Genotypic Analysis of the Virulence and Antibiotic Resistance Genes in Campylobacter species in silico

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Abstract

Campylobacter species is responsible for 400-500 million diarrhea cases worldwide every year. Emergence of antibiotic resistance has further complicated the scenario. A wide range of virulence factors and resistance genes are present in Campylobacter species and it is hypothesized there are genotypic variations in the prevalence of these genes. The study was conducted to investigate the presence of virulence and antibiotic resistance genes as well as to investigate difference in prevalence rate based on genotype through in silico tools. Among 28 species studied, sixteen isolates (61.54%) had the cdtB gene that breaks the double helix bonds. The cdtA genes were detected in ten (36.46%) C. jejuni strains while fifty percent (n=13) isolates harbored the cdtC genes. Ten isolates that harboured all three adjacent cdt genes were most toxigenic. The lipo-oligosaccharides associated genes, cgtB and wlan, responsible for β-1,3 galactosyltransferase production, were found in 7.69% and 30.77% of the isolates, respectively. About 57.69% isolates expressed waaC genes. Invasion protein (ciaB), outer membrane phospholipase (pldA), and IV secretory protein (virB11) were found in 53.85%, 34.62% and 7.69% of the isolates, respectively. Six isolates (23.08%) expressed both tetO and tetA genes while one isolate expressed only tetA resistance gene. Seven isolates (26.9%) had changes in gyrB genes that conferred the fluoroquinolone resistance. In silico PFGE typing found that genotype 3 contained all the virulence genes except cgtB gene while genotype 3 and 4 contained mutated gyrB gene. Genotype 1 and 5 contained no virulence and antibiotic resistance genes. Our data helps to predict the possibility of the presence of virulence and antibiotic resistance genes and helps to select appropriate antibiotic that are more efficacious.

Keywords: Antibiotic resistance genes; Campylobacter; Genotype; PCR, PFGE; Virulence genes

Introduction

Gram-negative Campylobacter species is responsible for traveler’s diarrhea and gastroenteritis in humans [1,2]. Campylobacter is responsible for 400-500 million diarrhea cases worldwide every year [3]. Guillain–Barré syndrome is caused by C. jejuni [4] but virulence mechanisms are not well understood. Several studies found that contaminated food, raw milk, water, vegetables, seafood, atmospheric modified packed meat transmitted the zoonotic pathogen Campylobacter to humans during consumption or handling process [1,2,5]. Several investigations found that main source of food-borne human campylobacteriosis is poultry and poultry products [6,7]. During slaughtering, the intestinal tract of healthy birds and raw meat are contaminated with thermophilic Campylobacter species. In slaughter house, faeces and processing facilities during the evisceration process contaminated swine carcasses that ultimately leads to contaminated food products [8-10].

The mechanism of Campylobacter gastroenteritis in humans is not well known that limits the prevention of campylobacteriosis. Virulence factors such as motility and adherence of bacteria to the intestinal mucosa, invasion of enterocyte and toxin production may contribute to the pathogenicity of campylobacteriosis infections [11,12]. The most common virulence factor in Campylobacter species, the Cytolethal Distending Toxin (CDT) causes cellular distension which eventually leads to cell death [13]. The CDT has 3 subunits namely CdtA, CdtB, and CdtC. The CdtB is the active subunit whereas CdtA and CdtC makes up the B subunit responsible for binding to susceptible cells [14]. Active toxin component of cdtB gene disrupted the double helix bonds in the nucleus and blocked the cell cycle [15,16]. The wlan, cgtB and waaC are LOS (lipo-oligosaccharides) associated genes while wlan and cgtB are involved in β-1,3 galactosyltransferase production [17]. These two genes are associated with waaC gene (which encodes heptosyltransferase I) and connected with the Guillain-Barre’ and Miller-Fischer syndromes [18,19]. GBS and other neuropathic conditions are caused because of molecular mimicry of Campylobacter lipooligosaccharide (LOS) with the carbohydrate moiety of gangliosides. The waaC gene, which encodes heptosyltransferase I, is responsible for transferring the first l-glycerod-manno-heptose residue to the inner core of LOS [20]. The wlan gene, which encodes a beta-1,3 galactosyltransferase, is responsible for biosynthesis GM1-like structure [18] whereas cgtB (which encodes another beta-1,3 galactosyltransferase) catalyzes the biosynthesis of the carbohydrate moieties analogous to GM2 [18,21].

Invasion protein (ciaB), outer membrane phospholipase A (pldA), and type IV secretory protein (virB11) genes associated with bacterial invasion on epithelial cells, were found by previous study [22] but their functions are not well known. Phospholipase A, pldA was found to be expressed in invasive strains [23].

Several studies found that multidrug-resistant Campylobacter has increased worldwide due to misuse of antibiotics [24,25]. For the treatment of systemic campylobacteriosis infections, other antibiotics such as gentamicin, tetracycline and ampicillin have been used [26,27].

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Fluoroquinolones, followed by tetracyclines, are the most commonly used antibiotics in the poultry industry [28] where Campylobacter is ubiquitous. It can be inferred that over use of tetracyclines and fluoroquinolones have contributed to their resistance. Mutations in gyrA and gyrB regions of DNA gyrase and been held accountable for resistance against fluoroquinolones such as ciprofloxacin [29]. Majority of fluoroquinolone resistance of Campylobacter is developed by amino acid substitution at Thr-86 to Ile though alternative mutation at Asp-90 and double mutation at Thr-86 and Pro-104 were also reported [30].

Resistance to tetracycline might be due to efflux, enzymatic alteration of antibiotic or by ribosomal protection. The tetA gene encodes efflux protein associated with pumping out tetracyclines out of the cell [31]. The tetO causes resistance by ribosomal protection whereas tetX gene encodes enzymes responsible for target modification [31]. Scientists identified that tetO protein recognized and bound with an open A site of the bacterial ribosome and conformational changes occurred that released bound tetracycline molecules [32]. Previous findings found that tetO also inhibited tRNA accommodation into the ribosomal A site and inhibited protein elongation phase [33].

Pulsed Field Gel Electrophoresis (PFGE) is considered as the gold standard for genotyping [34] and in silico data matched to that obtained in conventional method [35]. Our data helps to predict virulence and antimicrobial resistance profile of twenty-six Campylobacter species through in silico tools and also analyzes how pulsed-field gel electrophoresis (PFGE) typing distributed the virulence and resistance genes within the genotypes. The virulence and resistance profile of future isolates with known genotype can be predicted according to our data.

Materials and Methods

Strains used in the study

Strains used in the study are summarized in Table 1. All strains were of human/ animal origin.

Primers used in the study

Primers used for detection of virulence and antibiotic resistance genes are summarized in Tables 2 and 3 [36-43].

PCR amplification

In silico PCR amplification was done in the website http://insilico.ehu.eus/PCR/ [35,44].

PFGE digestion

An online software http://insilico.ehu.eus/digest/wasdesigned for insilico pulsed-field gel electrophoresis (PFGE) digestion [35,44]. Restriction enzyme KpnI recognized the restriction sequence G\_GTAC\_C of Campylobacter species. Dendrogram construction was done in the website.

Results and Discussion

Genetic diversity of isolates

A total of 26 isolates were subjected to in silico pulsed-field gel electrophoresis (PFGE) analysis with KpnI restriction digestion that recognized the sequence G\_GTAC\_C of Campylobacter species. Band fragments were separated in 1.2% agarose gel and lambda ladder compared the band size. In silico PFGE typing grouped 26 isolates into five genotypes using 80% as a cutoff value (Figure 1). Genotype 3 was the most prevalent followed by genotype 4 and 2. Fifty percent (n=13) isolates were present in genotype 3 whereas genotype 4 contained about 26.92% (n=7) of the isolates. Genotype 1 and 3 and harboured about 3.85% of the isolates (Figure 2).

Genotypic distribution of Cytotoxic Distending Toxin associated cdt genes

Toxicogenic activity of Campylobacter species is determined by cytotoxic distending toxin which is composed of the cdt gene cluster containing three adjacent genes (cdtA, cdtB and cdtC). It was found that
three cdt genes determined the functional activity of CDT toxin [15]. Previously researchers identified that cdtA and cdtC are responsible for binding to target whereas the cdtB encoded active subunit of the toxin [15,45]. Sixteen isolates (61.54%) had the cdtB gene with 620 bp PCR product (Figure 3). Only Campylobacter jejuni subsp. doylei 269.97 didn’t harbour cdtB gene out of the isolates studied. The cdtA gene was detected in ten (38.46%) C. jejuni strains with 370 bp PCR product (Figure 4). These isolates gave a 182 bp amplicon (Figure 5). The present study found ten isolates that harboured all three adjacent cdt genes and these were considered as most toxigenic strains. Genotype 1 and 5 contained no cdt genes (Figure 6) and hence such isolates are unlikely to cause gastroenteritis. On the other hand, genotype 2, 3 and 4 contained all three cdt genes. All the isolates present in genotype 3 carried cdtB genes while about 61.54% and 76.92% isolates present in genotype 3 expressed cdtA and cdtC genes, respectively. About 28.58% in genotype 4 expressed cdtB and cdtC genes while 14.29% isolates present in genotype 4 carried all three cdt genes. It must be noted that cdtB alone can cause cytotoxicity [46]. Hence isolates with only cdtB gene can cause gastroenteritis irrespective of the cdtA and cdtC although potency of toxin might be compromised. Thus all isolates from genotype 3 will cause diarrhea.

Genotypic distribution of genes associated with Guillain–Barré syndrome

The genes cgtB, wlaN and waaC are associated with GBS and other neuropathic conditions through molecular mimicry of LOS with gangliosides. The cgtB gene was detected in only two isolates and hence such isolates are unlikely to cause guillain barré syndrome.

Table 3: Primers for detection of antibiotic resistance genes.

| Gene | Primer sequence (5’-3’) | Amplicon size (bp) | References |
|------|------------------------|--------------------|------------|
| tetO | GGGCGTTTTGTTATATGGCC ATGGGACAGCGACAGAAGC | 559 | [42] |
| tetA | GCTCAGCTGACGACGAGAAG ACGCTACGATTGCG TAC | 486 | [43] |
| gyrA | ATTCGACAGCAGGAGGAAC AGCGATGCGATTACTA GCA | 270 | [30] |
| gyrB | ATTCGACAGCAGGAGGAAC AGCGATGCGATTACTA GCA | 382 | [30] |

Figure 1: Phylogenetic diversity of Campylobacter species identified by PFGE analysis.
produced 562 bp gene product (Figure 7) while 30.77% isolates had the \textit{wlaN} genes with 330 bp gene product (Figure 8). About 57.69% isolates expressed \textit{waaC} genes and gave 971 bp gene product (Figure 9). These three LOS associated genes were found in only \textit{C. jejuni} strains. The \textit{wlaN} and \textit{cgtB} genes were not found in the same isolates. Similar results were also found in previous study [17]. Not all LOS associated genes were present in same genotype (Figure 10). Twenty-five percent isolates in genotype 2 expressed \textit{cgtB} and \textit{waaC} genes. All the isolates in genotype 3 carried the \textit{waaC} gene while about 61.54% isolates in genotype 3 expressed \textit{wlaN} genes. About 14.29% isolates in genotype 4 carried both \textit{cgtB} and \textit{waaC} genes. Hence isolates from genotype 3 are more likely to exert their pathology through the mimicry of GM1 rather than GM2 whereas the reverse is true for isolates from genotype 2 and 4. All isolates from genotype 3 would exhibit heptosyltransferase...
I activity and would transfer first l-glycero-d-manno-heptose residue to the inner core of LOS.

**Genotypic distribution of adherence and invasion genes**

Invasion-associated marker (iam), genetic marker of *Campylobacter* species, identified in diarrhea and symptom free patients, was reported by previous study [47]. Previous study found that clinical samples in children didn’t harbor *iam* gene but isolates from older patients had the *iam* genes [48]. The present study found no invasion associated marker, *iam* gene. In Brazil, diarrhoeagenic *C. coli* isolates were found in children that had the *iam* gene while low prevalence of *iam* genes were found in *C. jejuni* strains [49]. Invasion protein (ciaB),

![Figure 5: Detection of cdtC gene. Isolates harbouring the gene gives a 182 bp amplicon.](image)

![Figure 6: Genotypic distribution of cdt genes. Genes encoding virulence genes are as follows: cdtA: Cytolethal Distending Toxin A; cdtB: Cytolethal Distending Toxin B; cdtC: Cytolethal Distending Toxin C.](image)

![Figure 7: Detection of cgtB gene. Isolates harbouring the gene gives a 562 bp amplicon.](image)
outer membrane phospholipase A (\textit{pldA}), and IV secretory protein (\textit{virB11}) genes associated with bacterial invasion on epithelial cells, were found by previous study [22] but their functions are not well known. Phospholipase A, \textit{pldA} was found to be involved in expression of invasion [23]. A study found that cattle isolates didn’t harbour phospholipase A, \textit{pldA} gene while sheep samples were potentially more virulent since carrying more \textit{iam} genes than that of cattle isolates [50]. About 34.62% isolates (n=9) had the \textit{pldA} genes and produced 385 bp gene product (Figure 11) in \textit{C. jejuni} strains. Previous study found that \textit{pldA} gene was encountered in high number (88-100%) in broiler samples [51]. High percentage (91.7%) isolates from poultry feces contained \textit{pldA} genes [52]. Several studies described that \textit{ciaB} gene is associated with invasiveness and a play role in progression of the disease [22,53]. About 53.85% isolates (n=14) had the \textit{ciaB} genes with 527 bp gene product (Figure 12). Previously 98.80% and 61% isolates were found to harbor \textit{ciaB} and \textit{pldA} genes, respectively.
[54]. Broiler meat samples and human strains equally harboured the plasmid associated virulence marker virB11 gene that could invade the human intestine [55]. Researchers found that very small subset of C. jejuni strains carried virB11 genes [39]. The virB11 gene was detected in two isolates (7.69%) with 708 bp gene product (Figure 13). Nature of the plasmid and geographical differences are responsible for low level of virB11 gene [52]. Several studies also found very low number of virB11 genes in the tested isolates [51,56]. Genotype 3 contained all three genes described above (Figure 14). About 84.62% isolates in genotype 3 expressed ciaB genes while about 61.54% and 7.69% isolates in genotype 3 harboured pldA and virB11 genes, respectively. About 14.29% isolates in genotype 4 carried pldA while about 28.58% isolates in genotype 4 carried ciaB genes. Twenty-five percent isolates in genotype 2 harboured virB11 and ciaB genes. Hence it can be inferred...
that invasion and adherence by isolates from genotype 2 are unlikely to be mediated by phospholipase A. The same can be stated invasion via virB11 invasion-associated marker for genotype 4.

Genotypic distribution of tetracycline resistance genes

Tetracycline resistance gene tetO was detected by using two primers named DMT1 and DMT2 as described previously [57]. Six isolates were found to harbor tetO resistance gene and gave 559 bp gene product (Figure 15). Hence the prevalence was 23.08%. Forty-six kilodalton membrane-bound efflux protein tetA exported tetracycline antibiotic from the cell [58]. Seven isolates (26.92%) expressed tetA gene with an approximate amplicon length of 486 bp (Figure 16). Six isolates expressed both tetO and tetA genes while Campylobacter jejuni subsp. jejuni M1 expressed only tetA resistance genes. Our data suggests that tetracycline resistance is more likely to be mediated due to tetA rather than tetO. Our data agrees with a study also found that more Kenyan isolates harboured tetA resistance genes rather than tetO [43]. Twenty-five percent isolates in genotype 2 carried tetO and tetA.
Genotypic distribution of tetracycline and fluoroquinolone resistance genes

The gyrA gene that conferred resistance to nalidixic acid or fluoroquinolone resistance was examined [30]. Present study found no gyrA gene positive isolates (not shown). Seven isolates (26.92%) had the gyrB genes that conferred the resistance of fluoroquinolone antibiotics and gave 382 bp gene products (Figure 18). Our study is a contrast to previous report which states that fewer isolates (4.17%) were found to have silent mutations in gyrB gene when compared to gyrA gene [30]. Genotype 4 contained the highest number of gyrB genes (42.86%) followed by genotype 2 (25%) and genotype 3 (23.08%) (Figure 17). Hence resistance to fluoroquinolone in Campylobacter is more likely due to mutations in the gyrB subunit of DNA gyrase rather than the gyrA subunit.

Genotype 3 contains the highest number of tetO genes (Figure 17). About 23.08% isolates in genotype 3 expressed tetO while 30.77% isolates in genotype 3 expressed tetA genes. About 28.57% isolates in genotype 4 harboured tetO and tetA genes. Hence isolates from genotype 2 and genotype 4 are equally likely to be resistant to tetracycline due to efflux pump and ribosomal protection. However isolates from genotype 3 are more likely to be resistant due to efflux pump rather than ribosomal protection.

Conclusion

Our study used bioinformatics aided approach to genotype and detect virulence and antibiotic resistance genes. Pulsed Field Gel Electrophoresis (PFGE) is considered as the gold standard for genotyping [34] and in silico data matched to that obtained in conventional method [35]. The summary of the results have been illustrated in Table 4. We concluded that there is variation in prevalence across the genotypes. Certain genotypes are more threatening than others. Our data isolates from genotype 1 and 5 are not pathogenic since it didn’t harbor any of gastroenteritis toxin genes (cdtA, cdtB, cdtC), genes associated with Guillain–Barré syndrome (wlaN, cgtB, waaC) or genes associated with adherence and invasiveness (iam, pldA, virB11, ciaB). These genotypes did not have genes associated with fluoroquinolone and tetracycline resistance. The cytolethal distending toxin genes has a higher prevalence in genotype 3. Since cdtB alone can exert cytotoxicity, all isolates from genotype 3 are capable of causing gastroenteritis. Based on the difference in the prevalence of Guillain–Barré syndrome associated genes among the genotypes, isolates from genotype 3 are more likely to exert their pathology through the mimicry of GM1 rather than GM2 whereas the reverse is true for isolates from genotype 2 and 4. All isolates from genotype 3 would exhibit heptosyltransferase I activity and would modify the inner core of LOS. Our data also suggests that that invasion and adherence by isolates from genotype 2 are unlikely to be mediated by phospholipase A. The same can be stated invasion via virB11 invasion-associated marker for genotype 4. The mechanism of drug resistance also varies across the genotype. Isolates from genotype 2 and genotype 4 are equally likely to be resistant to tetracycline due to efflux pump and ribosomal protection. However isolates from genotype 3 are more likely to be resistant due to efflux pump rather than ribosomal protection. As for resistance to fluoroquinolone resistance isolates...
from genotype 4 are the most resistant. All resistant isolates carry a mutated gyrB subunit of DNA gyrase rather than a mutated gyrA subunit. Campylobacter shows resistance to the clinically important antibiotics and this rising trend is a concern for public health. Therefore, enhanced research efforts are needed to understand the transmission, persistence and prevention of antibiotic-resistant Campylobacter. Campylobacter species acquired antibiotic resistance gene by horizontal gene transfer from either Streptomyces, Streptococcus, or Enterococcus species [59]. Seventy-five to seventy-six percent sequence homology was found between tetO gene of Campylobacter and tetM of Streptococcus pneumoniae. The virulence and resistance profile of future isolates with known genotype can be predicted according to our data. Additional studies are needed to understand how antibiotic resistant Campylobacter emerge under selective pressure. Application of advanced approaches, such as genomics and proteomics, is expected to provide new insights into the molecular mechanisms involved in the development of antibiotic resistance in Campylobacter. Good hygiene practice and food safety assurance programs should be implemented to reduce the contamination risk during handling and processing. Misuse of antibiotics in animal feed must be reduced to control antibiotic-resistant Campylobacter.

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