there was a slight enrichment of AICC in females, but this did not reach significance (Table 2).

The prevalence of Campylobacter concisus amongst different patient age groups showed that infection of 65/117 patients (56%) aged 0–9 years, 19/46 (41%) aged 10–19 years, 17/42 patients (40%) aged 20–29 years, 26/49 (53%) patients aged 30–39 years, 21/33 (64%) patients aged 40–49 years, 28/45 (62%) patients aged 50–59 years and 72/165 (44%) patients aged 60 years and over were positive for Campylobacter concisus (Fig. 2a).

The distribution of pathotypes in these age groups was then examined. In the clinically relevant subgroup, AICC were significantly higher in individuals 0–49 years (30/55, 54.5%) as compared with those 50 years or older (7/35, 20.0%, P = 0.0019). In contrast, AToCC were significantly more enriched in individuals 50 years or older (18/35, 51.4%) as compared with those 0–49 years (14/55, 25.4%, P = 0.0144) (Fig. 2b).

**DISCUSSION**

Over recent years the high prevalence of Campylobacter concisus in patients with gastroenteritis has become increasingly documented (Collado et al., 2013; Nielsen et al., 2013a; Ferreira et al., 2014). In fact, some studies have shown Campylobacter concisus to have a prevalence comparable to that of Campylobacter jejuni in patients with gastroenteritis. For example, in South Africa, Lastovica and colleagues reported that out of 5443 cases of gastroenteritis in which a Campylobacter species was isolated, 32% were identified as Campylobacter jejuni subsp. jejuni and 25% as Campylobacter concisus (Lastovica, 2006, 2009). In Denmark, Nielsen et al. (2013a) reported that Campylobacter jejuni/coli and Campylobacter concisus were isolated from approximately 11 and 9% of 8302 patients with gastroenteritis, respectively. These findings are supported by additional studies originating from Chile and Portugal (Collado et al., 2013; Ferreira et al., 2014).

Despite this, reports of the isolation of Campylobacter concisus from faecal samples of healthy individuals (Engberg et al., 2000; Inglis et al., 2011) have led to significant controversy regarding the role of this bacterium in human disease. However, recent studies showing that Campylobacter concisus strains can be divided into two pathotypes that are genetically different to commensal strains offer a possible explanation for this finding (Kaakoush et al., 2014b).

In the present study, we found a high prevalence of Campylobacter concisus in patients with gastroenteritis (49.7%) in an Australian population resident in Sydney. While previous studies have employed both cultivation and molecular detection techniques such as standard PCR to determine the prevalence of Campylobacter concisus
in patients with gastroenteritis, none has employed qPCR, a highly sensitive technique that can detect very low levels of DNA. The high prevalence of *Campylobacter concisus* observed in our study is probably due to the detection of the transient passage of *Campylobacter concisus* from the oral cavity, given that the oral cavity is a natural reservoir for the bacterium (Zhang *et al.*, 2010; Kaakoush & Mitchell, 2012).

In light of this, the abundance of *Campylobacter concisus* was calculated in order to examine the level of *Campylobacter concisus* DNA in the patients’ stools. A threshold to determine which levels of *Campylobacter concisus* were clinically relevant and which were potentially transient was established by examining the levels of *Campylobacter jejuni* being shed into the faeces of patients only positive for *Campylobacter jejuni*. Thus, *Campylobacter concisus* samples that had DNA abundance levels similar to those found in *Campylobacter jejuni* samples were considered to be clinically relevant, while those under this threshold were considered to result from transient passage of *Campylobacter concisus* from the oral cavity. While this threshold has its limitations, given the lack of human experimental models of *Campylobacter concisus* infection and the presence of human commensal *Campylobacter concisus* strains, we considered that comparison of levels to a highly related pathogen would provide a practical preliminary threshold. Based on this threshold, we found 90 of the 248 (36.3%) *Campylobacter concisus*-positive samples to be clinically relevant. Interestingly, the overall prevalence of *Campylobacter concisus* in this study. Thus, further studies would be required to obtain a more accurate threshold by which *Campylobacter concisus* levels could be classified as clinically relevant.

To date, there has been no study detailing the prevalence and abundance of *Campylobacter concisus* with markers of pathogenesis in patients with gastroenteritis. In this study, we putatively classified *Campylobacter concisus* pathotypes through abundance levels of exotoxin 9 and ZOT DNA in *Campylobacter concisus*-positive samples. Interestingly, we found AICC to be significantly more enriched in the clinically relevant subgroup as compared with the potentially transient subgroup (*P*=0.0497), and AToCC to be more enriched in the potentially transient subgroup as compared with the clinically relevant subgroup (*P*=0.0628). This could indicate that AICC are potentially more involved in the aetiology of gastroenteritis in comparison with AToCC, which are more likely to pass transiently to the stool. However, given that a significant number of AToCC were observed in the clinically relevant subgroup, this suggested that AToCC may require more specific conditions (e.g. presence of factors that activate ZOT) to cause disease.

### Table 1. Prevalence of other gastrointestinal pathogens in *Campylobacter concisus*-positive and -negative samples

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Patients positive in <em>Campylobacter concisus</em>-positive patients</th>
<th>Percentage (total n=248)</th>
<th>Patients positive in <em>Campylobacter concisus</em>-negative patients</th>
<th>Percentage (total n=251)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus 40/41</td>
<td>2</td>
<td>0.8</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Astrovirus</td>
<td>1</td>
<td>0.4</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td><em>Campylobacter coli</em></td>
<td>3</td>
<td>1.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>6</td>
<td>2.4</td>
<td>12</td>
<td>4.8</td>
</tr>
<tr>
<td><em>Campylobacter jejuni/coli</em></td>
<td>3</td>
<td>1.2</td>
<td>6</td>
<td>2.4</td>
</tr>
<tr>
<td>Clostridium difficile</td>
<td>19</td>
<td>7.7</td>
<td>16</td>
<td>6.4</td>
</tr>
<tr>
<td>Giardia lamblia</td>
<td>2</td>
<td>0.8</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Norovirus II</td>
<td>7</td>
<td>2.8</td>
<td>7</td>
<td>2.8</td>
</tr>
<tr>
<td>Rotavirus A</td>
<td>6</td>
<td>2.4</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>Salmonella</td>
<td>3</td>
<td>1.2</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>Shigella</td>
<td>2</td>
<td>0.8</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Yersinia enterolitica</td>
<td>2</td>
<td>0.8</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>22.6</td>
<td>58</td>
<td>23.1</td>
</tr>
</tbody>
</table>

### Table 2. Prevalence and percentage of *Campylobacter concisus* pathotypes in males and females of the clinically relevant subgroup

<table>
<thead>
<tr>
<th>Pathotype</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICC</td>
<td>15 (37.5 %)</td>
<td>22 (44.0 %)</td>
</tr>
<tr>
<td>AToCC</td>
<td>14 (35.0 %)</td>
<td>18 (36.0 %)</td>
</tr>
<tr>
<td>Mixed</td>
<td>8 (20.0 %)</td>
<td>9 (18.0 %)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (7.5 %)</td>
<td>1 (2.0 %)</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>50</td>
</tr>
</tbody>
</table>
Previously, Nielsen et al. (2013a) reported that in 10% of patients positive for Campylobacter concisus, a co-infection was present with either Clostridium difficile or Salmonella enterica. In this study, while we observed an overall high number of toxigenic Clostridium difficile in Campylobacter concisus-positive stools, toxigenic Clostridium difficile were also highly prevalent in Campylobacter concisus-negative stools. Indeed, we found no significant enrichment of Campylobacter concisus in samples positive for any other gastrointestinal pathogen. However, we did observe a negative trend between Campylobacter concisus and Campylobacter jejuni, the majority of Campylobacter jejuni-positive patients being negative for Campylobacter concisus (n=12), and the majority of those positive for Campylobacter concisus having very low levels of the bacterium [n=5/6 (83.3%)]. Due to the small sample size of Campylobacter jejuni-positive patients (n=18) in our study, this negative trend requires further investigation.

Most importantly, we found a large number of clinically relevant Campylobacter concisus samples (n=70) to have no other pathogen identified in the stool. Thus, it is possible that in these cases, Campylobacter concisus is the causative agent or at least a contributor to the symptoms of gastroenteritis. This finding emphasizes the need for diagnostic laboratories to employ protocols that identify emerging Campylobacter species in patients with gastroenteritis, so that in cases where no other pathogen is identified, the role of emerging Campylobacter species can be clarified. By employing these protocols, it is plausible that a reduction in the number of undiagnosed cases of gastroenteritis worldwide would be observed. Indeed, improved culture conditions suitable for Campylobacter concisus isolation (H2 enrichment) resulted in a pseudo-outbreak of this bacterium in Bern University Hospital, Switzerland (Casanova et al., 2015).

Based on the distribution of pathotypes amongst the clinically relevant subgroup, we found that AICC were significantly more prevalent amongst patients 49 years or younger (P=0.0019) and AToCC were significantly more prevalent amongst patients 50 years or older (P=0.0144). Given that Campylobacter concisus has been reported to be highly prevalent amongst immune deficient patients (Aabenhus et al., 2002), this latter observation may be accounted for by the reduction in immune system function with age. It would seem that patients with a less functional immune system may provide a more suitable environment for AToCC to colonize the gastrointestinal tract. This is of particular interest, given that AICC strains have been shown to manipulate autophagy, a process that protects host cells against intracellular pathogens (Burgos-Portugal et al., 2014). However, given that patients in the age group 20–29 also showed high levels of colonization by AToCC, other factors may be involved in the distribution of these pathotypes. One possible explanation for this could be that potential age-related shifts in the gut microbiota might facilitate colonization by AToCC. Thus, further investigations into the age-associated differences in pathogenesis between Campylobacter concisus pathotypes are required.

No significant difference in the overall prevalence of Campylobacter concisus was observed between males and females. While there was a slightly higher percentage of AICC amongst females in the clinically relevant subgroup, this did not reach significance, which suggests that the presence and abundance of Campylobacter concisus may not be influenced by gender.
This study is not without limitations. Larger epidemiological studies enrolling patients from multiple sites are required to truly ascertain the prevalence of *Campylobacter concisus* in cases of gastroenteritis in Australia. In addition, correlation of the abundance of *Campylobacter concisus* and the presence of the different pathotypes with patient clinical information such as severity of disease would add weight to the role of *Campylobacter concisus* in the aetiology of acute gastroenteritis. Further, the threshold established for clinical relevance of *Campylobacter concisus* levels is preliminary in nature and requires confirmation. Given that *Campylobacter concisus* presents with a milder but more prolonged form of the disease as compared with *Campylobacter jejuni* (Nielsen et al., 2012, 2013b), bacterial levels that may be considered clinically relevant may differ between the two species. Moreover, exotoxin 9 and ZOT are only putative indicators of *Campylobacter concisus* pathotypes, and these genes have been reported in other organisms. Thus, optimized approaches to identify *Campylobacter concisus* pathotypes in patients are required, including isolation of the bacterium and experimental verification of its pathogenic potential and the presence of these genes within its genome.

In conclusion, over recent years it has become increasingly more evident that *Campylobacter concisus* is involved in the aetiology of gastroenteritis. In support of this, our results provide further evidence as to the possible involvement of *Campylobacter concisus* in cases of gastroenteritis and the need for diagnostic laboratories to employ identification protocols for *Campylobacter concisus* and other emerging *Campylobacter* species, in order to reduce the number of undiagnosed cases of gastroenteritis. Given that following symptoms of gastroenteritis, patients with *Campylobacter concisus* have been reported to later develop more chronic sequelae (Nielsen et al., 2012, 2013b, 2014), and the association of this bacterium with IBD (Kaakoush et al., 2014), clinical follow-up studies in patients presenting with high levels of this bacterium in the intestinal tract are urgently needed. In particular, a prospective population-based study examining the long-term effects of *Campylobacter concisus* infection, as was elegantly performed for *Campylobacter jejuni* by Gradel et al. (2009), would be of significant clinical importance.

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