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Original article

Determination of the cytotoxic activity of *Campylobacter* strains isolated from bovine and swine carcasses in north-eastern Poland

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Abstract

The study was carried out to determine the cytotoxin production by *Campylobacter* spp. isolated from slaughtered cattle and swine in north-eastern Poland. In total three commercial slaughterhouses were sampled during one year. Carcass swabs were taken to detect the level of *Campylobacter* spp. contamination. *Campylobacter* spp. was found in 50 (34%) out of 147 swine carcasses examined. PCR analysis revealed 4 (8%) isolates to be *C. jejuni*, and 46 (92%) to be *C. coli*. From a total of 373 bovine carcasses, *Campylobacter* spp. were isolated from 49 (13.1%) samples. The results regarding the occurrence of *cdt* genes associated with cytotoxicity indicated that 100% of *C. jejuni* and 67.4% *C. coli* obtained from pigs had all three *cdtA*, *cdtB* and *cdtC* genes. In case of *C. jejuni* strains isolated from cattle all *cdt* genes were confirmed in 93.9% isolates. The isolates possessing all *cdt* genes had higher cytotoxic activity against cell lines used. The isolates both from cattle and swine were characterized by the highest cytotoxicity against HeLa cells. The values obtained reached 80.8% for *C. jejuni* isolates from cattle and 76.2% for *C. jejuni* and 69.0% for *C. coli* isolates from swine. High prevalence of cytotoxicity in *Campylobacter* spp. indicates a significant epidemiological role of this pathogen in human infections.

Key words: *Campylobacter* spp., livestock, cytolethal distending toxin, cytotoxic assay

Introduction

Campylobacter species is considered to be the most common cause of bacterial foodborne illnesses causing gastroenteritis in humans (Nic Fhogartaigh and Dance 2013). The wide prevalence of *Campylobacter* spp. in animal population carries the risk of contamination of food products such as raw or uncooked meat, milk and water (Wieczorek and Osek 2013). Poultry and poultry meat are described as the

common source of infection (Habib et al. 2012, Wai et al. 2012). The studies conducted by Petruzzelli et al. (2014) indicate that *Campylobacter* infections in humans are associated with the consumption of raw or undercooked beef or pork. Sheppard et al. (2009) found the poultry and cattle isolates based on clinical cases of campylobacteriosis.

During colonization of the gastrointestinal tract, the bacteria of *Campylobacter* spp. are predicted to express several putative virulence factors. The ability

Table 1. PCR primers used in the study.

Target Gene	Sequences (5' – 3')	PCR product size (bp)	Annealing temperature °C	References
<i>16S rRNA</i> for <i>Campylobacter</i> spp.	F – ATCTAATGGCTTAACCATTAAC R – GGACGGTAACTAGTTTAGTATT	857	58	Wieczorek and Osek (2005)
<i>mapA</i> for <i>C. jejuni</i>	F – CTATTTTATTTTGGAGTGCTTGTG R – GCTTTATTTGCCATTTGTTTTATTA	589	58	Wieczorek and Osek (2005)
<i>ceuE</i> for <i>C. coli</i>	F – AATTGAAAATTGCTCCAACATG R – TGATTTTATTATTTGTAGCAGCG	462	58	Wieczorek and Osek (2005)
<i>cdtA</i>	F – CCTTGTGATGCAAGCAATC R – AACTCCATTTGCTTTCTG	370	49	Datta et al. (2003)
<i>cdtB</i>	F – CAGAAAGCAAATGGAGTGTT R – AGCTAAAAGCGGTGGAGTAT	620	51	Datta et al. (2003)
<i>cdtC</i>	F – CGATGAGTTAAAACAAAAAGATA R – TTGGCATTATAGAAAATACAGTT	182	47	Datta et al. (2003)

of toxin production is the one that plays a special role in the pathogenesis of bacterial infections. Cytolethal distending toxin (CDT) is the only validated toxin of *Campylobacter* spp. The CDT is composed of three subunits CdtA, CdtB, and CdtC encoded by adjacent genes *cdtA*, *cdtB* and *cdtC* (Chae et al. 2012). Expression of all three genes is required to obtain full toxic activity. The CDT blocks cell proliferation by activation of damage of double-stranded DNA and the cell cycle arrest induced by this toxin is similar to that induced by ionizing radiation (Ripabelli et al. 2010). Except from the confirmation of pathogenic nature of *Campylobacter* spp. strains by the methods of molecular biology for the presence of *cdtA*, *cdtB* and *cdtC* genes responsible for the production of cytotoxins, *in vitro* cell culture methods are used. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay is confirmed to be an effective indicator of the toxicity of *Campylobacter* while detecting the effects of the cytotoxins in *in vitro* cell models (Malagon et al. 2010).

The aim of the study was to evaluate the cytotoxic activity among *Campylobacter* isolates from bovine and swine carcasses. PCR was applied to determine the prevalence of genes responsible for cytotoxin production among the strains isolated from cattle and swine. Furthermore, the expression of CDT activity in the culture supernatants of *Campylobacter* spp. isolates was tested for cytotoxicity assay with CHO, Vero and HeLa cells.

Materials and Methods

Bacterial isolates

The samples were taken in three slaughterhouses in the north-east of Poland from January to Decem-

ber 2012. The study material consisted of 147 swine and 373 prechill bovine carcasses after washing. The swabs from four different sites (neck, arm, loin and ham) with a total area of 400 cm² were taken from each carcass. The swabs were placed in a sterile bag with 400 ml of 0.1% sterile peptone water. The suspension was transported to the laboratory at 0 – 5°C and cultured immediately. In order to detect the presence of *Campylobacter* in the test samples 2 ml of the suspension was transferred to 18 ml of Bolton broth (Oxoid). The enrichment cultures were grown for 18 h at 42°C under microaerobic conditions (5% O₂, 10% CO₂ and 85% N) and then plated on to Karmali agar (Oxoid) and mCCDA (charcoal cefoperazone deoxycholate modified agar, Oxoid). All isolates were incubated under previously described conditions for 24 – 48 h. Plates were examined for morphologically typical *Campylobacter* colonies which were confirmed by microscopic morphology, motility, microaerobic growth at 25°C and the presence of oxidase. The isolates were subcultured only once in order to minimize cultural changes and then stored at -80°C in the defibrinated horse blood (Oxoid) with the addition of glycerol (80:20 v/v).

PCR reaction conditions

Species identification of *Campylobacter* spp. isolates from cattle and swine and the confirmation of the presence of *cdtA*, *cdtB* and *cdtC* genes responsible for cytolethal distending toxin production were performed based on PCR method. *Campylobacter* spp. isolates cultured on Columbia agar medium with blood (Oxoid) were suspended in 1 ml of sterile water, and centrifuged at 13 000 x g for 1 minute. The precipitate was suspended in Tris buffer. DNA isolation

was performed using Genomic – Mini Kit (A&A Biotechnology) according to the manufacturer's instructions. Purity and concentration of DNA obtained was determined spectrophotometrically. After appropriate dilution it was used in the PCR assay. Table 1 shows the characteristics of the primers used in the study.

Amplification was performed in a reaction mixture containing 5 µl of the PCR buffer (10 – times concentrated), 5 µl of dNTPs (final concentration of 200 µM), 0.5 µl of each primer (final concentration 0.1 µM), 10 µl MgCl₂ (final concentration of 5 mM), 2 µl (2 U) thermostable Taq polymerase (Fermentas), 5 µl of template DNA and DNase- and RNase-free deionised water to a final volume of 50 µl. All PCR reactions were carried out using the following conditions: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation for 1 min at 95°C, annealing at a temperature specific to the primer pair for 1 min and extension for 1 min at 72°C. The final elongation step was carried out at 72°C for 5 min. A positive control consisting of DNA extracted from *C. jejuni* ATCC 33291 and *C. coli* ATCC 43478 as well as a negative PCR control consisting of PCR-grade water were included in each PCR run. The PCR product was identified on a 2% agarose gel stained with ethidium bromide at a concentration of 5 µg/ml. Sizes of the amplification products obtained were compared with the 100 bp molecular weight marker.

Cytotoxicity assay

Determination of cytotoxicity of *Campylobacter* spp. isolates obtained from cattle and swine was performed on the following cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA): HeLa, Vero and CHO. Penicillin and streptomycin (both at 100 IU ml⁻¹, Sigma Aldrich) were added to protect against bacterial contamination. Incubations were conducted at 37°C in the incubator with 5% CO₂ flow and 95% humidity. All medium from the culture vessel was pulled down every 24 – 48 hours and the culture was supplemented with fresh medium. Cell monolayers were detached by addition of a trypsinizing solution (0.05% (w/v) trypsin + 0.02% (w/v) EDTA, (Sigma Aldrich) and gentle tapping of the flask. Cell aggregates in the suspension were dispersed by sterile – pipetting before the cells were washed in fresh medium.

The cell-free bacterial culture supernatants were used in the cytotoxicity assay. Isolates of *Campylobacter* spp. grown on mCCDA medium under microaerobic conditions at 37°C for 48 hours were suspended in PBS (phosphate buffered saline) and standardized spectrophotometrically to a concentration of

1 x 10⁹ bacteria ml⁻¹. The concentration obtained was confirmed by counting the colonies grown on solid media prepared by successive culture of the subsequent decimal dilutions. After the suspension concentration was standardized, 15% (w/v) of polymyxin B sulfate was added, in order to stimulate the release of cell-associated material. The suspension was incubated at 37°C for 30 min. and then centrifuged at 2 500 x g for 20 min. The supernatant was passed through a 0.45 µm filter (Millipore), and the filtrate was spent directly for further study. Each *Campylobacter* spp. isolate was tested in duplicate in three separately performed cytotoxicity tests.

Cytotoxicity assay was performed on HeLa, Vero and CHO cells lines according to the method described by Gilbert and Slavik (2004). Freshly trypsinized cells were suspended in the flat bottom 96 – well plates at a density of 2 x 10⁵ cells per well. After incubation, previously prepared polymyxin B extracts were added to each well. Then the MTT dye reduction assay was conducted in order to determine the percentage cells death by the measurement of absorbance in each well at a wavelength of A₅₄₀ with the use of Sunrise microtitre plate reader (Tekan). Cytotoxicity was expressed as percentage cell death and calculated base on formula given by Gilbert and Slavik (2004).

Statistical analysis

Tukey and Chi-square tests were used as appropriate. Statistical significance was defined as p<0.05.

Results

During the study period, a total of 147 swine carcasses were examined for the presence of *Campylobacter* spp. In total, 50 (34%) samples were positive for this pathogen. PCR analysis revealed affiliation of 4 (8%) isolates to *C. jejuni*, and 46 (92%) to *C. coli*. The results demonstrated that all *C. jejuni* isolates possessed genes *cdtA*, *cdtB* and *cdtC* involved in cytotoxicity (Fig. 1). Within *C. coli* isolates all *cdt* genes were found in 31 out of 46 (67.4%) isolates. Statistical differences in the prevalence of these genes were observed among both *Campylobacter* species (P < 0.05). All *Campylobacter* strains isolated from swine were tested for toxin production capacity using three cell lines. The CDT activity was defined when the cell – free bacterial culture supernatants caused destruction of over 30% cells. *C. jejuni* isolates showed cytotoxicity against CHO, Vero and HeLa cell lines at the average levels of 52.7%, 62.7% and 76.2%,

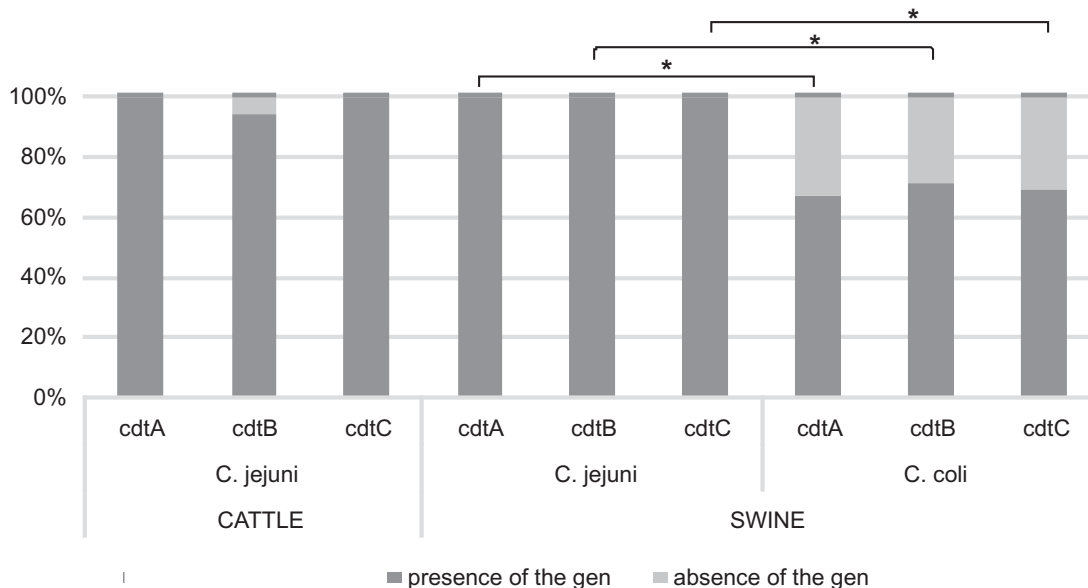


Fig. 1. The presence (%) of *cdt* genes in *Campylobacter* spp. isolated from cattle and swine. Asterisks indicate the significant differences obtained by Chi – square test ($p < 0.05$).

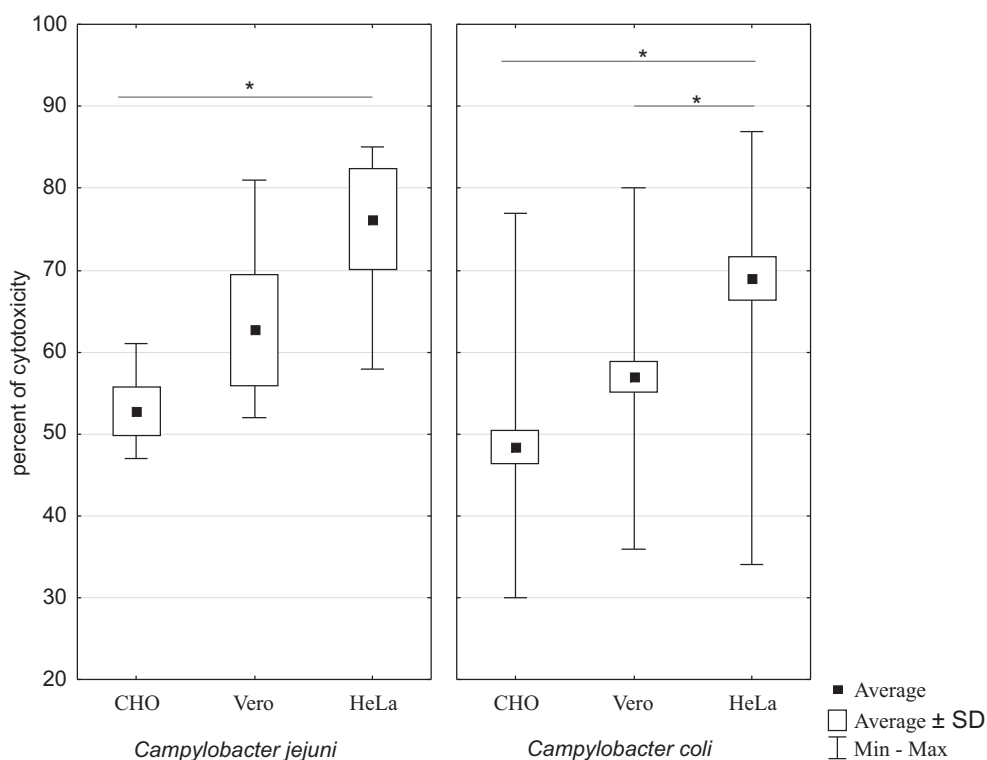


Fig. 2. The cytotoxicity (%) of *Campylobacter* spp. isolated from swine. Asterisks indicate the significant differences obtained by Tukey test ($p < 0.05$).

respectively. Statistically significant differences ($p < 0.05$) were noted between of HeLa and CHO cells. The cytotoxic activity of *C. coli* isolates was 48.4%, 57.0% and 69.0%, respectively to CHO, Vero and HeLa cells (Fig. 2). Remarkable differences ($p < 0.05$) were observed between cytotoxicity to

HeLa against CHO and Vero cells. None of the assays caused 100% cytotoxicity to the test cells over the 24 incubation period. The highest recorded values for *C. jejuni* isolates were 61% for CHO cells, 81% for Vero cells and 85% for HeLa cells. For *C. coli* isolates they were 77%, 80% and 87%, respectively.

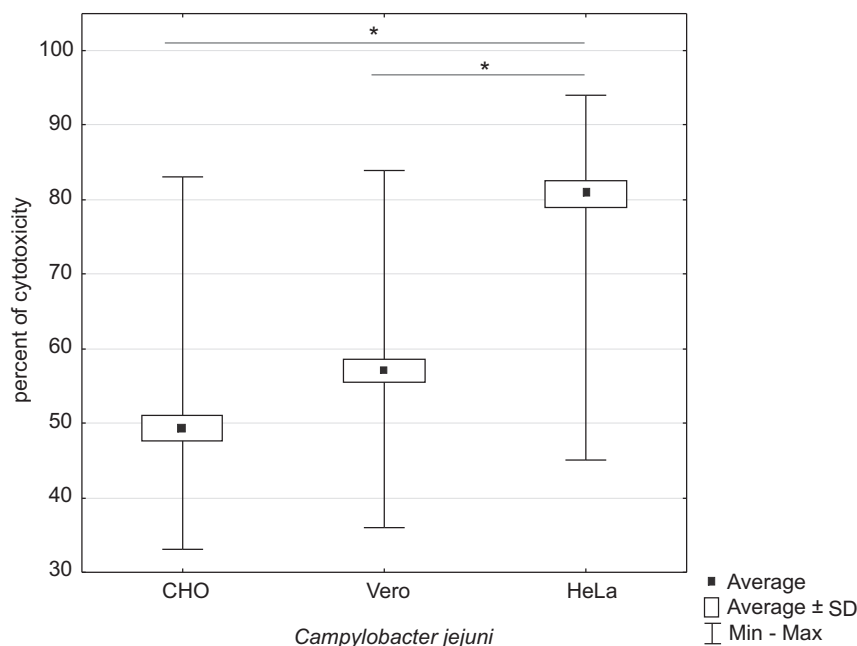


Fig. 3. The cytotoxicity (%) of *Campylobacter* spp. isolated from cattle. Asterisks indicate significant differences obtained by Tukey test ($p < 0.05$).

From a total of 373 bovine carcasses, *Campylobacter* spp. were isolated in 49 (13.1%) samples. All isolates were identified as *C. jejuni*. The presence of all three *cdtA*, *cdtB* and *cdtC* genes was confirmed in 46 (93.9%) isolates (Fig. 1). After examining cattle isolates in the *in vitro* assay, a significant difference ($p < 0.05$) in toxicity levels was observed to HeLa, CHO and Vero cells. Cytotoxic activity to HeLa cells was 80.8%, and to CHO and Vero lines the values were lower, reaching respectively 49.3% and 57.0% (Fig. 3). None of the tests performed showed death of all cells due to cell-free bacterial culture supernatants that were used. The highest noted cytotoxicity reached 94% for HeLa cells, 84% for Vero cells and 83% for CHO cells.

Discussion

An increasing number of recorded cases of campylobacteriosis in humans contributes to a greater interest of researchers in detecting potential reservoirs of *Campylobacter* spp. and its pathogenic mechanisms. Despite the high rate of *Campylobacter* spp. isolation from the intestine contents reaching 53.9% in cattle (Nesbakken et al. 2003) and up to 100% in swine (Pearce et al. 2003), the level of contamination of bovine and swine carcasses stands at a much lower level. Wiczorek and Osek (2010) confirmed the presence of *Campylobacter* spp. in 29.9% and 14.9% of the samples collected from swine and bovine carcasses.

These results are in line with own studies confirming the presence of *Campylobacter* spp. in 34% swine and in 13.1% bovine carcasses. However, research conducted by Ghafir et al. (2007) in Finland and Hakkinen et al. (2007) in Belgium showed lower coefficients of contamination of the surface of bovine carcasses, reaching respectively 3.55% and 3.3%. According to Borch et al. (1996), a significant reduction in contamination of carcasses by *Campylobacter* spp. is correlated with respect to strict procedures for the slaughter, proper scalding, skinning and cooling, and ensuring the avoidance of fecal contamination. Although the isolation rate of *Campylobacter* spp. in the samples obtained from cattle and swine is lower compared to the samples from poultry, showing up to 100% contamination, livestock can not be underestimated as a source of *Campylobacter* infection.

Species identification showed that 100% of bovine isolates belonged to *C. jejuni*. Among swine 8% of isolates were identified as *C. jejuni* and 92% as *C. coli*. The results obtained are in line with studies of other authors, which showed the dominance of *C. jejuni* in cattle and *C. coli* in swine (Ghafir et al. 2007, Wiczorek and Osek 2013).

Tissue culture techniques are successfully used in the studies of bacterial toxins. The research conducted showed a correlation between the presence of the genes responsible for CDT production and *in vitro* cytotoxic effect against cell lines (Fig. 4). Analysis of the pathogenicity of 49 *C. jejuni* isolates derived from cattle showed the presence of *cdtA*, *cdtB* and *cdtC*

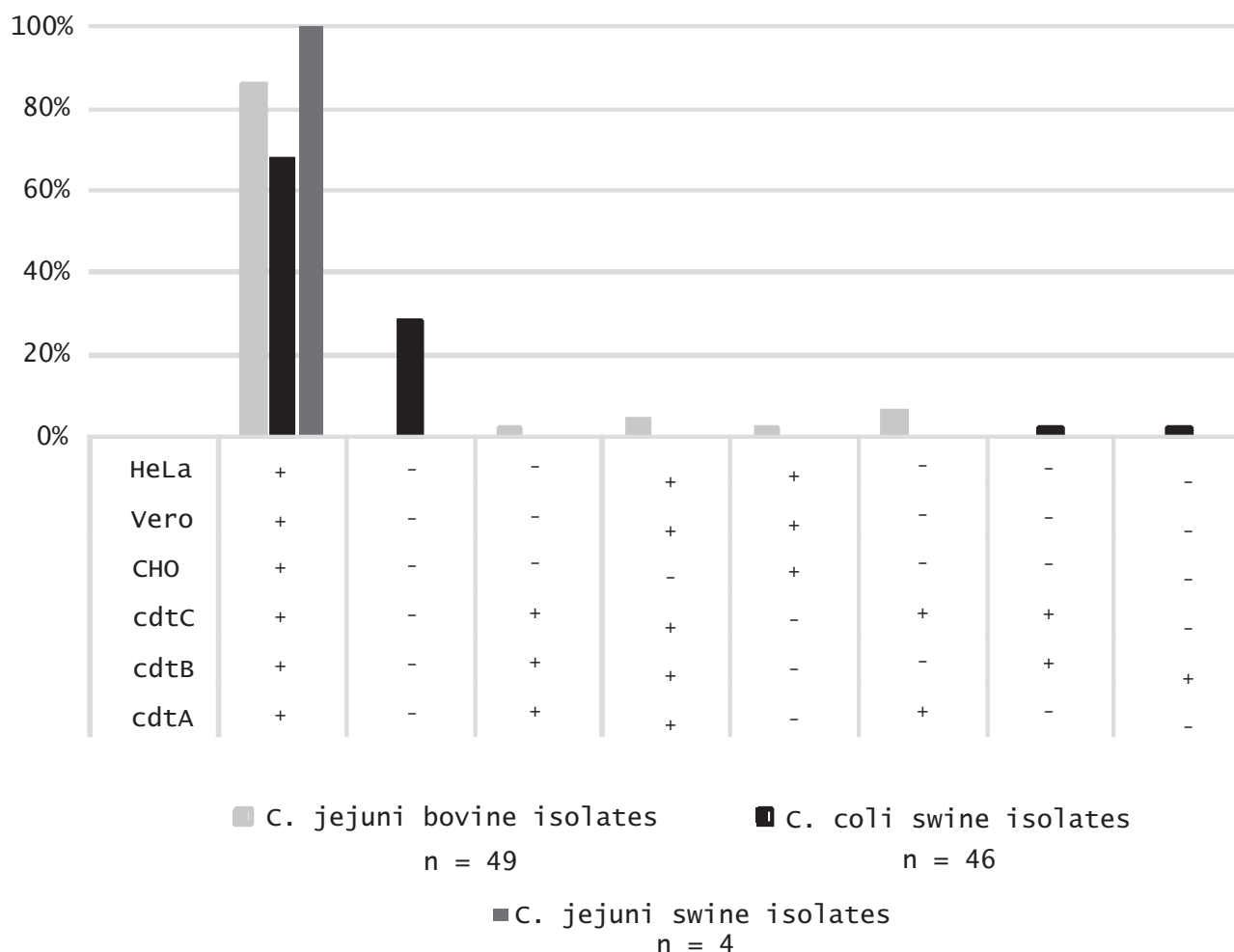


Fig. 4. Determination of cytotoxic activity of bovine and swine *Campylobacter* isolates. The presence (+) or absence (-) of *cdtA*, *cdtB*, *cdtC* genes intact cytotoxic activity on HeLa, Vero and CHO cells.

genes in 46 (93.9%) cases, among which cytotoxicity to at least one cell line was confirmed in 45 (97.8%) isolates. A similar correlation was observed regarding *Campylobacter* strains isolated from swine. All four isolates of *C. jejuni* were characterized by the presence of both *cdtA*, *cdtB* and *cdtC* genes and cytotoxic effect to the cell lines used. Among the 46 *C. coli* isolates the presence of all *cdt* genes was confirmed in 31 (67.4%) isolates, that all showed cytotoxic activity. However, not all researchers have obtained such a clear correlation between the presence of *cdt* genes and cytotoxic activity. Despite the high prevalence of all three, *cdtA*, *cdtB* and *cdtC*, genes in *C. jejuni* (80.0%) and *C. coli* (87.5%) strains isolated from slaughtered animals Ripabelli et al. (2010) reported low ability for toxin production in the cell lines running at 27.6% and 2.8% for *C. jejuni* and *C. coli* isolates, respectively. These authors point out that the lack of cytotoxicity found among strains with pathogenicity determinants may result from low toxin production. According to Jain et al. (2008) and

Gonzalez-Hein et al. (2014), this is CdtB cytotoxin subunit encoded by *cdtB* gene that plays an essential role in exerting a toxic effect on cells. This is reflected by own studies, which reported no cytotoxic effect of the cell-free supernatants of three *C. jejuni* isolates from cattle and having only *cdtA* and *cdtC* genes. Moreover, the above mentioned authors point out that the full toxic activity is conditioned by the presence of all three CDT subunits, because CdtA and CdtC are binding proteins for delivering CdtB into target cells. This is confirmed by the presented studies showing a lack of cytotoxic activity of two *C. coli* isolates from swine, that were missing at least one of these genes (Fig. 4).

Our findings confirm the relative higher prevalence of *cdt* genes and cytotoxin production in *C. jejuni* in comparison to *C. coli*, but these pathogenicity determinants are widespread among both strains of *Campylobacter*. In agreement with Ripabelli et al. (2010) and Zheng et al. (2006) all *C. jejuni* isolates from cattle and swine were positive for *cdtA*, *cdtB* and

cdtC genes. Furthermore, these isolates demonstrated a high cytotoxicity, showing no statistically significant differences between bovine and swine isolates ($p > 0.05$).

Various *in vitro* models have been, and are presently, used. HeLa, CHO and Vero are three commonly used cell lines. Moreover, Gilbert and Slavik (2004) showed that these lines are characterized by significant sensitivity to a majority of toxins produced by *Campylobacter* spp. Ripabelli et al. (2010) emphasized the legitimacy of the use of HeLa cells in cytotoxic assay. This observation is confirmed in the research conducted by Friis et al. (2005), that describe cell lines of human intestinal epithelium origin as the most appropriate for studying *Campylobacter* toxicity. However Al-Delaimi (2009) and Florin and Antillon (1992) suggest that Vero cells were more sensitive than HeLa and HEP-2 cells in detecting *Campylobacter* cytotoxin, Johnson and Lior (1988) reported that CHO cells were one of the most sensitive cell lines. Based on the above conclusions own research was focused on the problem of cell type-dependent cytotoxicity of *Campylobacter* spp. The results obtained show that cytotoxic activity was better observed in HeLa cells versus Vero and CHO cells ($p < 0.05$) in the isolates both from cattle and swine. However, it should be noted that the study was carried out on isolates obtained from cattle and pigs in comparison with the studies by Wassenaar (1997), conducted on poultry isolates that showed the highest incidence of cytotoxicity to CHO cells.

The studies presented have shown common prevalence of *cdt* genes and ability to produce a substance having cytotoxic effect in cell lines among bovine and swine isolates. Thus, the results obtained may suggest a significant role of cattle and swine as a source of cytotoxic *Campylobacter* spp. isolates that play significant role in development of infection. However, further research on other virulence factors involved in human infection seems to be advisable.

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