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# SUBTYPING OF *Campylobacter* spp. STRAINS AND THEIR INCIDENCE IN PIGLETS

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**Abstract**

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*Campylobacter* spp. are common cause of enteritis in humans, but the routes of transmission and the subtypes of these bacteria responsible for infection are not clearly known. Pigs, which are infected early in life, are known to be symptomless carriers of *Campylobacter* spp. We evaluated and subtyped 225 samples of rectal swabs from piglets with and without disease by polymerase chain reaction (PCR) and restriction fragment length polymorphism. Little difference was found in the presence of *Campylobacter* spp. in healthy and diseased piglets: *Campylobacter* sp. was found in 49% of 225 samples of rectal swabs from piglets with enteric disease, *C. coli*, *C. jejuni* and atypical strains representing 81%, 17% and 2%, respectively, and in 41% of rectal swabs from healthy piglets, *C. coli* and *C. jejuni* representing 85% and 15%, respectively. *C. coli* strains were subtyped from the PCR product with the *HinfI* restriction enzyme, and four subtypes were found and confirmed by sequencing. The most frequent were types III (46%) and I (31 %), whereas type II was found in 6% of strains only, and 17% of strains had no cutting site (type IV). Thus, both healthy and sick piglets had a high incidence of *Campylobacter* spp., which might result in contamination of pork during slaughter. No link was demonstrated between the incidence of individual subtypes of *C. coli* and the frequency or severity of disease on pig farms.

*Campylobacter coli, Campylobacter jejuni,* typing, *PCR-RFLP*

*Campylobacter jejuni* and *C. coli* are among the most common causes of acute bacterial enteritic diseases in humans. *Campylobacter* spp. commonly occur in the normal intestinal flora of a number of domestic as well as wild animals, and regularly reported sources of *Campylobacter* spp. infections in humans include inadequately cooked poultry and cross-contamination of cooked food or water. Frequent sources of *Campylobacter* spp. include not only poultry but also pigs. Like poultry, adult pigs have *Campylobacter* spp. as a part of their intestinal microflora without exhibiting any symptoms of disease. These bacteria are found at relatively frequently in the intestinal contents of pigs in slaughterhouses, with incidences of up to 92% (Harvey et al. 1999), but varying from 0 to 60% (Svedhem et al. 1981, Oosterom et al. 1985).

*C. jejuni*, *C. coli* and other *Campylobacter* spp. are found relatively frequently in animals and in the environment, as well as in human infections (Oosterom et al. 1985). The occurrence of *C. jejuni* in pigs appears to be increasing. In the older literature (Svedhem et al. 1981; Oosterom et al. 1985), *C. coli* was described as the dominant species in pigs, while *C. jejuni* was reported in connection with poultry; however, recent reports indicate that although *C. coli* is still the predominant species, *C. jejuni* accounts for 30–50% of findings in both healthy and sick pigs (Harvey et al. 1999). Other *Campylobacter* spp. particularly *C. lari* and *C. hyointestinalis*, have been isolated in pigs only sporadically. We (Steinhauserová 1998) have found that the incidence of infection with *Campylobacter*

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spp. among pigs on farms in the Czech Republic varies between 20 and 40% and that *C. coli* accounts for up to 90% of all strains isolated.

Colonization of the mucous membrane of piglet intestines occurs very early after birth. Because adult pigs carry *Campylobacter* spp., the pathogen is maintained in the environment and piglets come into contact with it very early in life. Harvey et al. (1999) found that intestinal colonization occurs 24–48 days after birth and that piglets 9–14 days of age may already excrete *Campylobacter* spp. It is not clear yet whether transmission is from faeces or associated with suckling. Weijetens et al. (1997) showed that 9 out of 10 sows carried *Campylobacter* spp. 1 week before farrowing, and up to 85% of piglets are infected with *Campylobacter* spp. during the first 4 weeks of life, with $\log_{10}$ 4.1 CFU/g of intestinal contents (Weijtens et al. 1993). The bacteria then survive throughout the animals' lifespan.

Epidemiological studies are needed to identify the routes and mechanisms by which *Campylobacter* spp. spread among animals, in order to design measures to limit or stop the spread (Svedhem and Kaijser 1981). Currently, only the species of isolated strains is identified, without more detailed description. Because *Campylobacter* strains differ in their pathogenicity and virulence, however, they must be subtyped for epidemiological purposes. The available typing techniques, including serotyping, phagotyping and biotyping, are neither suitable as routine procedures nor do they have the necessary discriminatory power. Therefore, various molecular-based genotyping techniques have been designed and tested.

The purpose of this study was to ascertain the incidence of *Campylobacter* sp. among piglets up to the age of 8 weeks and to subtype the *C. coli* strains by molecular methods.

## Materials and Methods

### Bacterial strains

A total of 225 samples of rectal swabs and intestinal mucous membrane from sick or dead piglets (up to 8 weeks of age) with clinical symptoms of enteritis and 75 samples from piglets without diarrhoea or other clinical symptoms of disease were examined. The samples were obtained in cooperation with the Department of Microbiology and Immunology, University of Veterinary and Pharmaceutical Sciences, Brno, from 23 farms in the Czech Republic in 1999, were transported in an Amies transport medium (CCM425, Oxoid Ltd, United Kingdom) and were tested for the presence of pathogenic agents within 48 h after collection.

Strains *C. jejuni* CCM 6212, *C. coli* CCM 6211, *C. upsaliensis* ATCC 43954 and *C. lari* CCUG 23947 were used as references.

### Culture and biochemical tests

The specimens were inoculated directly and after enrichment (BHI broth CM225) onto CCDA selective medium (Campylobacter Blood-free Agar Base CM739) with CCDA supplement (SR155). The cultures were incubated in a micro-aerophilic atmosphere (Gas pack, BR 60) at 37 °C for 48 h. All the media were obtained from Oxoid Ltd, United Kingdom. The species were identifed with the oxidase test, by catalase production, natrium hippurate and indoxyl acetate hydrolysis, hydrogen sulfite production on TSI agar, nalidixic acid and cephalothin sensitivity tests and growth at 42, 30 and 25 °C.

### DNA extraction

Bacterial DNA was extracted by a slight modification of the method of Sambrook et al. (1989). Briefly, colonies identified as *Campylobacter* spp. were suspended in Tris–HCl– EDTA buffer, pH 8.00, frozen and thawed repeatedly and digested with proteinase K at 37 °C. The DNA released was purified by extraction with phenol:chloroform:isoamylalcohol (Serva, Germany) and chloroform and then precipitated with absolute ethanol. The DNA pellet was dissolved in 40 ml of Tris-Hcl- EDTA buffer and stored at -20 °C.

### Polymerase chain reaction

All strains classified biochemically as *Campylobacter* spp. were tested by the polymerase chain reaction (PCR). The CJF1 and CJF2 oligonucleotide primers from the flagelline A gene sequences (*fla A*) described by Itoh *et al.* (1995) were used. The assumed length of the product was 410 base pairs.

The reaction mixture (25 µ1) contained 10 mmol.l$^{-1}$ Tris-HCl (pH 9.0 at 25 °C), 50 mmol.l$^{-1}$ KCl, 1.5 mmol.l$^{-1}$ $MgCl_2$, 0.1% Triton X-100, 200 µmol.l$^{-1}$ each of the deoxynucleotides dATP, dCTP, dGTP and dTTP, 20 µmol.l$^{-1}$ each of CJF1 and CJF2 primers (Sigma-Aldrich, USA), 1.25 U of Taq polymerase (Promega, USA) and 1 µg of template DNA, made up to a final volume of 25 µ1 with water. PCR amplification was performed in a 200PTC

thermocycler (MJ Research, USA) under the following conditions: 94 °C for 4 min, 30 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 30 s and final amplification at 72 °C for 10 min. A negative control with no template DNA was prepared. After cycling, the products were visualized by electrophoresis (12 $V.cm^{-1}$) in a 1.5% (w/v) agarose gel (Serva, Germany) with 1 $g.l^{-1}$ ethidium bromide. *pUC/Hae III* (Sigma, USA) was used as the molecular-size marker.

PCR-restriction fragment length polymorphism (RFLP) was used for differentiation of thermophilic *Campylobacter* spp. The PCR–RFLP reaction mixture contained 4 µl of PCR product and 1 U of restriction enzyme *HinfI* (TaKaRa, Japan) and was made up to a final volume of 20 ml. The reaction mixture was incubated at 37 °C overnight, and the products were analysed on polyacrylamide gel (FMC BioProducts, USA).

### Sequencing of amplified products

Restriction sites on *C. coli* for *Hinf* I were confirmed by sequencing, and four strains representing the RFLP types found were selected. The Original TA Cloning® Kit (Version G, Invitrogen, USA) was used for cloning. After checking the amplification on agarose gel, DNA was ligated with Topo TA vector, which was then used to transform the Top 10 F' cells. The transformed cells were placed on Petri containing Luria-Bertoni agar, ampicillin (100 $\mu g.ml^{-1}$) and a mixture of X-gal and IPTG and incubated at 37 °C for 16-20 h. After incubation, four white colonies with an insert were placed in Luria-Bertoni broth and incubated for 16 h. Plasmids were isolated from a portion of the cells by rapid alkaline lysis. Positive transformants were further confirmed according to the size of split inserts on agarose gel. Plasmids for sequencing were isolated with the Qiagen Plasmid Mini Kit.

The sequences of the two DNA chains were ascertained on an ABI PRISM 310, Version 3.0 (Perkin Elmer, USA).

## Results

We identified *Campylobacter* spp. in 111 of 225 samples from sick piglets (49%). Biochemical typing showed that 90 were *C. coli* strains (81%), and 19 were *C. jejuni* (17%), of which 18 strains were typed as *C. jejuni* susp. *jejuni* and one as *C. jejuni* subsp. *doylei*. Two of the isolated strains could not be identified by biochemical methods. In the set of 75 samples from piglets with no clinical signs of disease, the incidence of *Campylobacter* sp. was 41%, comprising 31 strains, 26 of which (85%) were identified as *C. coli* and 5 as *C. jejuni* (Table 1).

Table 1
Occurrence of thermophilic *Campylobacter* spp. in piglets

| | Piglets with enteritis | | Piglets without enteritis | |
|---|---|---|---|---|
| | No. | % | No. | % |
| *C. coli* | 90 | 81 | 26 | 85 |
| *C. jejuni* subsp. *jejuni* | 18 | 17 | 5 | 15 |
| *C. jejuni* subp. *doylei* | 1 | | 0 | |
| Atypical strains | 2 | 2 | 0 | |
| Total | 111 | 100 | 31 | 100 |

The *C. coli* strains isolated by the PCR reaction were cut with the *HinfI* restriction enzyme and classified into one of four groups according to the size of the fragments obtained (Fig. 1). Type I strains contained restriction site A with fragments of 100 and 310 bp, and type II strains contained restriction site B with fragments of 145 and 265 bp. Type III strains had both A and B restriction sites and were cut to fragments of 100, 45 and 265 bp. Type IV strains contained neither restriction site, and the PCR product (410 bp) was not cut (Plate XI, Fig. 2).

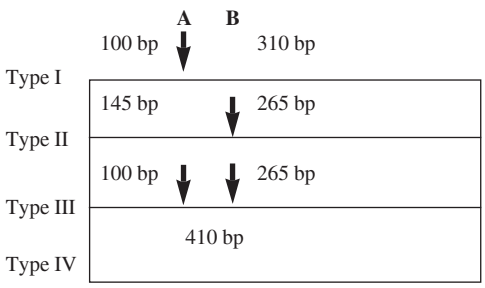


Fig. 1. PCR-RFLP patterns of *Campylobacter coli* observed with *HinfI*

The groups of *C. coli* strains were not of equal size: the most frequent was type III (46%), with two cutting sites. The A site (type I) was found in 31% of strains and the B site (type II) in 6% of strains; only 17% of *C. coli* strains (type IV) were not digested with *HinfI* restrictase.

No relationship was found between the incidence of a specific type and the frequency of infection on the farms. Usually one, or at most two, types of *C. coli* were identified on each farm. In the case of repeated samplings, however, another type or a different frequency of the two types was usually found.

## Discussion

The incidence of *Campylobacter* spp. was 49% in the 225 samples from sick piglets and 41% in the samples from piglets without clinical signs of disease. *C. coli* was the most frequent species in both sick and healthy piglets, representing 81% and 85% of samples, respectively.

Evaluation of the incidence of the various types of *C. coli* on different farms showed no relationship between the number of sick piglets and the prevalent subtype of *C. coli*. A wide diversity among isolated strains of *Campylobacter* spp. was also reported by Weitens et al. (1997), who demonstrated the existence of identical subtype profiles in sows and piglets, which seems to support the assumption of maternal transmission of *Campylobacter* spp. Similar subtyping profiles were not found, however, among *Campylobacter* strains isolated on different farms.

We used the flagellin A part of the gene, as flagellin genes have already been sequenced, and all studies point to the presence of two (*fla A* and *fla B*), positioned in tandem. These genes appear to be sufficiently conserved to be good candidates for genotyping (Guerry et al. 2000). On the basis of information obtained from the gene bank, we assumed that cutting with *HinfI* restrictase would produce one cutting position (278 bp) that would be specific to *C. coli* and another cutting position (100 bp) in other strains. Our preliminary results show, however, that the cut was not specific to *C. coli* and that there are one or two cutting positions elsewhere (145 bp and 100 bp). DNA sequencing of *C. coli* samples also confirmed the division into four types, while failing to provide proof of the existence of a cutting position at 278 bp.

As *C. coli* and *C. jejuni* occur normally in the intestinal content of pigs without clinical symptoms, it is evident that pigs represent a possible source of human infection with *Campylobacter* spp. Both healthy and sick piglets had a high incidence of *Campylobacter* spp., which might result in contamination of pork during slaughter. The high incidence rate of *C. coli* indicates the importance of subtyping. No link was demonstrated between the incidence of individual subtypes of *C. coli* and the frequency or severity of disease on pig farms.

## Incidence a subtypizace kmenů *Campylobacter* sp. izolovaných od selat

*Campylobacter* sp. je častou příčinou enteritid u člověka, ale cesty přenosu nejsou dosud zcela spolehlivě objasněny. Jedním z častých nositelů *Campylobacter* sp. jsou i prasata, jejichž střevo bývá kolonizováno *Campylobacter* sp. již brzy po narození. Bylo hodnoceno 225 vzorků rektálních výtěrů selat s výskytem průjmů i bez klinických příznaků. Byly zjištěny jen malé rozdíly mezi nálezy *Campylobacter* sp. u klinicky zdravých a nemocných selat. Ze vzorků rektálních výtěrů selat trpících enteritidami byl zjištěn výskyt *Campylobacter* sp. u 49 %, z toho tvořil *Campylobacter* coli 81%, *Campylobacter jejuni* 17 % a 2 % byly atypické kmeny. Z rektálních výtěrů zdravých selat byla zjištěna incidence 41 % *Campylobacter* sp.; z toho 85 % *Campylobacter coli* a 15 % *Campylobacter jejuni*.

U kmenů *Campylobacter coli* byla provedena subtypizace štěpením PCR (polymerase chain reaction) produktu restrikčním enzymem *HinfI*. Byly zjištěny a sekvenováním potvrzeny 4 subtypy *C. coli*. Nejvyšší frekvence byla u typu III (46%) a I (31 %). Typ II se vyskytoval jen u 6% kmenů a 17 % kmenů nemělo žádné štěpné místo (typ IV). Při hodnocení výskytu jednotlivých subtypů *C. coli* na farmách nebyla zjištěna vazba výskytu určitého typu na frekvenci nebo průběh onemocnění. Klinicky zdravá a nemocná selata jsou zdrojem *Campylobacter* sp. a může docházet ke kontaminaci svaloviny během porážení prasat.

**Acknowledgements**

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Plate XI
Steinhauserová I. et al.: Subtyping... pp. 197–201

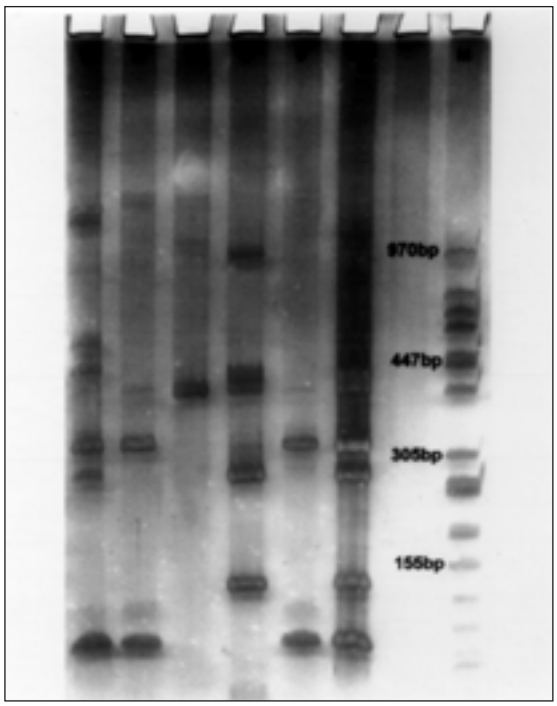


Fig. 2. RFLP analysis of PCR products. Line 1, type III (265, 100, 45 bp); line 2, type III (265, 100, 45 bp); line 3, type IV (410 bp); line 4, type II (265, 145 bp); line 5, type I (310, 100 bp); line 6, type III (265, 100, 45 bp); M, marker (TopBio)