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Molecular and epidemiological characterization of thermophilic *Campylobacter* species

Brief Summary of Doctoral Thesis

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Introduction, aims of the study

The thermophilic campylobacters are the major cause of human bacterial gastroenteritis throughout the world and in Hungary after salmonella enteritis. These species are present in the gut of birds and most mammal species as part of the normal flora. Knowledge regarding the distribution and molecular epidemiology of the zoonotic *Campylobacter* species within Hungarian livestock and poultry is scarce. The aim of this study was to identify the distribution, genotypes and antimicrobial susceptibility of *Campylobacter* species in the most important food-producing animals in Hungary.

Directive 2003/99/EC of the European Parliament and of the Council on the monitoring of zoonoses and zoonotic agents provides for the collection of data on the occurrence of zoonoses and zoonotic agents including *Campylobacter* spp. in animals, food, feed and humans to determine the trends and sources of zoonoses. Furthermore, the alarming emergence of resistance to antimicrobial agents is a characteristic that should be monitored on a harmonized basis.

Our institute, the National Reference Laboratory for Antimicrobial Resistance has been organizing the monitoring program for antimicrobial resistance including campylobacters in Hungary since 2001. Every month, official veterinarians of all the 19 Hungarian counties submitted a set of three intestine samples to our laboratory collected from three individuals per herd or flock of each main food-producing animal species (swine, cattle, broiler and occasionally turkey). Based on this sampling protocol, the aims of this study were the following:

1. Isolation of thermophilic campylobacters by selective culture in order to determine the presence and distribution of these species in different food producing animals.
2. As identification of the isolates at species level by the traditional culturing and biochemical tests is laborious, our aim was to develop a real-time PCR method for the identification of *C. jejuni* and *C. coli*, which is faster and has better reliability and reproducibility, in order to facilitate the examinations of the monitoring program.
3. Comparison of the species identification results obtained by the traditional culturing and biochemical tests and by PCR methods.
4. Comparison of efficacy of selective culture of campylobacters and the sensitivity of PCR to detect thermofilic campylobacters direct from samples.
5. To detect the rate of resistant strains for different antibiotics and highlight the trends, which are important in human treatment and to show the spread of resistance.
6. Genetic characterization of *Campylobacter* isolates by *fla*A SVR sequencing, PFGE and MLST in order to be able to compare the strains, to reveal their origin and spreading. Comparison of the methods.

7. Examination of ways how chicken flocks can be raised up without campylobacter infection.

Surveillance studies, epidemiologic investigations and laboratory research focus primarily on *C. jejuni* and *C. coli* as the causative agents of infections and on poultry as a source of infection, however, several more unusual *Campylobacter* species have been associated with human disease. As these emerging campylobacteria are under-isolated not only because of their lower prevalence, but due to the isolation protocols favoring *C. jejuni* subsp. *jejuni* or *C. coli*. Their role in human disease remains a question, and their importance may be currently underestimated.

The culture method used in this study was designed first of all for detection of thermophilic campylobacters, although as we were monitoring the prevalence and distribution of campylobacters in Hungarian food-producing animals, *C. lanienae* was detected nationwide for the first time in Hungary in pig herds.

We genotyped these strains by partial 16S rRNA gene sequencing and PFGE analysis in order to be able to compare them with those strains described in literature and to provide further details on the properties of *C. lanienae*.

Materials and methods

An ongoing national monitoring program investigating antimicrobial resistance has been conducted to isolate thermophilic *Campylobacter* species from intestine samples collected at slaughterhouses. The samples were ligated sections (~15 cm) of colon (swine, cattle) or caecum (poultry). A set of three intestine samples were examined collected from three individuals per herd or flock. The three subsamples were considered as one sample representing one herd. *Campylobacter* species were isolated by direct plating onto mCCDA (Oxoid, Drogen, Belgium). The isolate was considered to be *Campylobacter* if it did not grow microaerobically at 25 °C and at 41.5 °C aerobically, but after 48-72 hours of incubation in microaerobic atmosphere at 41.5 °C typical colonies appeared, which were oxidase positive and typical morphology and motility of the cells was observed under microscopy.

The Total RNA Isolation Kit Nucleospin 96 RNA (Macherey-Nagel, Düren, Germany) was used for DNA extraction as it was the most effective method.

A new real-time PCR method was developed for the identification of *C. jejuni* and *C. coli.* We have designed primers for the *hip*O gene of *C. jejuni*. After traditional agar gel based PCR optimalization we compared our method with some other traditional PCR methods for the species identification. After all we have chosen the primers for the detection of *C. coli* described by Wang et al. (2002) annealing to the *gly*A gen and the primers designed by us for the detection of *C. jejuni,* because these two PCR systems were optimized to run on real-time PCR platform under the same temperature profile allowing simultaneous identification of the two species. EvaGreen (Biotium, California, USA) fluorescent DNA-binding dye was used and discrimination of the two species was done by melting curve analysis.

Isolates from the monitoring program were first examined with our newly developed real-time PCR method for the identification of *C. jejuni* and *C. coli,* the most frequent species. Samples that were negative for *C. jejuni* and *C. coli* were further amplified by a genus specific PCR, using the primers (C412F and C1228R) and protocol described by Linton et al. (1996). Forty five *Campylobacter* isolates could be determined by sequencing the fragment amplified with the genus specific primers.

Based on preliminary phylogenetic analysis results, fifteen *C. lanienae* isolates from the main subclusters were selected and further sequenced with the *C. lanienae* species specific primers CLAN76F and CLAN1021R described by Logan et al. (2000). The sequences generated by the genus and species specific primers were overlapping on the 16S rRNA gene (the 816 and 920 bp long amplicons cover together 1152 nucleotide of the gen). Sequences of these 15 strains were used for further analyses. The partial 16S rRNA gene sequences were submitted to the GenBank database (accession numbers: HM462449 to HM462455, HM462460 and HM462464 to HM462470).

Analysis of the partial 16S rRNA gene sequence patterns was performed according to the protocol described by Gorkiewicz et al. (2003). They marked different types of the variable regions (Vc1, Vc2, Vc5 and Vc6) with capital letters (1A-H, 2A-L, 5A-G, 6A-E) according to sequence patterns and described the typical combinations for each *Campylobacter* species. One of the four (Vc1) variable regions was omitted from comparison analyses, due to the shorter length of the Hungarian *C. lanienae* sequences.

Altogether 73 *C. jejuni* and *C. coli* isolates were selected and the *fla*A gene template encompassing the short variable region (SVR) was generated by PCR with the primer pair FLA4F and FLA1728 previously described. Sequencing reactions of the *fla*A SVR were performed with the forward and reverse primers FLA242FU and FLA625RU, respectively. *Fla*A allele types were identified using the *C. jejuni* MLST website (http://pubmlst.org/campylobacter/, 12th June 2010.).

The phylogenetic tree (based on partial 16S rRNA gene and *fla*A gene SVR) was built using the Molecular Evolutionary Genetics Analysis (MEGA) 4.0 software. The evolutionary distances were computed using the Neighbour-Joining method, implemented with the Maximum Composite Likelihood model. The topology of trees was confirmed by 1000 bootstrap replicates.

PFGE analysis was performed on 122 *C. jejuni* and *C. coli* isolates and on 9 *C. lanienae* and 1 *C. hyointestinalis* subsp. *hyointestinalis* isolates. In case of *C. jejuni* and *C. coli* isolates, *Sma*I restriction endonuclease was used. *Kpn*I was used as a second enzyme on isolates with identical or closely related *Sma*I profiles. In case of other *Campylobacter* species three different restriction endonucleases *Sma*I, *Sal*I and *Kpn*I (MBI Fermentas, Vilnius, Lithuania) were used. DNA relatedness was computationally analysed by the Fingerprinting II software (Bio-Rad, Marnes-La-Coquette, France) using the band-based dice similarity coefficient and the unweighted pairs geometric-matched analysis (UPGMA) dendrogram with a position tolerance of 1% for optimization and band comparison. Clonally related isolates were suspected at 95% (*C. jejuni* and *C. coli)* or 90% (other *Campylobacter*) similarity between macrorestriction patterns. *C. jejuni* ATCC 33560 was included as a reference strain.

The *Campylobacter* MLST is based on the examination of 7 house-keeping genes. In case of *C. jejuni* the primer pairs used for the PCR amplification of internal fragments of these genes amplify approx. 1000 bp fragments. Separate nested primer pairs were used to sequence 400-600 bp fragments. In case of *C. coli* the primers used for amplification and sequencing were the same. After correction of the sequences the sequence type (ST) and the clonal complex (CC) was identified using the *C. jejuni* MLST homepage (http://pubmlst.org/campylobacter/).

The phenotype of the isolates was determined based on the following tests: catalase and H_2S production, hippurate and indoxil acetate hydrolyses growth on *Brucella* agar containing 1% glycine and their sensitivities to cephalothin and nalidixic acid (30 µg; Oxoid).

The antimicrobial susceptibility testing method used was microtiter broth dilution using EUCAMP plates (TREK Diagnostic Systems Ltd., Cleveland, USA) according to the recommendations of the Community Reference Laboratory for Antibiotic Resistance. Whenever the plates were not available, E-test strips (AB Biodisk, Solna, Sweden) were applied to determine the minimal inhibition concentration (MIC; µg/ml). Data were interpreted using EUCAST (European Committee on Antimicrobial Susceptibility Testing) epidemiological cut-off values and if not available, the EUCAST or NARMS clinical breakpoints were applied. Pearson's Chi-squared statistical method was used to determine the differences in antimicrobial resistance rates.

In feeding experiments the *Campylobacter*-reducing effect of Immunofort and Sangrovit natural feeding additives were tested at farm and experimental environments.

Results

During 2008 and 2009, out of 1110 samples 441 were positive (39.7%) for *Campylobacter* spp. by culture. Out of 441 positive samples examined with the newly developed real-time PCR assay, 266 were identified as *C. coli* (60.3%) and 143 *C. jejuni* (32.4%). Samples resulted negative for *C. jejuni* and *C. coli* (45 isolates) were amplified with genus specific primers and sequenced in order to determine the species. The prevalence of Campylobacter species by animal host is presented in Table 1. *Campylobacter* species other than *C. jejuni* or *C. coli* were isolated only from pigs. Multiple infections were found in one case of bovine samples, in 4 cases of pig samples and in 8 cases of broiler samples.

The sensitivity of the culture and the direct PCR methods were compared through parallel examinations of some intestine samples but the sensitivity of the PCR method was much lower than that of the culture. At the same time isolates with ambiguous results by the traditional biochemical tests (23 out of 69) could be identified by PCR.

Table 1. Number of samples from cattle, broiler chickens and swine examined for campylobacters. Number and percentage of samples positive for campylobacters and the distribution of species.

2008-2009	No. of samples	No. of positive samples (%)	No. of isolates				
			C. jejuni (%)	*C. coli* (%)	*C. lanienae* (%)	*C. hyoint.* subsp. *hyoint.* (%)	*C. hyoint* subsp. *lawsonii* (%)
Cattle	267	18 (6.7)	14 (77.8)	5(27.8)	0 (0.0)	0 (0.0)	0 (0.0)
Pig	480	208 (43.3)	11 (5.3)	156 (75.0)	43 (20.7)	1 (0.5)	1 (0.5)
Broiler	348	209 (60.1)	115 (55.0)	102 (48.8)	0 (0.0)	0 (0.0)	0 (0.0)
Turkey	15	6 (40.0)	3 (50.0)	3 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	1110	441 (39.7)	143 (32.4)	266 (60.3)	43 (9.8)	1 (0.2)	1 (0.2)

The seasonality was typical of the *Campylobacter* infection. Increasing prevalence of campylobacters was found from April to October and the prevalence was higher in autumn and winter. Significant correlation was found with Pearson's product-moment correlation test between the relative humidity and the total number of *Campylobacter* positive samples ($p=0.00161$).

C. lanienae occurs nationwide on swine rearing farms. With the help of the *C. lanienae* specific PCR method based on 16S rRNA we have successfully amplified all of 43 *C. lanienae* strains. There was one strain (6555) that produced an amplicon that was approximately 200 bp longer than the expected size. Both sizes of amplicons were found in case of three strains (5172, 24639, 17459).

Phylogenetic examinations were carried out on the selected 15 *C. lanienae* strains. The 15 partial 16S rRNA gene sequences were aligned to all *C. lanienae*, *C. hyointestinalis* subsp. *lawsonii* sequences available in GenBank from the corresponding region. One sequence of representative strains of *C. hyointestinalis* subsp. *hyointestinalis*, *C. fetus*, *C. jejuni* and *C. coli* were also included in the alignment. The *C. lanienae* clade was disparate from the *C. hyointestinalis* strains. Its genetic relationship was most marked to the *C. hyointestinalis* subsp. *lawsonii* clade.

The Hungarian *C. lanienae* sequences covered 3 variable regions (Vc2, Vc5 and Vc6) out of 4 characterized by Gorkiewicz et al. (2003). In Vc2 four of the Hungarian strains showed a sequence pattern different from *C. lanienae* 2C. In fact, these sequences in this region suit sequence pattern 2A typical for *C. hyointestinalis* or *C. fetus*. In the Vc5 variable region four strains showed sequence pattern 5B and 11 strains 5C as expected of *C. lanienae*. In the Vc6 variable region ten out of 15 strains showed a sequence pattern different from that described by Gorkiewicz et al. (2003) for *C. lanieanae* (marked as 6Da), containing three point mutations in this variable region (marked as 6Db).

One *C. hyointestinalis* subsp. *hyointestinalis* and nine *C. lanienae* strains were selected for macrorestriction profile analysis using three different restriction enzymes. After repeated digestions six and five *C. lanienae* isolates could be typed by *Sma*I and *Kpn*I enzymes, respectively. Unfortunately digestion by *Sal*I was not successful. Typable isolates yielded at least eight well differentiated restriction fragments. All isolates shared unique profiles.

A total of 73 *fla*A SVR sequences were determined for *C. jejuni* (37) and *C. coli* (36) isolated from cattle, swine, broilers and turkeys. A total of 47 different *fla*A SVR sequences were identified, corresponding to 18 peptide alleles. Out of 47, 35 *fla*A types were detected only once. The identical nucleotide allele types formed 12 clusters on the phylogenetic tree including isolates from different animal origins but not all peptide alleles clustered on the same branches of the phylogenetic tree. *C. jejuni* and *C. coli* isolates did not form unified clusters either. Out of the 12 clusters epidemiological connections were found in seven cases among the strains from the same cluster.

A total of 122 isolates (60 *C. jejuni* and 62 *C. coli*) recovered from bovine, swine, turkey and poultry samples were subjected to macrorestriction profile analysis with *Sma*I restriction endonuclease. Forty *C. jejuni* and 40 *C. coli* isolates produced unique *Sma*I profiles. The remaining 42 isolates formed 18 small PFGE clusters of two to three isolates with identical profiles, including isolates from different animal origins. These 42 isolates were further typed using *Kpn*I. Of these, 24 were assigned to 10 different *Kpn*I clusters comprised of two to four isolates with identical profiles. There were no isolates from different animal

species with identical patterns after *Kpn*I digestion. Within these 10 *Kpn*I clusters, seven epidemiological connections were found.

In five cases (*Kpn*I clusters 1, 5, 6, 7, and 9), broilers originated from different flocks of the same farm. In the case of *Kpn*I-cluster 10 the pigs originated from the same farm but reared in different places. Isolates in *Kpn*I cluster 4 originated from bovine from geographically distant herds, but the transport trailer to the slaughterhouse was the same. In cases where either PFGE patterns or *fla*A types differed, no epidemiological connections were found.

One *C. jejuni* and one *C. coli* MLST profile were identified. *C. jejuni* was isolated from bovine and it belonged to clonal complex 42 (ST 42) on the basis of our MLST sequence results. *C. coli* was isolated from swine and it belonged to clonal complex 828 (ST 899).

Resistance to nalidixic acid (p=0.0465) and enrofloxacin/ciprofloxacin (p=0.0114) increased from 2008 to 2009. The ratio of nalidixic acid (p=4.626e-05) and fluoroquinolone-resistant (p=3.403e-07) *Campylobacter* strains was higher in broilers than in pigs in 2009. Fluoroquinolone resistance was significant, especially in *C. coli* from broilers, 89.0% in 2009. Erythromycin resistance showed decreasing tendency. Lower erythromycin (p=0.02509) resistance rates were observed in *C. jejuni* than in *C. coli* isolates, it was more frequent in pigs. The ratio of tetracycline-resistant *C. coli* strains was much higher in pigs than in broilers in 2009 (p=1.046e-08). Resistance to tetracycline among the *C. coli* isolates from swine was 90.7%. Considerable streptomycin-resistance was found among *C. coli* isolates from swine (75.9%) in 2009, while from broilers this was only 8.5%. The clindamycin resistance also increased among *C. coli* strains from swine. Most of the tested strains of both species proved to be chloramphenicol and gentamycin sensitive.

Investigating resistance to fluoroquinolones, macrolides and tetracycline, 55.2% of *C. coli* and 21.6% of *C. jejuni* isolates were found resistant to more than one of these antimicrobial classes. Resistance to all three antimicrobial classes was relatively rare: 1.4% in *C. jejuni* and 4.1% in *C. coli* in 2009.

The 23 *C. lanienae* isolates tested showed similar tendency as the previous *Campylobacter* species. Out of the 5 erythromycin resistant strains 4 were tetracycline resistant, and all enrofloxacin/ciprofloxacin resistant strains were tetracycline resistant. One strain (3894) was resistant to erythromycin, enrofloxacin, nalidixic acid, tetracycline and clindamycin as well.

We could not prove the *Campylobacter*-reducing effect of Immunofort nor that of Sangrovit.

Discussion

The prevalence of campylobacters in Hungarian broiler chicken flocks (60.1%) and pig herds (43.3%) at the time of slaughter was high. Conversely, cattle herds (6.7% positive) are not common carriers of campylobacters. Perhaps these rates would be a bit higher if we had had used enrichment broth during the isolation but we omitted the enrichment step in order to simplify the routine work. Broiler chickens carry the bacteria in high number, so presumably the sensitivity of the culture method influences the results less but in case of pigs and bovine the difference could be higher.

A seasonality of broiler flock colonization has been observed in some countries, leading to a prevalence peak during the warm summer months (June-September). It is of interest to note that in Hungary higher colonization rate was found during the cold autumn and winter months with a peak in October and November. Examining the causes of the seasonality significant correlation was found between the relative humidity and the total number of *Campylobacter* positive samples.

Our isolation method was developed for the detection of *C. jejuni*, *C. coli* and *C. lari* but we succeeded in isolating of *C. lanienae* strains, too. *C. lanienae* seems to be part of the normal gut flora in Hungarian pigs. It is of particular interest, that *C. lanienae* was the second most common (20.7%) species isolated from pig intestines in this study. *C. jejuni* was isolated in only 5.3 % of the positive pig samples.

C. lanienae could be isolated only from pigs. The isolation failure of *C. lanienae* from cattle samples could be explained by either the absence of the organism or probably *C. lanienae* colonizing bovine have different culture preferences. Inglis et al. (2003, 2004) detected *C. lanienae* in bovine feces by dilution plating on Karmali agar at 40 °C in Canada, the CCDA was not appropriate in the same study. It is remarkable that there are no reports available on the detection of *C. lanienae* from cattle in Europe.

The disadvantage of the identification of the strains at the species level with the traditional culturing and biochemical tests that it gives uncertain results in several cases, so our aim was to develop a real-time PCR method for the identification of *C. jejuni* and *C. coli*, which is faster and has better reliability and reproducibility, to facilitate the examinations of the monitoring program. The real-time PCR method developed in this study is able to identify *C. jejuni* and *C. coli* isolates simultaneously, and is suitable for the examination of at least 45 samples within 4 hours. The sensitivity of the direct PCR was far behind the sensitivity of the culture method but for the examination of antibiotic-resistance the isolation of the bacteria was essential. At the same time the real-time PCR replaces the biochemical tests after the isolation, it makes the species identification quicker and more reliable.

PCR, sequence and phylogenetic analysis are the most widely used molecular techniques applied for the identification, differentiation, and further characterization of *Campylobacter* species. On the basis of the genetic examinations it turned out that *Campylobacter* spp. have extensive genetic diversity and genome plasticity that can reflect quick adaptation during infection cycle. Different mechanisms can play role in the development of the new variations, such as transformation**,** intra- and intergenomic recombination, genome rearrangements and chromosomal point mutations.

Similarly to other species enlarged gene size was observed also in *C. lanienae*. The phenomenon is attributed to the existence of intervening sequences (IVS) of different lengths located after nucleotide 213 (*C. jejuni*, ATCC 43431 numbering) in the 16S rRNA genes of certain strains. The 6555 strain contained only the enlarged gene, whereas the 5172, 24639, 17459 strains resulted with the species specific PCR method two amplicons, one at the expected size and one approximately 200 bp longer than that. All the other strains contained only the gene without IVS. The role of the 16S rRNA IVS is not elucidated.

On the phylogenetic tree based on partial 16S rRNA gene the *C. lanienae* species clade showed several clusters, confirming the genetic diversity of this species. *C. lanienae* originating from humans do not form a disparate group; instead they associated with different pig clusters. However, *C. lanianae* strains originating from cattle and sheep form a distinct monophyletic cluster. Isolates of bovine and swine origin have not only genotypical, but also phenotypical differences. All *C. lanienae* strains isolated from cattle by Inglis et al. (2003) were susceptible to cephalothin, unlike the reference human strain and all Hungarian *C. lanienae* strains from swine samples that were cephalothin resistant.

Considerable genetic diversity of the porcine isolates was observed whereas all ruminant isolates showed an identical 16S rRNA gene sequence pattern of 2A/5C/6Db in the 3 analyzed variable regions. This particular pattern was shared by 3 porcine *C. lanienae* strains (designated 25491, 31051 and FK176), however, porcine *C. lanienae* strains display larger variability in the 3 variable regions. These raise the possibility that porcine strains may have served as progenitors in the origin of ruminant strains. Pattern 2A was originally described in *C. hyointestinalis* and *C. fetus* strains, but not in *C. lanienae* strains (Gorkiewicz et al., 2003), demonstrating that additional variations exist in this variable region of *C. lanienae* strains. In addition, 2 distinct patterns characterized by 3 nucleotide substitutions within the Vc6 region were identified in some of the *C. lanienae* strains; to be able to differentiate these variants we split pattern 6D into 6Da and 6Db.

Phylogenetic analysis of *C. jejuni* and *C. coli* was performed based on *fla*A gene SVR sequences. Due to the small size of the SVR (321 bp), the *fla*A SVR sequencing method is quick, accurate, reproducible and comparable among laboratories. SVR comparisons have

been used to study the spread of *Campylobacter* populations within the poultry industry, including differentiating between poultry and environmental isolates.

PFGE provides excellent discrimination between isolates of Campylobacter, and PFGE is often regarded as the “gold standard” for *C. jejuni* typing. After examination of *C. jejuni* and *C. coli* isolates using *Sma*I enzyme, ten out of 18 *Sma*I clusters included isolates from different animal origins and/or with different *fla*A SVR type grouped together. Contrary, after *Kpn*I digestion isolates with identical restriction profiles had identical *fla*A SVR types and animal origin, thus represented real genetic clones.

As a result of *fla*A SVR and PFGE typing different epidemiological connections were discovered. In the case of poultry isolates recovered from 2 or 3 different flocks of the same farm, persistence of stable *C. jejuni* clones for at least eight months was observed. Probably the inappropriate cleaning or disinfection before placing of the new flock into the stable was the cause of the contamination with the same strain. Presence of genetic clones isolated from pigs originated from the same farm but reared at different places can imply that the pigs were infected with the same strain during the suckling-age. Isolates from cattle originated from geographically distant farms but transported to the same slaughterhouse could be stable clones occurring in the country or the animals were contaminated on the transport trailer. Moreover, considering identical poultry isolates without any epidemiological connection or obvious external contamination source, it seems that highly stable genetic clones of both *Campylobacter* species circulate among Hungarian broiler farms as well. Similarly, genomic stability of certain *C. jejuni* strains from a variety of hosts and geographic areas have been reported. Further studies involving broiler and human isolates need to be conducted for elucidating the importance of these stable clones in human infections.

In this study we made attempts to characterize *C. lanienae* by PFGE for the first time. Three different restriction enzymes - *Sma*I, *Kpn*I and *Sal*I - were used. *Sma*I seems to be useful for typing *C. lanienae* isolates if epidemiological studies are required.

PFGE is the most sensitive method among the ones discussed above but at the same time problems can arise because it could detect considerable differences among strains that in fact belong to the same clone. Its standardization is a further difficulty that obstructs the comparison of the examinations at different places. Because of these characteristics MLST is being used instead. Great advantage of MLST is its reliability, the results are comparable if the determination of the sequences is carried out with the appropriate technique. Thus, the MLST is the most suitable method for nationwide or international comparative typing both for short and long-term studies. Whereas it is expensive thus the MLST method is used only in limited number, for the most important strains in an epidemiological examination.

The number of quinolone-resistant and macrolide-resistant *Campylobacter* strains causing human infections has increased markedly in the past decade. Fluoroquinolones are

generally advocated as first-line drugs for antimicrobial treatment of *Campylobacter* enteritis but because of increasing resistance to fluoroquinolones this therapy got in danger.

In food-producing animals, the prevalence of resistance to quinolones has reached very high rates (74.3% of *C. jejuni* and 70.1% of *C. coli* in 2009) and is growing both in *C. jejuni* and *C. coli* isolated from pigs, bovine and broilers. The ratio of quinolone-resistant *C. jejuni* (p=0.00159) and *C. coli* (p=4.475e-06) strains is even higher in broilers than in swine, similarly to the results reported in Italy, Malta, Romania, Poland and Netherlands.

In both *Campylobacter* species, both in broiler flocks and pig herds the macrolide resistance has decreased in Hungary in recent years. One of the reasons could be that the usage of macrolide antibiotics has been banned as yield booster in the EU since July, 1999.

However our feeding experiments were not successful, in place of antibiotics different natural feeding additives and probiotics come into prominence in the control of campylobacters.

The significant tetracycline resistance and the presence of erythromycin- enrofloxacin- and multiresistant *C. lanienae* strains pose the question could *C. lanienae* play a role in the spread of antimicrobial resistance. Although *C. lanienae* is considered to be non-pathogenic, its relatively high prevalence in pigs and the fact that it has been isolated from humans could raise concerns regarding its zoonotic potential.

New scientific results

1. We have developed a new real time PCR method that detects the most frequent thermophilic *Campylobacter* species (*C. jejuni* and *C. coli*) from mixed infections as well. For the identification of *C. jejuni* we have developed new primers ourselves. With the help of this new real-time PCR method identification of 45 samples within 4 hours could be done. It was reached through the automation of the extraction and the pipetting processes of the PCR reactions by robots.
2. We surveyed the prevalence and distribution of *Campylobacter* species occurring in food producing animals. The findings of this study reflect that the prevalence of campylobacters in Hungarian broilers (60.1%) and pigs (43.3%) at the time of slaughter was high. Conversely, Hungarian cattle (6.7% positive) are not common carriers of campylobacters. Significant correlation was found between the relative humidity and the total number of *Campylobacter* positive samples.
3. We isolated and identified *C. lanienae* for the first time in Hungary. We reported the nationwide occurrence of the bacteria in pigs. We found that *C. lanienae* was the second most common (20.7%) *Campylobacter* species in pigs, while only 5.3% of positive pig intestine samples were contaminated with *C. jejuni.*
4. During the partial 16S rRNA gene sequence analysis we described the occurrence of 2A sequence pattern also in *C. lanienae* on the Vc2 variable region. Within the Vc6 region 2 distinct patterns were identified characterized by 3 nucleotide substitutions; to be able to differentiate these variants we split pattern 6D into 6Da and 6Db.
5. In this study we characterized *C. lanienae* by PFGE using three different restriction enzymes - *Sma*I, *Kpn*I and *Sal*I -, for the first time. *Sma*I seems to be useful for typing *C. lanienae* isolates if epidemiological studies are required.
6. We determined by PFGE and *fla*A SVR sequence the *C. jejuni* and *C. coli* genotypes occurring in food producing animals in Hungary. Stable genetic clones of both *C. jejuni* and *C. coli* were found.
7. For the first time in Hungary we examined one *C. jejuni* and one *C. coli* strain from animals with MLST method.
8. We observed significant resistance to enrofloxacin/ciprofloxacin and to nalidixic acid among the isolates especially in *C. jejuni* (73.3%) and *C. coli* (77.2%) isolated from broiler chickens. Higher erythromycin- (p=0.043) and tetracycline-(p=1.865e-14) resistance rates were found among *C. coli* (9.7% and 74.1%), compared to *C. jejuni* (3.1% and 36.6%) isolates, thus it was more frequent in pigs.

Scientific publications

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