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Genotyping and PCR Detection of Potential Virulence Genes in *Campylobacter jejuni* and *Campylobacter coli* Isolates from Different Sources in Poland

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ABSTRACT. The prevalence of potential virulence markers was determined among the population of Polish *Campylobacter jejuni* and *Campylobacter coli* isolates from children, chickens, pigs and dogs. The presence of the *flaA*, *flaB*, *cdtA*, *cdtB*, *cdtC*, *cdtABC*, *virB11*, and *cj0588* genes among 74 *C. jejuni* and 15 *C. coli* isolates was detected by PCR. High prevalence of five different putative virulence and toxin genes (*flaA*, *cdtA*, *cdtB*, *cdtC*, and *cj0588*) was found among isolates obtained from children, chickens and dogs. The occurrence of these genes among isolates obtained from pigs was significantly different than for strains isolated from other sources. Two methods for genotyping *Campylobacter* spp. strains were applied – *flaA*-typing, and ADSRRS-fingerprinting method, which was used for the first time for *Campylobacter* spp. strains. Similarity of the genetic profiles was demonstrated in strains isolated from chickens and dogs, and in isolates from chickens and children. Strains isolated from pigs, both *C. jejuni* as well as *C. coli*, did not group with isolates from other sources.

Abbreviations

ADSRRS	amplification of DNA fragments surrounding rare restriction sites	CDT	cytolethal distending toxin
		PCR	polymerase chain reaction

Campylobacter jejuni and *Campylobacter coli* are recognized as one of the most frequent bacterial causes of food-borne gastroenteritis in humans (Allos 2001). Poultry is established as a common source of human infection caused by C*ampylobacter*, though other numerous reservoirs of infection, such as pets, unpasteurized milk and water, have been noted. Origins of human *Campylobacter* infection other than poultry meat have been suggested based on the different antimicrobial susceptibility patterns found between human and poultry strains (Luber *et al*. 2003). Specific virulence mechanisms of *Campylobacter* infection in humans are not yet well defined. The molecular genetics of *Campylobacter* has been extensively studied but the pathogenesis of *Campylobacter* infections is not clearly understood. Publication of the *C. jejuni* NCTC 11168 genome sequence (Parkhill *et al.* 2000) revealed the presence of several genes encoding proteins with potential virulence properties. Flagella-mediated motility, adherence to intestinal epithelial cells, invasion and survival in the host cells as well as the ability to produce toxins are important virulence factors which are involved in the pathogenesis of *Campylobacter* infections (Bang *et al.* 2003). The flagellin genes are the best-characterized virulence determinants of *Campylobacter* spp. The involvement of the *flaA* gene in *Campylobacter* colonization has been shown (Nachamkin *et al*. 1993). Mutants in the *flaA* gene are characterized by reduced motility and short flagella while *flaB* gene mutants have flagella of almost normal length, maintain their motility, but are characterized by a reduced virulence potential (Nachamkin *et al.* 1993). Several *Campylobacter* cytotoxins have been identified (Hänel *et al*. 1998) and the cytolethal distending toxin (CDT) has been characterized in detail (Pickett *et al*. 1996; Whitehouse *et al.*1998). CDT consists of three protein subunits, CdtA, CdtB, and CdtC, with CdtB recently identified as a nuclease. However, little is known about the functions of CdtA or CdtC. Expression of all three *cdt* genes is required for producing an active CDT which is lethal for host enterocytes (Lee *et al*. 2003; Lara-Tejero *et al*. 2001).

It was shown that 19–53 % of *Campylobacter* spp. strains contain plasmids of various sizes. Among them is the pVir plasmid with a G+C content of 26 %, which is lower than in the whole sequenced genome of the NCTC 11168 strain, suggesting acquisition of the plasmid *via* horizontal transfer (Bacon *et al.* 2002). The plasmid-encoded *virB11* gene is a marker potentially associated with the virulence of C*ampylobacter* spp. Bacon (2000) showed that a mutation in one of the four genes located in the plasmid – the *virB11*

homologue genes – resulted in a 6-fold reduction in adherence and an 11-fold reduction in invasion compared to the wild type. Another potential gene encoding a virulence factor is gene *cj0588*. Wooldridge *et al.* (2003) reported expression of this gene *in vivo* during infection. They have detected the presence of anti-Cj0588 antibodies in the blood serum of patients after *C. jejuni* infection. The *cj0588* gene was determined to encode a protein involved in adherence to Caco-2 cells (Sałamaszyńska-Guz and Klimuszko 2008).

Since *Campylobacter* may be transferred from animals to humans, it was interesting to know whether all *Campylobacter* isolates obtained from different sources have the same virulence factors. Subgrouping *Campylobacter* strains with respect to their virulence factors could be an important step in understanding the pathogenesis of the human disease (Nadeau *et al.* 2003). To address this matter, 74 *C. jejuni* and 15 *C. coli* isolates from chickens, pigs, dogs and children were tested. The presence of different putative virulence and toxin genes was detected by PCR assays. *Campylobacter* strains were analyzed using two genotyping methods, *fla*A-typing and ADSRRS-fingerprinting (amplification of DNA fragments surrounding rare restriction sites). The relationship between bacterial genotypes and the prevalence of selected genes among *C. jejuni* and *C. coli* isolates from different sources in Poland has not yet been studied.

MATERIALS AND METHODS

Bacterial strains. Isolates analyzed were collected in Poland in 2006–2007; in total, 89 isolates (obtained from chickens, pigs, dogs and children) were examined. Each of the twelve children isolates (11 *C. jejuni* and 1 *C. coli* stain) corresponded to patients with gastroenteritis. The 77 isolates of animal origin came from: chicken samples, collected from farms (44 *C. jejuni* and 11 *C. coli*), pig samples, from diarrheal stool samples (12 *C. jejuni* and 2 *C. coli*), dogs samples, from diarrheal stool samples (7 *C. jejuni* and 1 *C. coli*).

Growth conditions and identification of isolates. Samples were spread onto brain heart infusion agar BHI (*bioMérieux*) containing 5 % sheep blood and incubated in a microaerobic atmosphere for 24 h at 42 °C. Suspected colonies were confirmed as thermophilic *Campylobacter* species based on their characteristic cell morphology and Gram staining. Species identification was confirmed by API Campy test (*bioMérieux*) and multiplex PCR for simultaneous identification of *C. jejuni* and *C. coli*. *C. jejuni* ATCC 29428 reference strain was used as a control.

Detection of virulence and toxin genes by PCR. Primer sequences of *flaA*, *flaB*, *cdtA*, *cdtB*, *cdtC*, *virB11*, *cj0588* genes and the *cdt* cluster and sizes of the PCR products used are shown in Table I. PCR-negative and -positive controls with DNA from reference strain were used. All the negative samples were further tested.

Table I. Primers used for detection of virulence and toxin genes

Gene	Sequence (5′→3′)	Product, bp	Reference
flaA	ATG GGA TTT CGT ATT AAC AC CTG TAG TAA ATC TTA AAA CAT TTT G	1700	Hänel *et al*. (2007)
flaB	AAG GAT TTA AAA TGG GTT TTA GAA TAA ACA CC GCT CAT CCA TAG CTT TAT CTG C	260	Goon *et al*. (2003)
cdtA	TCG ACT GTT CTT CTA AAT TTG AAA ATG TAA ATC C CGG CCG CTC ATC GTA CCT CTC CTT GGC G	765	Lee *et al*. (2003)
cdtB	CGA CAA TTT TAA TGT TGG CAC TTG G CGG CCG CAT GTC CTA AAA TTT TCT AAA ATT TAC TGG	750	Lee *et al*. (2003)
cdtC	TCG ACG CAA CTC CTA CTG GAG ATT TGA AAG CGG CCG CAT CTT ATT CTA AAG GGG TAG CAG C	495	Lee *et al*. (2003)
cdtABC	GGA AAT TGG ATT TGG GGC TAT ACT TTG CAC ATA ACC AAA AGG AAG	1215	Bang *et al*. (2003)
virB11	GAA CAG GAA GTG GAA AAA CTA GC TTC CGC ATT GGG CTA TAT G	708	Bacon *et al*. (2000)
cj0588	ATG AGA TTT GAT TTT TTT GTT TCA ATT TTT GAT ATA GTA GTA AA	770	Sałamaszyńska-Guz and Klimuszko (2008)

flaA-*typing and RFLP analysis*. Chromosomal DNA was extracted from *C. jejuni* and *C. coli* cells using a Genomic Mini kit (*A&A Biotechnology*, Poland). PCR-RFLP profiles of the *flaA* gene were performed according to Nachamkin *et al*. (1993) with modifications (Petersen and On 2000). PCR products (1.7 kb) were digested with *Alu*I and analyzed by electrophoresis.

ADSRRS-fingerprinting was done according to Krawczyk *et al.* (2003) using two restriction enzymes: *Xba*I and *Bgl*II, the adapters assembled from two oligonucleotides (Table II). PCR was done using the cycling conditions specified by Krawczyk *et al.* (2003). The amplified products were electrophoresed in polyacrylamide gels. Dendrograms were generated using the nearest neighbor method, squared Euclidean using the Statistica 6.0 program.

Table II. Adapters and primers used in ADSRRS-fingerprinting

Adapters and primers	Sequences (5′→3′)
*Xba*I short adapter	CTA GGT CGA CGT T CCT TCA TCC ACC AAC GTC GAC
*Bgl*II long adapter	GAT CCG TCG ACA ACG GCG TTC CTT CGT CTA CCA TCC GGA TGG TAG ACG AAG GAA CGC CGT TGT CGA CG
*Xba*I short primer	CCT TCA TCC ACC AAC GTC GAC
*Bgl*II long primer	CGC AAG GAA GCA GAT GGT AGG

RESULTS

Detection of different virulence and toxin genes by PCR. Results of PCR detection of eight putative virulence and toxin genes among *C. jejuni* and *C. coli* strains isolated from chickens, pigs, dogs and children stool samples are summarized in Table III. A single PCR amplicon of the *flaA* gene with expected size of 1700 bp was observed in all *C. jejuni* and *C. coli* strains. The *flaB* gene was found in 100 % isolates from dogs, 87.3 % from chickens, 75 % from children and 71.4 % from pigs.

Among isolates from children with diarrhea the presence of *cdt* genes and the *cdt* gene cluster was detected in 100 % of the samples. This concerns both – *C. jejuni* strains as well as the one strain of *C. coli.* Among *C. jejuni* and *C. coli* strains isolated from pigs the presence of *cdtA*, *cdtB*, *cdtC* and the *cdt* gene cluster was at a surprisingly low level (28.6, 50, 57.1 and 50 %, respectively). One *C. jejuni* pig isolate was positive for of all the *cdt* genes, and for all eight putative virulence genes detected in this study. A similar frequency of *cdtA*, *cdtB*, *cdtC* and the *cdt* gene cluster was observed in dog and chicken isolates (75, 87.5, 75, 87.5 % for dog isolates and 70.9, 83.6, 87.3, 87.3 % for chicken isolates).

The highest prevalence of the *virB11* gene was observed in *Campylobacter* isolates from pigs (35.7 %), dog (25) and chicken (12.7).

Analysis of the prevalence of the *cj0588* gene revealed that all of the *Campylobacter* strains isolated from children carried this marker. Only 7.1 % of pig isolates possessed this gene. For dog and chicken isolates, a similar frequency (87.5 and 72.7 %) of these genes was noted.

flaA-*typing* (Fig. 1). The presence of eleven main phylogenetic groups of *C. jejuni* strains isolated from different sources was demonstrated at 0 % variability, indicating complete homogeneity. The remaining five groups contained isolates representing individual genetic profiles (Fig. 2A).

The characteristic features of the *C. jejuni* isolates from sixteen groups (I–XVI) are presented in Table IV. The presence of six main phylogenetic groups (from A to F), including one containing a strain with an individual genetic profile, was determined for *C. coli* isolates (Fig. 2B; Table IV). Two phylogenetic groups of *C. coli* strains exhibiting complete homogeneity were identified among isolates obtained from different sources: chickens + dogs and chickens + children. Strains isolated from pigs both *C. jejuni* and *C. coli*, differed compared to isolates from other sources.

ADSRRS-fingerprinting patterns (Fig. 3) of *C. jejuni* and *C. coli* isolates revealed the presence of many scarcely numbered phylogenetic groups (from 1Cj to 29Cj for *C. jejuni* and from 1Cc to 9Cc for *C. coli*). Strains isolated from different sources were classified as distinct types. None of the patterns indicated a predominant genotype. Dendrograms based on the genetic profiles are shown in Fig. 4.

DISCUSSION

High percentage of the *flaA* gene among the tested strains isolated from different sources indicates the important role of the respective gene product in *Campylobacter* pathogenesis. Similar observations have been reported by Bang *et al*. (2003). Molecular genetic approaches with defined mutants showed that *flaA* is essential for colonization (Nachamkim *et al.* 1993). The complex flagellum of *Campylobacter* spp. is encoded by two tandem-oriented flagellin genes (*flaA* and *flaB*). While the function of the *flaA* gene seems to be fully elucidated, there are many speculations as to the *flaB* gene function, *e.g*., a role in antigenic variation or influence on motility in various environmental conditions (Wassenaar *et al*. 1994).

Other potential virulence markers analyzed in this study were the *cdt* genes and the *cdt* gene cluster. Cytolethal distending toxin causes direct DNA damage leading to induction of DNA damage checkpoint pathways (Lee *et al*. 2003). Different combinations of *cdt* gene primers were applied to detect the presence of *cdtA*, *cdtB*, *cdtC* and the *cdt* gene cluster sequences in the DNA isolated from all strains. The *cdt* genes were shown to be conserved among different *Campylobacter* strains (Fouts *et al*. 2005). Bang *et al*. (2003) observed that the prevalence of these genes in isolates from different sources exceeds 90 %. Based on results obtained in this study (Table III), the presence of the examined genes depended on the source from which the isolates were obtained. The presence of *cdt* genes and the *cdt* gene cluster was detected in 100 % of *Campylobacter* isolates from children with diarrhea. Rożynek *et al*. (2005) obtained similar results for *C. jejuni* strains isolated from children with diarrhea, with the percentage of strains containing *cdtA*, *cdtB*, *cdtC* and the *cdt* gene cluster accordingly 98.4, 97, 98 and 98 %. However, in *C. coli* isolates from children with diarrhea (n = 18) the detection rates of these genes were much lower (5.6, 83, 5.6, and 98 %) (Rożynek *et al.* 2005). On the other hand, a similar frequency of *cdt* genes and the *cdt* gene cluster which was observed in dog and chicken isolates may suggest that contaminated chicken meat is the source of *Campylobacter* for dogs.

Bacon *et al*. (2000) was the first to investigate the possible involvement of a plasmid in the virulence of *C. jejuni*. The occurrence of the *virB11* gene was determined in the genomes and it was found that *virB11* is localized on the pVir virulence plasmid. The 16.7 % prevalence of the *virB11* gene in children isolates

Table III. PCR detection of putative virulence and toxin genes[a] in *C. jejuni* and *C. coli* isolates (n = number of isolates)

Source	Species	n	*flaA*		*flaB*		*cdtA*		*cdtB*		*cdtC*		*cdtABC*		*virB11*		*cj0588*	
Chickens	*C. jejuni*	44	44	100	38	86.4	28	63.6	36	81.8	37	84.1	37	84.1	6	13.6	36	81.8
	C. coli	11	11	100	10	90.9	11	100	10	90.9	11	100	11	100	1	9.1	4	36.4
	total	55	55	100	48	87.3	39	70.9	46	83.6	48	87.3	48	87.3	7	12.7	40	72.7
Pigs	*C. jejuni*	12	12	100	8	66.7	3	25.0	6	50.0	6	50.0	6	50	5	41.7	1	8.3
	C. coli	2	2	100	2	100	1	50	1	50	2	100	1	50	0	0	0	0
	total	14	14	100	10	71.4	4	28.6	7	50	8	57.1	7	50	5	35.7	1	7.1
Dogs	*C. jejuni*	7	7	100	7	100	6	85.7	7	100	6	85.7	7	100	1	14.3	7	100
	C. coli	1	1	100	1	100	0	0	0	0	0	0	0	0	1	100	0	0
	total	8	8	100	8	100	6	75	7	87.5	6	75	7	87.5	2	25	7	87.5
Children	*C. jejuni*	11	11	100	9	75	11	100	11	100	11	100	11	100	2	16.7	11	100
	C. coli	1	1	100	0	0	1	100	1	100	1	100	1	100	0	0	1	100
	total	12	12	100	9	75	12	100	12	100	12	100	12	100	2	16.7	12	100

[a]*Left columns* – number of positively tested isolates, *right columns* – percentage (in *rows* evaluating a total of findings the right columns represent an average of positive detections in %); for further details *see* text.

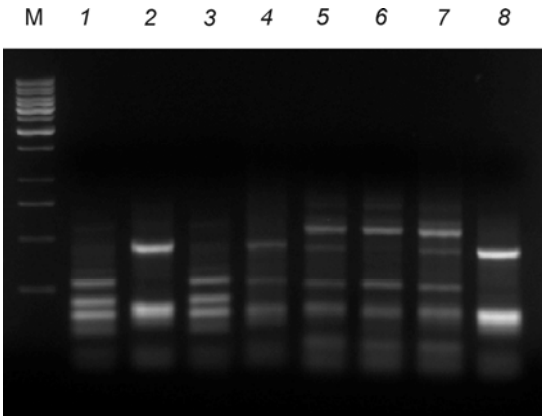

Fig. 1. *flaA*-typing for representative *Campylobacter* sp. isolates. M – molecular size marker (250–10000 bp), strains isolated: *1*, *5*, *6* – from dogs, *2*, *4* – from children, *3*, *7* – from chickens; *8* – from pig.

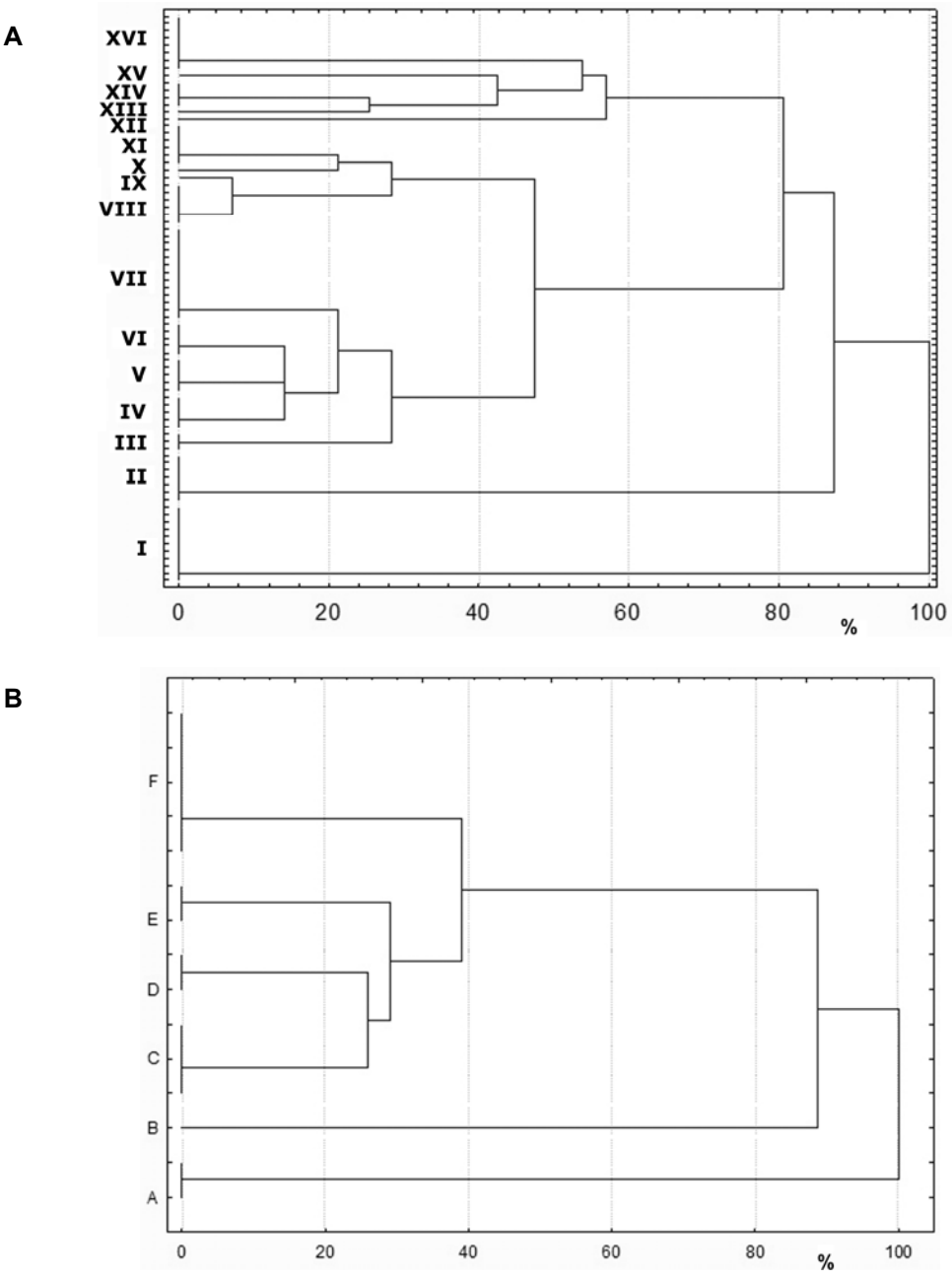

Fig. 2. Dendrogram generated using the nearest neighbor method, squared Euclidean distance measure, based on quantitative and qualitative differences between products obtained using *flaA*-typing for *C. jejuni* (**A**) and *C. coli* (**B**) isolates.

Table IV. PCR-RFLP analysis of *flaA* gene in isolates of *C. jejunii* (*flaA*-typing groups I–XVI) and *C. coli* (*flaA*-typing groups A–F)

Source of isolates	Number of *C. jejuni* isolates															
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
Chicken	–	7	3	–	–	3	8	5	1	1	6	1	–	–	1	8
Children	–	–	–	6	5	–	–	–	–	–	–	–	–	–	–	–
Dogs	–	–	–	–	–	2	3	–	–	–	–	–	2	–	–	–
Pigs	8	–	–	–	–	–	–	–	–	–	–	–	–	4	–	–

	Number of *C. coli* isolates					
	A	B	C	D	E	F
Chicken	2	1	3	1	–	4
Children	–	–	–	1	–	–
Dogs	–	–	–	–	–	1
Pigs	–	–	–	–	2	–

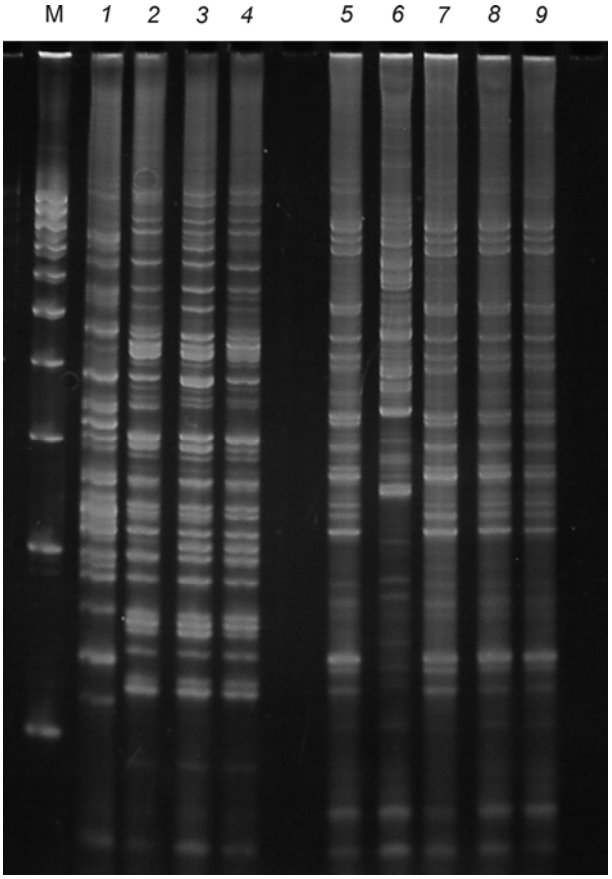

Fig. 3. ADSRRS-fingerprinting for representative *Campylobacter* sp. isolates. M – molecular size marker (100–1000 bp), *1–4* – strains isolated from chickens, *5–9* – strains isolated from dogs.

in this study is comparable with 10.3 % in humans reported by Bacon *et al*. (2000) but much lower than in pigs isolates (35.7 %). Until now, the role of the protein encoded by the *virB11* gene in the invasion and colonization process of eukaryotic cells by *Campylobacter* spp. rods has not been elucidated.

Another marker analyzed in our study was the *cj0588* gene. The product of this gene is homologous to TlyA proteins (found in *Brachyspira hyodysenteriae*, *Helicobacter pylori* and *Mycobacterium tuberculosis*) which were determined to play role in bacterial virulence. No difference in the hemolytic activity between *cj0588* gene mutant and the wild strain was found (Sałamaszyńska-Guz and Klimuszko 2008); however, involvement of the Cj0588 protein in adherence to Caco-2 cells was confirmed. The occurrence varied, depending on the origin of *Campylobacter* isolates and sources. It is difficult to elucidate such a sporadic occurrence of the *cj0588* gene among strains isolated from pigs. To determine the role of the Cj0588 protein in *Campylobacter* spp. virulence further studies will be needed.

flaA-*typing and RFLP analysis*. The majority of isolates, for which the typing indicated identity, were derived from the same source except for two groups, in which isolates had identical patterns but were from different sources, *i.e*. chicken and dogs. Isolates from chickens and children demonstrated 78 % of similarity, implicating chickens as potential source of sporadic human and dog infections. Strains from children and dogs exhibited 85 % of similarity. This suggests a transmission of *C. jejuni* from children with diarrhea to dogs or *vice versa*.

Zorman *et al*. (2006) reported identical or very similar PFGE-profile types among isolates from chicken, retail chickens meat and human samples, which indicates a transmission of *C. jejuni* and *C. coli* from farm chickens to retail chicken meat and humans.

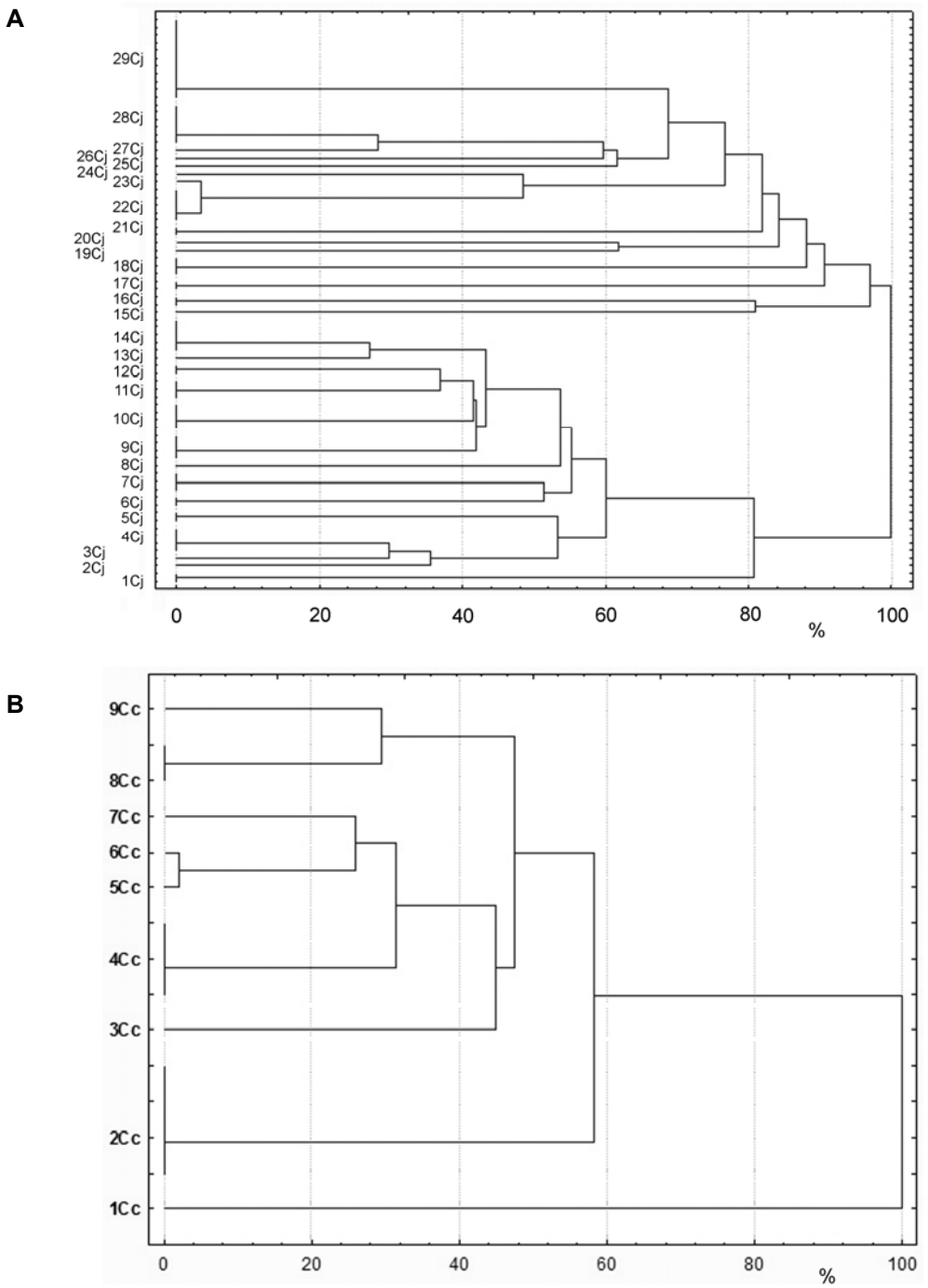

Fig. 4. Dendrogram generated using the nearest neighbor method, squared Euclidean distance measure, based on quantitative and qualitative differences between products obtained by ADSRRS-fingerprinting for *C. jejuni* (**A**) and *C. coli* (**B**) isolates. **A**: strains isolated from chicken: 6Cj, 10Cj, 12Cj, 14Cj–21Cj, 24Cj, 26Cj, and 29Cj, isolates from pigs: 5Cj, 11Cj, 27Cj, and 28Cj, isolates from dogs: 4Cj, 13Cj, and 25Cj, isolates from children: 1Cj–3Cj, 7Cj–9Cj, 22Cj, and 23Cj. **B**: strains isolated from chicken belong to 2Cc–7Cc groups, isolates from pigs – group 8Cc, dog isolate – 9Cc, children isolate – 1Cc.

ADSRRS-fingerprinting analysis. Strains isolated from children and dogs demonstrated 75 % of similarity. The AFLP study of animal sources of human campylobacteriosis (Siemer *et al*. 2001) showed a similarity between human profile of a *C. jejuni* strain and a dog profile described by Hald *et al*. (1996) in study on healthy puppies. Although such studies do not indicate the direction of transmission, the route of the spread is from pets to humans, as healthy human-*Campylobacter* carriers are uncommon (Jimenez *et al.* 1999).

Strains isolated from pigs differed in the genetic profiles compared to other isolates. They demonstrated 85 % of differences between strains isolated from chicken and almost 80 % of differences in respect to strains isolated from children. Such divergent genetic profiles could account for the fact that strains isolated from pigs are characterized by different virulence mechanisms.

ADSRRS-fingerprinting analysis of *C. coli* isolates showed a primary division of the dendrogram branches into two initial groups at the level of ≈60 % of differences for the first group. The single strain isolated from a child with diarrhea constituted already from the beginning a distinct group, demonstrating lack of homology compared to other groups.

The presence was noted of three phylogenetic groups of *C. coli* strains isolated from chickens and pigs. The remaining six isolates represented an individual genetic profile. *C. coli* strains isolated from pigs and dogs demonstrated 25 % of differences.

Besides biochemical (insufficiently accurate) and metabolite-based (*e.g.*, MALDI-ToF, reliable and highly specific; *cf.* Kolínská *et al*. 2008) identification method for distinguishing *Campylobacter* spp. strains, several genotyping methods have been developed on order to differentiate *Campylobacter* isolates (Wassenaar and Newell 2000; Fitzgerald *et al*. 2001). In this study, for the first time the ADSRRS-fingerprinting analysis was applied for genotyping *Campylobacter* spp. strains. We found that this method is rapid and offers good discriminatory power. Krawczyk *et al*. (2003) suggested that this novel method had a power of discrimination similar to "gold standard" for molecular typing – PFGE, and may appear to be more complex than the RAPD technique. We found this method to be fast and reproducible.

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