ISOLATION AND IDENTIFICATION OF THERMOPHILIC *CAMPYLOBACTER* SPP. BY PCR-RFLP IN BROILER FLOCKS

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Summary. The aim of the present work was to isolate and identify thermophilic *Campylobacter* spp. in broiler flocks using Polymerase Chain Reaction based Restriction Fragment Length Polymorphism (PCR- RFLP) method. Forty cloacal samples were taken and examined from four randomly selected poultry flocks in different poultry farms in Lithuania. Thermophilic *Campylobacter* spp. were isolated by both direct inoculation on mCCDA selective medium and by selective enrichment in Bolton enrichment broth. The results of our study showed that all four examined poultry flocks were contaminated with *Campylobacter* spp. Using PCR assay amplification of 491 bp amplicon of a highly polymorphic part of the 23S rRNA gene out of 40 broiler cloacal samples examined *Campylobacter* spp. were detected and confirmed in 37 samples (92.5 %). After subsequent digestion of the PCR products with restriction enzymes *AluI* and *TspEI*, *C. jejuni* was identified in 32 (86.5 %) and *C. coli* in 5 (13.5 %) out of 37 isolates. Three broiler flocks of four examined were contaminated only with *C. jejuni* and one flock carried out mixed infection with *C. jejuni* and *C. coli*. To our knowledge, this is the first report of thermophilic *Campylobacter* isolation and identification from broilers in Lithuania. The results obtained in present study could serve for future surveillance on *Campylobacter* bacteria.
Key words: *Campylobacter* spp., broilers, PCR-RFLP, identification.

TERMOFILINIŲ *CAMPYLOBACTER* SPP. BROILERIŲ PULKUOSE APTIKIMAS IR IDENTIFIKAVIMAS PGR-RFIP METODU

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Santrauka. Tyrimo tikslas buvo aptikti ir identifikuoti termofilines *Campylobacter* spp. broilerių pulkuose, naudojant polimerazės grandininę reakciją restrikcijos fermentų ilgio polimorfizmo (PGR-RFIP) metodu. Tyrimo metu ištirta 40 broilerių kloakos mėginių, paimtų iš atsitiktinai parinktų skirtingų broilerių pulkų skirtingose Lietuvos broilerių fermose. Termofilinės kampilobakterijos išskirtos tiesiogiai sėjant ant mCCDA agaro ir sėjant ant mCCDA agaro atlikus pagausinimą Bolton sultinyje. Tyrimo metu nustatėme, kad visi keturi broilerių pulkai buvo užsikrėtę *Campylobacter* spp. bakterijomis.

Alikus PGR reakciją, *Campylobacter* spp. nustatytos 37 (92,5 proc.) iš 40 tirtų broilerių kloakos mėginių. Atlikus PGR-RFIP reakciją su restrikcijos fermentais *AluI* ir *TspEI.* nustatyta, kad iš 37 broilerių kloakos mėginių 32 (86,5 proc.) buvo užsikrėtę *C. jejuni* ir 5 (13,5 proc.) – *C. coli* bakterijomis. Trijuose iš keturių tirtų broilerių pulkų buvo nustatytos *C. jejuni*, viename pulke broileriai buvo užsikrėtę tiek *C. jejuni,* tiek *C. coli* bakterijomis. Mūsų duomenimis, PCR-RFLP metodu Lietuvoje broilerių pulkų užsikrėtimas *Campylobacter* spp. bakterijomis iki šiol tirtas nebuvo. Tikimės, kad šio tyrimo rezultatai bus naudingi atliekant tolimesnius kampilobakterijų epidemiologinius tyrimus.
Raktažodžiai: *Campylobacter* spp., broileriai, PGR-RFIP, identifikavimas.

Introduction. *Campylobacter* spp. are the most common foodborne bacterial cause of human enteric disease in several industrialized countries (Friedman et al., 2000; Gillespie et al., 2002; Sandberg et al., 2006). The overall incidence of campylobacteriosis in the EU-25 was 47.6 per 100 000 population, and it is slightly higher than for *Salmonella* (European Food Safety Authority, 2005). This makes *Campylobacter* the most commonly reported gastrointestinal bacterial pathogen in human in the EU. Risk factors for *Campylobacter* infections include eating or preparing raw or undercooked meat, especially chicken meat, drinking unpasteurized milk or untreated water, contact with domestic animals and pets, and foreign travel. The ingestion and handling of contaminated poultry meat is supposed to be major infection route for humans (Corry and Atabay, 2001).

The most important *Campylobacter* species causing human infections are the thermophilic species *C. jejuni, C. coli, C. upsaliensis* and *C. lari*. In industrialized countries, 80 - 90 % of human campylobacter infections are induced by *C. jejuni*, while only about 7 % are induced by *C. coli* and *C. lari* and only about 1 % by *C. upsaliensis* (Vandamme, 2000). These species are often referred to as thermotolerant campylobacters because of their optimal growth at 42°C and are also associated prominently with poultry by colonizing preferentially the avian gastrointestinal tract (Corry and Atabay, 2001).

The incidence of *Campylobacter* spp. in poultry varies

considerably between farms and depends on a number of factors, primarily on zoo-technical and hygienic circumstances (Skov et al., 2004). The occurrence and spread of *Campylobacter* in conventional broiler flocks has been studied in several countries and demonstrated prevalence ranging from 2.9 % to 90 % of *Campylobacter* spp. in poultry flocks (Perko–Makela et.al., 1999; Padungton and Kaneene, 2003; Hald et al., 2004; Bouwknegt et al., 2004). A seasonal variation in the prevalence of *Campylobacter*-positive broiler-flocks has also been reported (Refrégier-Petton et al., 2001). Farm animals and poultry spread the bacteria through meat products that become contaminated with *Campylobacter* during the slaughter process (Allen et al., 2007). The bacteria may survive in the food chain, causing a risk in the kitchens where the food is finally prepared and consumed, unless strict hygiene measures are followed (Mazick et al., 2006).

The aim of the present study was to isolate and identify thermophilic *Campylobacter* spp. in broiler flocks in Lithuania using Polymerase Chain Reaction based Restriction Fragment Length Polymorphism (PCR-RFLP) method.

Materials and methods. *Sampling plan.* The minimum number of samples to detect an infection of *Campylobacter* spp. in broiler flocks, was determined with the Win Episcope 2.0 program (Blas et al., 1998). Based on the available data about broiler flocks size in Lithuania it was estimated that the average number of broilers per flock was 30,000 and the expected prevalence of *campylobacter*-positive broilers was 50 % (level of confidence 97.5 %).

Cloacal swab samples were collected at random from 40 individual broiler chickens representing 4 broiler flocks from four different poultry farms 7-3 days before slaughter from June to August period in 2007. Each broiler flock was sampled using cloacal swabs from 10 individual broilers.

Isolation of Campylobacter spp. by bacterial culture methods. Moistened with saline (0,9 % NaCl) sterile cotton swabs were used to collect cloaca content from broilers. Thermophilic *Campylobacter* spp. were isolated by direct plating and by plating after selective enrichment. For the direct plating, cloacal swabs were streaked onto Campylobacter Blood-Free Selective Agar Base (modified CCDA- Preston) (CM0739; Oxoid Ltd., England) with CCDA Selective Supplement (SR0155E; Oxoid Ltd., England) and for selective enrichment the same swabs were placed in a tube containing a Bolton selective enrichment broth (CM0983; Oxoid Ltd., England) with Bolton broth selective supplement (SR0183E; Oxoid Ltd., England) and 5 % Laked horse blood (SR0048; Oxoid Ltd., England) and transferred to the laboratory (LVA, Department of Food safety and animal hygiene).

Inoculated by direct plating mCCDA plates were incubated in microaerophilic atmosphere (85 % nitrogen, 10 % carbon dioxide and 5 % oxygen) generated by Campygen (CN25; Oxoid Ltd., England) at 37 ^{o}C for 48 h. After incubation from each plate a single colony suspected of being *Campylobacter* was examined by phase-contrast microscopy, catalase and oxidase reactions, tested for hydrolysis of hippurate according to standard procedure, and was further purified on blood agar plates (Blood Agar Base No. 2 (Liolfilchem, Italy) supplemented with 7 % Laked horse blood and incubated at 37 ^{o}C for 48 h for 1-2 days. Inoculated tubes containing Bolton enrichment broth were incubated microaerobically at 42 ^{o}C for 24 h, for selective enrichment. After incubation 10 µl of the broth from each tube was streaked onto plates with mCCDA agar and isolation of campylobacters was done as described in direct plating procedure. The purified isolates after growth on blood agar were subsequently stored at – 40 ^{o}C in special broth (Nutrient broth No. 2 (CM67, Oxoid Ltd., England), Agar No. 1 (Liolfilchem, Italy) with 10 % DMSO supplement (Merck, Germany) until further investigation.

DNA isolation. After growing the bacteria on blood agar plates, a loopful of bacterial culture was taken and suspended in 500 µl of 5 % of Chelex (Sigma- aldrich, USA) solution. The suspension was heated at 56 ^{o}C for 30 min and 95 ^{o}C for 10 min, and then centrifuged for 2,5 min at 10 000 rpm (two times). Supernant was transfered into a new tube. Extracted DNA were used immediatly for PCR amplification or stored at -20 ^{o}C until examination.

Detection and differentiation of thermophilic Campylobacter spp. by PCR-RFLP. All strains biochemically and morphologically classified as *Campylobacter* spp. were confirmed by the amplification of 491 bp amplicon of a highly polymorphic part of the 23S rRNA gene, and further species differentiation was accomplished by digestion of the PCR product with two restriction enzymes, *AluI* and *TspEI*, resulting in specific restriction fragments for each species (Fermer and Engvall 1999).

Two oligonucleotide primers of 23S rRNA gene were used: forward primer, THERM1 (5‘- TATTCCAATACCAACATTAGT - 3‘) and reverse primer THERM4 (5‘- CTTCGCTAATGCTAACCC - 3‘) (Fermer and Engvall 1999).

The PCR amplification was performed in a final volume of 100 µl containing 1 X reaction buffer (MBI Fermentas), 2.5 mM magnesium chloride (MBI Fermentas), 30 µM dNTP mix (MBI Fermentas), 0.025 U of Taq DNA polymerase (MBI Fermentas) and 0.1 µM of each of the oligonucleotide (Sigma Genosys, USA). The PCR amplification was performed on a PTC-100 Programmable thermal controller (MJ Research Inc., USA) at 94 °C for 5 min, 32 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min and the final amplification at 72 °C for 10 min. A negative control with sterile water instead of DNA template was also prepared. PCR products were analysed by electrophoresis: 10 µl volume of each PCR product was loaded onto a 3.5 % Top Vision LE GQ Agarose gel (MBI Fermentas) containing 0,5 µl/ml of ethidium bromide solution. The gel was visualised on an UV board. The GeneRuler^{TM} 50 bp DNA Ladder (MBI Fermentas) was used as the molecular size marker. The presence of 491 bp DNA fragment indicated a positive results for *Campylobacter* spp. isolate.

The PCR–RFLP reaction mixture contained: 5 µl of PCR product, 0.5 U of restriction enzyme *AluI* (MBI Fermentas) or *TspEI* (MBI Fermentas) and made up to 20

µl final volume. The reaction mixture with *AluI* was incubated at 37 °C for 2 h and with *Tsp509I* at 65 °C for 3 h. Products were analysed on 3.5 % Top Vision LE GQ Agarose gel (MBI Fermentas) containing 0.5 µl/ml of ethidium bromide solution and visualized on an UV board. The GeneRuler^{TM} 50 bp DNA Ladder (MBI Fermentas) was used as the molecular size marker.

Results. *Results of bacteriological cultivation*. One isolate from each cloacal sample was examined. During bacteriological examination 37 (92.5 %) isolates were identified as presumable *Campylobacter* spp. according to growth on mCCDA, motility and biochemical activity.

Detection and differentiation of Campylobacter spp. by PCR – RFLP. Out of 37 isolates presumably identified as *Campylobacter* spp. after bacteriological examination, all 37 (92.5 %) isolates were confirmed as *Campylobacter* spp. by PCR - a product of 492 bp was amplified. Digestion of PCR products with restriction enzyme *AluI* yielded different results for *C. jejuni* and *C. coli*. Digested PCR amplicon of *C.jejuni* gave a unique combination of fragments, approximately 202 bp, 166 bp and 124 bp of size and digestion of *C. coli* PCR amplicon resulted fragments of approximately 290 bp and 202 bp size.

Identification to the species level revealed that out of 37 isolates positive for thermophilic *Campylobacter* spp. by the PCR reaction, 32 (86.5 %) isolates were identified as *C. jejuni* and 5 (13.5 %) isolates were identified as *C. coli*. Three broiler flocks of four examined were contaminated only with *C. jejuni* and one flock carried out mixed infection with *C. jejuni* and *C. coli*.

Discussion. Poultry meat is considered to be the important source of zoonotic *Campylobacter* infections in developed countries. It has been evaluated that poultry is a source mainly of *C. jejuni* and *C. coli* infection and probably has a low rate of *C. upsaliensis* and *C. lari* colonization (Moreno et al., 1995). It has been postulated that cats are the main source of *C. upsaliensis* that produces disease in humans (Goossens et al., 1990). Wild birds have been considered natural vertebrate reservoirs of *Campylobacter lari.* However *C. upsaliensis* and *C. lari* could be passed on to chicken broilers from pets and wild-birds when they can get in the poultry farm. Wild birds contaminated with *C. lari* are frequently mentioned as possible vectors for transmission of these bacteria to poultry (Waldenström et al., 2002). Different molecular strategies and genetic targets, applied for the detection and identification of *Campylobacter* spp., are designed only for *C. jejuni* and *C. coli,* and only few methods for all - *C. jejuni, C. coli, C. upsaliensis* and *C. lari* - thermophilic *Campylobacter* species, causing campylobacteriosis to human (Goossens et al., 1990; Fermer and Engvall, 1999). Appliance of these methods for detection and differentiation of *Campylobacter* spp. in broiler flocks is useful to estimate the true incidence of broiler flocks contamination with *Campylobacter* spp.

The PCR-RFLP method used in the present study has been successfully used for examination of human, dogs, cats, and pigs (Steinhauserova et al., 2005; Malakauskas et al., 2006) contamination routes with thermophilic *Campylobacter* spp. Our study is the first which used PCR-RFLP method for examination of poultry flocks contamination with *Campylobacter* spp. After digestion of the PCR products with restriction enzymes, *AluI* yielded different results for *C. jejuni* and *C. coli,* while *TspEI* enzyme can be used to differentiate between *C. coli* and *C. upsaliensis* because it digests *C. lari* and *C. upsaliensis* differently and does not digest *C. coli* and *C. jejuni*. The results of our study were in agreement with the work of Fermer and Engvall (1999).

Prevalence of *Campylobacter* infections varies among and also within countries. It has been reported that only 27% of the broiler flocks were infected in the UK (Pearson et al., 1996), 41.1% in Germany (Atanassova and Ring, 1999), 47% in Denmark, 10–27% in Sweden (Friedman et al., 2000). The results obtained in the present study showed that at four poultry farms randomly selected four broiler flocks were contaminated with *Campylobacter* spp. This suggests that prevalence of *Campylobacter* spp. among poultry farms may be very high. However, these results are not unexpected, as in summer period contamination of broiler flocks with *Campylobacter* spp. can easily reach 100 %, as it has been showed in other countries (Vandeplas et.al., 2006). In our study sampling has been done in summer period and moreover one week or less before slaughter when high infection rate is expected (Jacobs-Reitsma et.al., 1995; Bouwknegt et.al., 2004). However, due to limited number of examined poultry farms further studies are needed to estimate the true prevalence in Lithuania.

C. jejuni was the most frequently (86.5 % of the cases) found of *Campylobacter* species in the present study. These results correlate with data in Ireland and Denmark studies, where *C. jejuni* has been found the most prevalent species as well: Whyte et al. (2004) found 83.4%, whereas Wedderkopp et al. (2003) reported that 89.5% of the positive samples were contaminated with *C. jejuni*. In the present study 3 broiler flocks of four examined were contaminated only with *C. jejuni* and one flock carried out mixed microflora with *C. jejuni* and *C. coli*. It is in agreement with study carried out by Bull et. al. (2006) where five broiler flocks were colonized exclusively with *C.jejuni* another exclusively with *C. coli* and one with both species.

The results obtained in the present study showed a high *Campylobacter* infection rate of the examined broiler flocks in Lithuania. Therefore, there is an increased importance of further investigations on the epidemiology of the *Campylobacter* spp. infection, mainly focusing on the possible contamination routes of infection and the possibilities of prevention. It is a strongly recommended that the main focus for the control of *campylobacter* in chickens should be on the farm, and it is important to improve our understanding of the epidemiology of campylobacter in poultry, in order to formulate control measures which can prevent flock colonization.

Conclusions. The results of our study showed that all four examined poultry flocks were contaminated with *Campylobacter* spp. indicating the high prevalence of these zoonotic bacteria among poultry farms. The most frequent species found in broiler cloacal samples is *C.*

jejuni 86.5 %, followed by *C. coli* 13.5 %. One flock was contaminated with dual infection of *C. jejuni* and *C. coli*, while the remaining flocks were contaminated only with *C. jejuni*.

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