IDENTIFICATION OF VIRULENCE GENES IN CAMPYLOBACTER JEJUNI AND C. COLI ISOLATES BY PCR

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Abstract

Using the PCR technique, the presence of the seven genotypic virulence markers of Campylobacter has been tested. It was found that all C. coli (92 strains) and C. jejuni (105 strains) possessed the flaA and cadF markers. Several strains were positive for the CDT toxin genes (76.6%, 85.3%, and 83.2% strains with the A, B, and C cdt gene variants, respectively). On the other hand, only 66.0% and 20.8% isolates had the iam and virB genes.

Key words: Campylobacter, virulence markers, detection, PCR.

Campylobacter sp. is now recognised as a leading bacterial cause of human gastroenteritis worldwide but their pathogenic mechanism has not been exactly explained yet. One of the main reasons of this state is genotypic, hence phenotypic diversity of the species belonging to the genus Campylobacter. Regarding consumers’ safety, it is essential to characterise pathogenic markers in strains that are found in food. It was discovered that some strains of C. jejuni are not pathogenic at all or elicit mild symptoms in humans, while other isolates are the cause of serious diseases (4). It is not sure, which factor is essential to the disease development. However, flagella-mediated motility, bacterial adherence to intestinal mucosa, invasive capability, and the ability to produce toxins have been proposed as the main virulence determinants (3, 10, 25, 27).

As mentioned above, motility is one of the most important factors of Campylobacter pathogenicity. The motion is determined by spiral form of the bacterium and flagella bundles placed on the cell tips. Due to these features, bacteria are able to move against peristaltic movements, penetrate epithelial mucus, and colonise intestinal cells. Flagellum is built from flagellinum, protein coded by the flaA and flaB genes, which both are 1.7 kbp in size. The flaA gene is expressed on the higher level than the flaB, thus it is crucial to bacterial motility.

Both markers have a role in the process of host cell invasion through the expressions of protein that influence cell membrane permeability and cytoskeleton rearrangements.

The ability to adhesion and penetration into host cells is one of the most important elements in the pathogenesis of intestinal form of campylobacteriosis. Extramembrane proteins (e.g. CadF of 37 kDa weight) take part in the process of adhesion and invasion. These proteins enable adhesion of Campylobacter to the erythrocyte fibronectine (13). It was demonstrated that the cadF mutants were not able to colonise chicken caecum (14, 30).

Many of genetic factors connected with Campylobacter invasiveness are placed on the pVir plasmid. For example, the virB11 gene that encodes the IV secretory system protein. Strains with mutation in the virB11 sequence have much lower adhesion and penetration ability in vitro in comparison to original strains, as well as lower pathogenicity in vivo (1).

A sequence that was present in 85% of invasive Campylobacter strains and only in 20% of non-invasive isolates was designed as iam (invasion associated marker). However, the function of the iam marker in the process of campylobacteriosis development has not been precisely explained yet (6).

Toxins produced by Campylobacter are also important factors of the pathogenicity. Knowledge about their structure is relatively small. They were described for the first time in 1988 by Johnson and Lior (12), who identified them in some species of Campylobacter - C. coli, C. jejuni, C. lari, and C. foetus. Cytotoxic distending toxin (CDT) consists of three subunits encoded by the cdtA, cdtB, and cdtC genes, which are 30, 29, and 21 kbp in size, respectively, and are also arranged as an operon. Specific function of CDT toxin proteins was not exactly determined. It is considered that CdtB, which is encoded by the cdtB gene, is the active subunit of the holotoxin and may block the cell cycle in G2 phase through inhibition of CDC2 kinase and causes cell distension (7, 9, 28). Different Campylobacter strains have various CDT toxin production abilities,
although *C. jejuni* shows higher cytotoxic activity in comparison to *C. coli* (21).

The aim of this study was to generate more knowledge regarding the pathogenicity of thermotolerant *Campylobacter* with focus on the examination of several genotypic pathogenic markers in 197 *Campylobacter jejuni* and *C. coli* strains, isolated from different sources by using polymerase chain reaction (PCR).

**Material and Methods**

**Bacterial strains.** A total of 197 *Campylobacter* strains isolated from faeces (n = 137) and poultry carcasses (n = 60) were used in this study. The following positive and negative reference strains were also included: *C. jejuni* ATCC 33291, *C. coli* ATCC 43478, *Escherichia coli* EDL 933, and *Salmonella* Typhimurium ATCC 14028.

**DNA preparation.** *Campylobacter* strains were grown at 42°C in Karmali agar for 24 h under microaerophilic condition. A bacterial colony was suspended in 1 ml of sterile water and centrifuged at 13 000 g for 1 min. Afterwards, DNA was extracted using the Genomic – Mini kit (A&A Biotechnology, Poland) according to the manufacturer’s instruction. The purity and concentration of the DNA preparations were estimated using spectrophotometry at 260 and 280 nm.

*Campylobacter* species determination was done using multiplex PCR (m-PCR) for the simultaneous detection of the *C. jejuni* and *C. coli* in a single reaction tube as described previously (29).

**PCR primers.** All primers used in the study are shown in Table 1. PCR primers were synthesised by Symbiosis (Poland).

### Amplification of virulence genes

The PCR mixture (50 μl) used for DNA amplification contained: 5 μl of the PCR buffer (10-times concentrated), 5 μl of dNTPs (Fermentas, Lithuania, final concentration 200 μM), 10 μl of MgCl₂ (final concentration 5 mM), 0.5 μl of each primer (final concentration of 0.1 μM each), 2 μl (1 U) of the Taq thermostable DNA polymerase (Fermentas), 5 μl of the bacterial template DNA, and DNase- and RNase-free deionised water (Biomedicals). All PCRs were carried out in a thermal cycler (PTC-100, MJ Research, USA) under the following conditions: initial DNA denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min (with the exception *flaA* gene - 48°C for 1 min) and 72°C for 1 min. The final extension step was performed at 72°C for 5 min. The analysis of the amplified products was performed in 2% agarose (Sigma) in Tris-Acetated–EDTA (TAE) buffer at 100 V. The DNA bands were visualised by staining with ethidium bromide, analysed under UV light (300 nm) and photographed using the Gel Doc 2000 documentation system (Bio-Rad). The size of the PCR amplicons was compared to the 100 bp DNA marker (Fermentas).

**Statistical analysis.** Statistical significance was calculated using the chi-square test.

### Results

During the study period, total 197 *Campylobacter* strains were tested. The distribution of the putative virulence genes in *Campylobacter jejuni* and *C. coli* isolates of different sources is shown in Table 2. The results of PCR detection of 7 virulence-associated genes are also summarised in Fig. 1.

### Table 1

**Characteristics of PCR primers used in the study**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ → 3’)</th>
<th>Target gene</th>
<th>Size of PCR amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDTAF</td>
<td>CCTTGTGATGCAAGCAATC</td>
<td>cdtA</td>
<td>370</td>
<td>11</td>
</tr>
<tr>
<td>CDTAR</td>
<td>ACATCCCCATTGTCTTICTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDTBF</td>
<td>CAGAAACCAATGGAGTGTT</td>
<td>cdtB</td>
<td>620</td>
<td>8</td>
</tr>
<tr>
<td>CDTBR</td>
<td>AGCTAAAAAGGCTGAGATAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDTCF</td>
<td>CGATGAGTTAAAACAAAAAGATA</td>
<td>cdtC</td>
<td>182</td>
<td>8</td>
</tr>
<tr>
<td>CDTCR</td>
<td>TTTGCATTATAGAAAAATACGGTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2B</td>
<td>TGGAGGGAATTTTAGATATG</td>
<td>cadF</td>
<td>400</td>
<td>13</td>
</tr>
<tr>
<td>R1B</td>
<td>CTAATCCCTAAAGTTGAAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAMF</td>
<td>GCCGCATATATATCGACC</td>
<td>iam</td>
<td>518</td>
<td>15</td>
</tr>
<tr>
<td>IAMR</td>
<td>TTCACGACTATATCGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VirBF</td>
<td>GAACAGGAAATGGAAAACACACTAGG</td>
<td>virB11</td>
<td>708</td>
<td>1</td>
</tr>
<tr>
<td>VirBR</td>
<td>TTCCCGATTGGGCTATATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flaAF</td>
<td>GATTTTGATTTTAAACACAATGGTGCG</td>
<td>flaA</td>
<td>1700</td>
<td>5</td>
</tr>
<tr>
<td>flaAR</td>
<td>CTGTAATATCTTAACATTTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The obtained results showed that all analysed isolates of *C. jejuni* and *C. coli* derived both from carcasses and poultry faeces possessed the *flaA* sequence and also the *cadF* gene (Table 2, Fig. 1).

Three toxin genes, the expression of which is necessary to activate CDT toxin synthesis, were also detected in several of the isolates tested. The majority of the evaluated isolates possessed the *cdtB* (85.3%) and *cdtC* (83.2%) genes whereas the *cdtA* marker was present in 76.6% of the isolates. It should be emphasised that more than a half of *Campylobacter* isolates possessed all three toxin subunit genes together. If species affinity was taken into consideration, a higher percentage of *C. jejuni* (from 85.9% to 92.4%) than *C. coli* (63.8% – 84.8%) isolates demonstrated positive reaction towards the *cdt* genes.

The next one of the virulence markers determining the invasiveness of *Campylobacter* isolates – the *virB11* gene, localised on the pVir plasmid was examined. This gene was present only in 41 from 197 (20.8%) analysed bacterial isolates, i.e. in 18.5% of *C. jejuni* and 22.9% strains of *C. coli*.

Another virulence gene linked with *Campylobacter* invasiveness – *iam* was detected in 66% of the investigated strains. It was found that the marker was predominant in *C. coli* (82.9% positive isolates), whereas only 46.7% *C. jejuni* strains possessed this gene (P<0.05).

![Fig. 1. The presence of the genotypic virulence markers in *C. jejuni* and *C. coli* isolates analysed.](image)

**Table 2**

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Tested</th>
<th>Number (%) of strains Found positive by PCR for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>cdtA</em></td>
</tr>
<tr>
<td>Poultry carcasses</td>
<td>C. jejuni</td>
<td>52</td>
<td>49/52</td>
</tr>
<tr>
<td></td>
<td>C. coli</td>
<td>85</td>
<td>58/85</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>137</td>
<td>107/137</td>
</tr>
<tr>
<td>Faeces</td>
<td>C. jejuni</td>
<td>40</td>
<td>35/40</td>
</tr>
<tr>
<td></td>
<td>C. coli</td>
<td>20</td>
<td>9/20</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>60</td>
<td>44/60</td>
</tr>
<tr>
<td>Together</td>
<td>C. jejuni</td>
<td>92</td>
<td>84/92*</td>
</tr>
<tr>
<td></td>
<td>C. coli</td>
<td>105</td>
<td>67/105*</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>197</td>
<td>151/197</td>
</tr>
</tbody>
</table>

* P<0.05

**Discussion**

The research on the presence of potentially pathogenic *Campylobacter* in domestic animals and in food of animal origin is fundamental to consumers’ safety. The analysis of risk caused by the prevalence of these microbes in broilers is particularly important. The identification and prevalence in food of *Campylobacter* strains possessing pathogenicity markers have not yet been a subject of broad analyses in Poland, although it is
a significant element of campylobacteriosis risk assessment. These markers and determinants show a potential Campylobacter pathogenicity for people; however, they do not determine direct bacterial virulence. The identification of specific nucleotide sequence encoding given virulence factor does not mean that the genes are expressed and their products are present. On the other hand, the negative result of PCR can show, apart from actual lack of determined sequence, also a defect of the chosen method or existing gene mutation that prevent the amplification with the used primers. Hence, when analysing the presence or lack of pathogenic markers in the examined group of Campylobacter isolates, one should take into consideration all the mentioned aspects.

In this study, seven genes important in the pathogenesis of campylobacteriosis were chosen on the basis of previously published data. Genetic virulence markers, participating in adhesion and colonisation (flaA, cadF), invasion (virB11), and toxins production (cdtA, cdtB, cdtC) were determined in a separated group of Campylobacter isolates belonging to two species: C. jejuni and C. coli, isolated from poultry faeces and carcasses. Moreover, the iut sequence that is probably connected with a diarrhoeal form of the disease was determined in the examined bacteria.

The involvement of the flaA gene in Campylobacter ability to colonise the mucosal surface was demonstrated previously (25, 27). The results of our research showed that this marker was present in all C. jejuni and C. coli strains tested. Bang et al. (2) examined the factor presence in C. jejuni and C. coli derived from swine and cattle (together 40 specimens), and obtained the same results, i.e. 100% positive isolates. Datta et al. (8) also determined this factor in the group of 111 C. jejuni strains isolated from human clinical specimens, poultry carcasses, faeces, and cattle, and found that all of them were positive. All these results may suggest that the flaA gene product is necessary in bacterial colonisation of animal alimentary tract and allows bacteria to adhere to the surface of contaminated poultry carcasses. Other virulence markers, e.g. the cadF gene product, may also participate in this process. A recent study showed that CadF protein was responsible for the initiation of interaction between bacterial cells and host epithelium surface receptors (20). The cadF gene is highly conservative among C. jejuni and C. coli species, which may suggest its crucial role in the campylobacteriosis development (14, 20). As detected in the present study, the cadF gene was identified in 100% of the examined C. jejuni and C. coli isolates, derived both from poultry carcasses and faeces. Other authors, who determined virulence markers in all or almost all strains isolated from poultry carcasses, faeces, and from human clinical specimens, obtained very similar data (2, 8, 14). A research conducted by Rozynek et al. (23) on Polish C. jejuni and C. coli isolates showed that all 92 analysed strains deriving from poultry carcasses possessed the cadF gene, and 79 from 80 Campylobacter strains, which probably were the cause of children diarrhoea, also had this virulence marker (23).

The next virulence marker examined in the present study was one of the markers determining the invasiveness of Campylobacter isolates – the virB11 gene, localised on the pVir plasmid. This gene was present in 41 from 197 (20.8%) analysed bacterial specimens, i.e. in 18.5% of C. jejuni and 22.9% strains of C. coli, respectively. These figures are slightly higher than the values described by other authors. Datta et al. (8), who analysed different Campylobacter strains found that the virB11 gene was present in 9.5% to 15.4% of isolates, depending on the source. Bang et al. (2), applying PCR to determine the presence of this genetic virulence factor, obtained 7.5% positive results. Tracz et al. (26) conducted research on 104 C. jejuni isolates derived from people and identified the pVir plasmid in 17% strains. A relationship between this marker and occurrence of blood in faeces of patients with campylobacteriosis symptoms was also determined. However, this correlation was absent when considering other clinical symptoms, such as diarrhoea, fever, or vomits. These authors suggested that products of the pVir plasmid genes may have a significant role in serious cases of disease caused by C. jejuni (26). On the other hand, research conducted by Louwen et al. (18) on the strains of the same species derived from 125 patients, did not confirm these observation because only a single patient among 48 persons with blood in faeces, was infected by the virB11-positive C. jejuni. In total, the marker was identified only in 3% of the examined Campylobacter isolates. Schmidt-Ott et al. (24) showed even a smaller percentage of virB11-positive strains (1.8%) among 56 C. jejuni strains isolated from patients with bloody or watery diarrhoea. The important role of the pVir plasmid and its virulence factors in the disease development might be questioned, especially in some isolates, when taking into consideration the above-mentioned results. Moreover, some authors showed that Campylobacter derived from symptomatic patients possessed more frequently other plasmids. The products of these plasmids may also play a role in the bacteria invasiveness. The reasons of this discrepancy are not known. It might be the result of geographical differences, progression of disease in particular patients, or the effect of different target sequence used by other authors, and different gene amplification conditions applied in the virB11 identification. According to these arguments, it is necessary to conduct further examinations of the virB11 gene in C. jejuni and C. coli, as well as the research on the role of pVir plasmid in the pathogenesis of campylobacteriosis.

Toxins produced by Campylobacter might be another factor, which potentially plays a role in the disease development. CDT toxin composed of three subunits - CdtA, CdtB, and CdtC, is one of the best analysed. All the mentioned proteins are necessary to induce cytotoxic effect in vitro. However, some authors suggest that only CdtB subunit has an enzymatic activity and mediates DNA degradation and cell cycle blocking. It is supposed that two remaining proteins take a role in CdtB subunit transport to host cell (11, 16, 17). During the present study, three genes, the expression of which is necessary to activate CDT toxin synthesis, were
the high prevalence of the widespread among the isolates from poultry. Especially, that these putative pathogenic determinants are chromosomal genetic marker is associated preferentially on a similar level (15).

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present study, a high genetic variation in the C. jejuni and C. coli population was found.

In conclusion, the high prevalence of the seven virulence and toxin Campylobacter genes demonstrated that these putative pathogenic determinants are widespread among the isolates from poultry. Especially, the high prevalence of the cdf, flaA, and cdt genes demonstrated that these markers may play an important role in C. jejuni and C. coli virulence. Moreover, in the present study, a high genetic variation in the C. jejuni and C. coli population was found.

References


5. CAMPYNET: http://campynet.vetinst.dk/Fla.htm


examined. The majority of the evaluated isolates possessed the cdtB (85.3%) and cdtC (83.2%) genes, whereas the cdtA marker was present in 76.6% isolates. It should be emphasised that more than a half of Campylobacter isolates possessed all three toxin subunit genes together. If species affinity was taken into consideration, a higher percentage of C. jejuni (from 85.9% to 92.4%) than C. coli (63.8% – 84.8%) isolates demonstrated positive reaction towards the cdt genes (Table 2). These results are in agreement with the data obtained by other authors, who examined the presence of three mentioned genes in C. jejuni and C. coli derived from different sources (2, 8). Very few data published in Poland showed that the cdtA, cdtB, and cdtC genes were present in 77.5%, 93.8%, and 77.5% of isolates, respectively, derived from children with diarrhoea (sample number - 80) and in 98.9% of isolates recovered from poultry carcasses (n = 92). These results were obtained, similarly as in the present study, by the amplification of the respective genes with the PCR method (23). Martinez et al. (19) developed multiplex PCR to determine the presence of CDT markers, which allowed testing all the variants of cdt during a single analysis and additionally as a reaction control – a part of the 23S rRNA Campylobacter gene. However, it has been proved that simultaneous amplification of four genes can cause a decrease in the specificity and sensitivity of the test. The multiplex PCR test developed by these authors was used to analyse 100 C. jejuni isolates derived from humans and animals, and the cdtA, cdtB and cdtC genes were determined in 98% of the examined strains. On the other hand, Eyigor et al. (9), using the PCR method, determined the presence of the cdtB gene in 100% of 105 C. jejuni and C. coli isolates originating from poultry carcasses. Moreover, almost all of the tested C. jejuni strains produced CDT toxin on a high level, in contrast to a small percentage of C. coli, as showed by the production of a cytotoxic effect on HeLa cells. Dassanayake et al. (7) confirmed these results by testing the presence of the cdtB gene and CDT toxin activity in C. jejuni and C. coli strains derived from monkeys.

The other putative virulence gene, i.e. iam connected with diarrhoea symptoms in patients infected with Campylobacter, was also detected in the present study. The studies in vitro (6) showed that this chromosomal genetic marker is associated preferentially with both adherence and invasion. We observed the iam PCR product in 130 of 197 tested strains. In the other PCR studies performed in Poland, this gene was found on a similar level (15).

In conclusion, the high prevalence of the seven virulence and toxin Campylobacter genes demonstrated that these putative pathogenic determinants are widespread among the isolates from poultry. Especially, the high prevalence of the cadF, flaA, and cdt genes demonstrated that these markers may play an important role in C. jejuni and C. coli virulence. Moreover, in the present study, a high genetic variation in the C. jejuni and C. coli population was found.


