CONTAMINATION OF POULTRY ENVIRONMENT WITH CAMPYLOBACTER SPP. AND SIGNIFICANCE FOR COLONISATION OF BROILERS

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Abstract. Various animal species, wild birds, rodents and pets are the main source of Campylobacter contamination in broiler farms. However, little is known about the ability of these bacteria to survive outside the host and their role in the colonisation of broilers. Therefore, in this study we aimed to identify the sources of Campylobacter in poultry farm environment and their significance for broiler colonisation. For this purpose, *Campylobacter* spp. were isolated from broilers and their environment (litter, water of puddle, concentric zones, tap water) by both direct inoculation on mCCDA selective medium and selective enrichment into Exeter broth. Campylobacter isolates were identified to the species level by multiplex polymerase chain reaction. The genetic diversity of *Campylobacter jejuni* strains isolated from broilers and the environment was examined by PCR-based restriction fragment length polymorphism analysis of PCR amplified portion of the flagellin-A (*fla*A) gene. The results revealed that 28.5% out of 752 samples were contaminated with *Campylobacter* spp. The *fla*A-RFLP analysis of 263 *C. jejuni* isolates revealed 21 different *fla*A genotypes. Each broiler flock had specific Campylobacter genotypes, which had not been transmitted to other broiler flocks reared in the same building.

Studies have shown that Campylobacter can survive in poultry environment. They can be transferred from the environment into broiler houses by shoes, cloths and wildlife. Therefore, strict bio-security and hygiene rules must be implemented at the farm level to prevent spread of this pathogen. Additionally, hostile environment for pathogenic bacteria must be established in the areas around a broiler house.

Keywords: *Campylobacter jejuni*, poultry, RFLP, campylobacteriosis

Introduction. *Campylobacter jejuni* continues to be the most common cause of bacterial gastroenteritis in the developed world (EFSA, 2015). In the European Union, the incidence of campylobacteriosis is 64.8 cases per 100,000 population (EFSA, 2015). In 2013 in Lithuania, campylobacteriosis was the most prevalent food-borne zoonosis in humans with the incidence of 38.3 cases per 100,000 population (EFSA, 2015). Compared with the Baltic States, campylobacteriosis incidence in Lithuania is higher than in Estonia (28.9 cases per 100,000) or Latvia (0.4 cases per 100,000) (EFSA, 2015). However, in neighbouring Poland, the incidence of this zoonosis is only 1.4 cases per 100,000 (EFSA, 2015).

The risk factors for Campylobacter infections include: ingestion of undercooked meat; contaminated food and water or raw milk; direct contact with children, pets and farm animals; swimming in lakes and also traveling abroad (Jakopanec et al., 2008; Richardson et al., 2007; Ellis-Iversen et al., 2012). However, poultry is still a major source of this organism (EFSA, 2015). Studies in Lithuania have revealed that up to 80.9% of broiler and 78.5% of cattle caecal samples were contaminated with campylobacters (Kudirkienė et al., 2010; Ramonaitė et al., 2013), whereas contamination of broiler wings and drumsticks was considerably lower, 46.6% respectively (Bunevičienė et al., 2010).

Molecular typing is one of essential components in many epidemiological studies, including the identification of food-borne outbreaks of infections. Surveys generating the molecular epidemiology data of *C. jejuni* are critical to understand the sources and routes of transmission (Levesque et al., 2008) and to develop control strategies in order to reduce the incidence of campylobacteriosis (Moffatt et al., 2010).

As control of campylobacters at poultry farms is complicated, it is important to get a better understanding of the *C. jejuni* population structure and phylogenetic relationship in association with broiler houses and environment. Therefore, this study was aimed to investigate the links between broilers and their environment at a broiler farm considering genetic diversity of *C. jejuni* within 1 year period.

Materials and methods

Flock sampling

The samples from flock were taken once a week from litter (before broiler placement day and during production for entire flock). To sample a broiler house, one pair of disposable fabric overshoes (boot socks) were worn over rubber boots as the collector and had to be used a minimum of 100 meters inside the house. Boot socks were pre-moistened with sterile physiological saline maximum recovery broth (610077, Liofilchem, Italy) before the use in order to allow maximum uptake of Campylobacter cells from the litter. After that, the boot socks were placed into a sterile plastic bag and transported to the laboratory. When the boot sock was shown to be positive by PCR, 10 faecal swabs from broiler cloacal samples were taken to confirm positivity once a week until the first depopulation event. The swabs were placed into tubes with 10 mL sterile modified Exeter broth, which was prepared from Bolton broth (CM985, Oxoid, England) with Campylobacter growth (SV61,

Mast Diagnostics, Merseyside U.K), Campylobacter enrichment (Exeter) (SV59 Mast Diagnostics, Merseyside UK) supplements, and 1% of the laked horse blood, as described in Williams et al., 2012. Before slaughter, 10 faecal samples were taken for the detection of the total *Campylobacter* spp. number and placed into sterile plastic bags.

Environmental sampling

The samples from the environment were also taken weekly. We took the samples from the zones around the poultry farm, puddles, tap water and flies, rat faeces, wild animals (cats, dogs) and birds. The environment around the poultry farm was divided into 5 zones (1 m, 40 m, 60 m, 80 m and 100 m). The samples were taken from boot socks as well as described above. The samples taken from puddles were put in sterile 100 mL bottles all seasons except winter. The tap water samples were taken from the study house and additional houses when broiler age was 20−25 days. They were put into sterile 100 mL bottles as well. Before taking the samples, we sterilised the tap and flushed water for 3−5 minutes. We put wild animals, birds and rat faeces into sterile bags. Flies were taken with a sterile pincer and put in sterile bags, too.

Isolation procedure and identification

The plastic bags with boot socks from litter and zones were filled with 200 mL of sterile saline maximum recovery broth (610077, Liofilchem, Italy) and palpated by hand to release faecal material. The procedure was used by the technique of Merga et al. The 10 µL of the same saline with boot socks was streaked onto a campylobacter blood-free medium base (mCCDA), (610130, Liofilchem, Italy) with mCCDA Selective Supplement (81037, Liofilchem, Italy). The plates were incubated in a microaerophilic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen) generated by Campygen (CN25; Oxoid, UK) at 37ºC for 48 hours. After the incubation, the colonies suspected in having Campylobacter were obtained from each plate, examined by microscopy and further purified on blood agar plates (610188, Liofilchem, Italy) supplemented with 5% laked horse blood (HBL100, E&O Laboratories, Scotland), and incubated at 37ºC for 48 hours in microaerophilic atmosphere. The subcultured isolates were subsequently stored at -80ºC in Brain Heart Infusion Broth (BHI) (610008, Liofilchem, Italy) with 30% glycerol (REACHEM, Slovakia) until further use.

Broiler cloacal samples were collected using sterile cotton swabs and directly plated on mCCDA. The plates were incubated in a microaerophilic atmosphere at 37ºC for 48 hours. The faecal samples from the broilers, which we used for the detection of the total *Campylobacter* spp. number, as well as wild animals, birds and rat faeces were mixed with modified Exeter broth with the proportion 1:10 (1 g of faecal with 10 mL of Exeter broth) and put into the BagMixer for 1 minute. We also prepared decimal dilutions until 10^{-10} for the broiler faecal samples. The Campylobacter counts (cfu/g) from the broiler faecal samples were calculated according to ISO 10272–2:2006. From every dilution, 100 µL were taken and put onto mCCDA agar. Saline (10 µL) from wild animals, birds and rat faeces was also streaked onto mCCDA agar. All the plates were incubated in a microaerophilic atmosphere at 37ºC for 48 hours. Each fly was macerated in a sterile mortar, suspended in 2 mL of 0.9% saline. The mixture was centrifuged at 14,000 rpm for 7 minutes. The pellet was re-suspended in 2 mL of Exeter broth and vortexed before incubation for enrichment at 37ºC for 24 hours. After the enrichment, the tube was again centrifuged and 10 µL were streaked onto mCCDA agar and incubated at 37ºC for 48 hours in microaerophilic atmosphere. The identification and the purification of Campylobacter isolates were further performed as described above.

Additionally, a selective enrichment procedure was performed for each sample to detect the low numbers of Campylobacter spp. For this purpose, broiler samples swabs were placed into 10 mL of modified Exeter broth. Also, 1 mL of clear fluid from the saline with boot socks from litter and zones was transferred into 10 mL of Exeter broth for enrichment. This Exeter broth was prepared from Bolton broth (CM985, Oxoid, England) with Campylobacter growth (SV61, Mast Diagnostics, Merseyside U.K), Campylobacter enrichment (Exeter) (SV59 Mast Diagnostics, Merseyside UK) supplements, and 1% of the laked horse blood, as described in Williams et al., 2012. The enrichment tubes were incubated into microaerophilic atmosphere at 37ºC for 48 hours. From other samples (boot socks, faecal, puddles, tap water) after the incubation, 10 µL of the broth were streaked onto mCCDA plates. The identification and the purification of Campylobacter isolates were further performed as described previously.

DNA extraction and multiplex – PCR

PureLink® Genomic DNA Kit (Thermo Scientific, Lithuania) was used to isolate DNA directly from the boot socks samples, and the presence of *Campylobacter* spp. was confirmed using PCR assay by Katzav et al. (2008).

The DNA isolation was carried out using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania). The supernatant was placed in a new tube and stored at -20ºC.

Campylobacter isolates were identified to the species level by the modification of the method and primers as described by Wang et al. (2002) and by Katzav et al. (2008) (Table 1). Primers C412F and CampR2 created a 857 bp fragment, which occurred in all *Campylobacter* spp. A 323-bp amplicon was generated for *C. jejuni*, and a 126-bp amplicon was generated for *C. coli* by using primers mix hybridising to the *C. jejuni* (primers CJF and CJR) and the *C. coli* (primers CCF and CCR). Each PCR mixture contained 2.0 µL of 2mM deoxynucleotide triphosphates (dNTPs) mixture (Thermo Scientific, Lithuania), 2.5 µL of 10xPCR buffer, 2.5 µL of 25 mM MgCl2, 0.25 µL of Maxima Hot-Start Taq DNA polymerase 5U/µL (Thermo Scientific, Lithuania), 1.0 µL of a 100 µmol 1-1 primer mix containing C412F, CampR2, *C. jejuni* and *C. coli* primers (Thermo Scientific, Lithuania), and 1.0 µL of DNA. PCR products were analysed by gel electrophoresis: 11 µL of each PCR product was loaded onto 1.9% TopVision Agarose gel (Thermo Scientific, Lithuania) containing 6.5 µL of

ethidium bromide solution. The gel was visualised on the UV board. The GeneRulerTM 100 bp DNA Ladder (Thermo Scientific, Lithuania) was used as the molecular size marker.

The *fla*A-RFLP genotyping

The isolates identified as *C. jejuni* were typed by PCR-RFLP for the *fla*A gene. A fragment of 1700 bp of the *fla*A gene was amplified in a PCR reaction using a pair of specific primers (Table 1). The PCR was carried out in a 50 µL (final volume) mixture containing 2.5 µL of DNA, 0.5 µL of A1 primer and 0.5 µL of A2 primer, 5 µL of 10xPCR buffer, 5 µL of 2mM dNTP's mix, 3 µL of 25 mM $MgCl_2$, 0.25 µL of Taq DNA polymerase (Thermo Scientific, Lithuania), and 33.25 µL of sterilised bidistilled water. First, the samples were incubated for 1 minute at 94°C and then cycled 35 times at 94°C for 15 seconds, at 45°C for 45 seconds, and at 72°C for 1 minute 45 seconds. The samples were then incubated at 72°C for 5 minutes and were maintained at 4°C until processed. The PCR product was digested by incubation at 37ºC for 3 hours in the reaction mix containing 21.8 µL of sterile bidistilled water, 3 µL of buffer, 0.2 µL of DdeI and 5 µL PCR product. Digested PCR products were run on 2.5% agarose at 90 V for 90 minutes. The *fla*A-RFLP gels were visualised on the UV board and photographed. The images in the TIFF format were performed using BioNumerics version 7.1 (Applied Maths, Belgium). After the pattern normalisation, the similarity matrix was calculated using the Dice similarity coefficient and the clustering by the Unweighted Paired Group Method with Arithmetic mean values (UPGMA). The band position tolerance and the optimisation coefficient were set to 2.0%. The GeneRulerTM 100 bp plus DNA Ladder (Thermo scientific, Lithuania) was used as the molecular size marker.

Table 1. **Primers used for multiplex – PCR and for *fla*A-RFLP typing of *C. jejuni* isolates**

Primer	Sequence	Amplicon bp	Gene
flaA (A1)	5'-GGA TTT CGT ATT AAC ACA AAT GGT GC-3'	1700	*fla*A
flaA (A2)	5'-CTG TAG TAA TCT TAA AAC ATT TTG-3'		
CJF	5'-ACTTCTTTATTGCTTGCTGC-3'	323	*C. jejuni hipO*
CJR	5'-GCCACAACAAGTAAAGAAGC-3'		
CCF	5'-GTAAAACCAAAGCTTATCGTG-3'	126	*C. coli glyA*
CCR	5'-TCCAGCAATGTGTGCAATG-3'		
C412F	5'-GGATGACACTTTTCGGAGC-3'	857	*Campylobacter* spp. 16S rRNA
CampR2	5'-GGCTTCATGCTCTCGAGTT-3'		

Results

Prevalence of Campylobacter in broilers and environmental sources

Campylobacter spp. bacteria were detected in 6 out of 7 examined broiler flocks (85.7%). Within 1 year period, 752 individual samples were collected covering 7 broiler flock rotations. In total, 214 (28.5%) samples were positive for Campylobacter. *C. jejuni* strains were found in 173 (80.8%) samples and *C. coli* in 21 (9.8%) samples (Table 2). Most often, *Campylobacter* spp. bacteria were isolated from broiler cloacae and puddle water samples. Also, the litter samples taken from broiler houses No. 2, 4 and 10 were very often contaminated with campylobacters. The lowest percentage of *Campylobacter* positive samples were found in the litter of broiler house No. 3, concentric zones around the broiler houses, wild bird and animal (cats, dogs) faeces. Only a few rat faecal samples were positive for *Campylobacter* spp. bacteria. And only 1 sample taken in the anteroom of broiler house No. 3 was contaminated with *Campylobacter jejuni*.

Table 2. **Prevalence of *Campylobacter* from broilers and farm environment in Lithuania, 2012-2013**

The type of source	No. of tested samples	No. (%) of positive samples		
		C. jejuni	*C. coli*	*Other Campylobacter spp.*
Litter, broiler house No. 3	44	6 (66.7)	1 (11.1)	2 (22.2)
Litter, broiler houses No.2,4,10	123	26 (68.4)	11 (28.9)	1 (2.6)
Anteroom No.3	36	1 (100.0)	0	0
Zones	130	20 (95.2)	1 (4.8)	0
Puddles	69	25 (67.6)	0	12 (32.4)
Wild birds	32	6 (100.0)	0	0
Rats	21	0	0	2 (100.0)
Wild animals faeces (cats, dogs)	5	1 (100.0)	0	0
Flies	44	0	0	0
Broiler cloacae	222	88 (88.9)	8 (8.1)	3 (3.0)
Tap water	26	0	0	0

Evaluation of *Campylobacter* counts (cfu/g) in broiler faeces before each slaughter of broiler flock revealed that higher *Campylobacter spp*. counts were found in the samples taken from broiler crop No. 4 during March−April period (6.86 $\log_{10}$ cfu/g) (Figure 1). Meanwhile, the lowest counts of *Campylobacter* were found in faeces samples taken from broilers of crop No. 2 (November−December) and crop No. 7 (September−October).

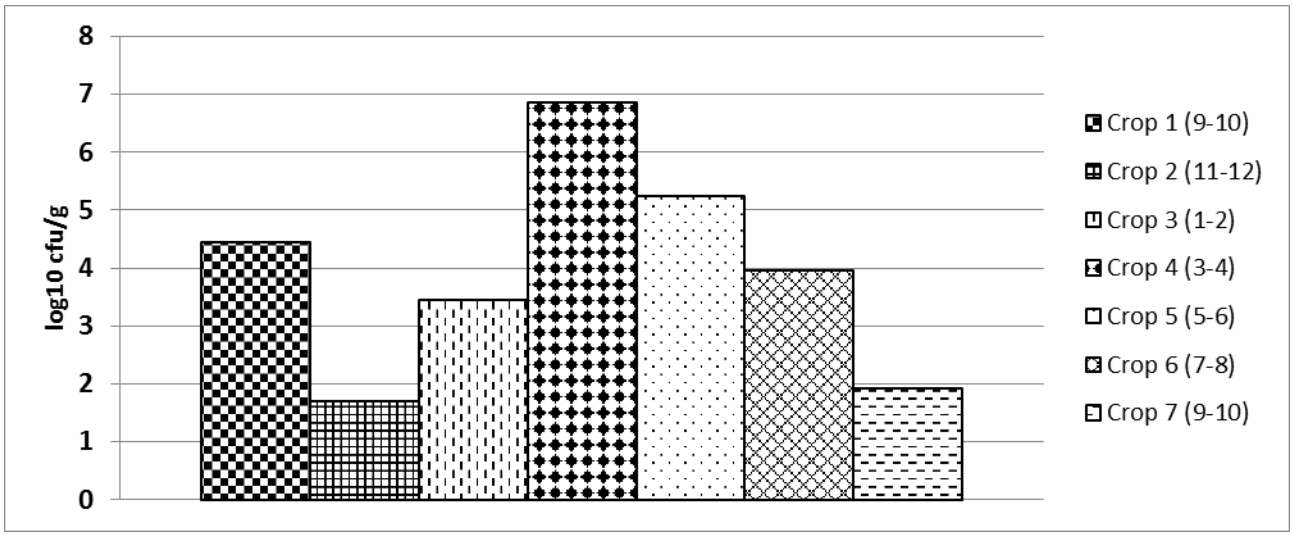

Figure 1. **The counts of *Campylobacter spp*. in broiler faeces at the farm**

Genotype diversity of *C. jejuni* isolates

The *fla*A-RFLP typing of 263 *C. jejuni* isolates generated 21 different *fla*A genotypes (Figure 2). The most frequent *fla*A genotype was XIV, and it was found in various samples taken from water of puddles, litter of broiler houses No. 3, 2, 4 and 10, broiler cloacal samples and concentric zones around broiler house No. 3. The same XIV *fla*A genotype was found in the samples of 6 broiler flocks. Campylobacters assigned to this *fla*A genotype were found in the samples taken up to a 100 m radius around the broiler house. The majority of all genotyped isolates were assigned to 11 identified *fla*A genotypes found in broilers and environment samples. In many cases, the *fla*A genotypes, which were found in broiler samples, were prevalent around the poultry houses and their surroundings up to a 40 m radius around the building and surrounding buildings in broiler flocks. *Fla*A genotypes I, V, IX, XI, XVII, XIX and XX were established in the environment and prevalent during rearing of first, third, sixth and seventh broiler flocks. Two *fla*A genotypes (IV and XXI) of *C. jejuni* strains were isolated only from broilers and were not found in any environmental sample. These Campylobacter *fla*A genotypes were found in samples of the last 2 broiler flock rotations.

Discussion

Horizontal transmission from the environment is considered to be the most likely source of *Campylobacter spp.* to broilers. It is widely accepted that horizontal transmission within a flock occurs rapidly once individual birds are colonised by *Campylobacter* (Carrillo et al., 2004; Horrocks et al., 2009). Thus, the risk of colonisation of broilers by campylobacters and their dissemination maybe increased by the high number of broilers in the flock, environmental water supplies, litter, insects, wild birds, rodents, faecal contact, people and other animals on a farm (Adkin et al., 2006; Horrocks et al., 2009).

This study revealed higher prevalence (85.7%) of *Campylobacter spp.* in broiler flocks compared with a previous study by Kudirkienė et al., 2009. Hungary and the United Kingdom have reported investigations with very high proportions of positive samples (from 74.2% to 80.0%) (EFSA, 2015). However, the prevalence of campylobacters in broiler farms can be even higher, as up to 96.5% of Campylobacter-positive broiler flocks were found in neighbouring Poland (Wieczorek and Osek, 2015).

Our study reveals that environmental sources contaminated with *Campylobacter* on the poultry farm are related to positive broiler flocks. The results of the *fla*A-RFLP typing showed that half of the *fla*A genotypes were the same in broilers and their environment. These *fla*A genotypes were found first in the environment, then in litter, and after that in broilers. The findings indicate that campylobacters can be transmitted from the environment to the broiler flock. There are several potential sources of Campylobacter that show a temporal correlation with the flock becoming Campylobacter positive. These include rodents (Umali et al., 2012; Nkogwe et al., 2011), humans (Messens et al., 2009; Ridley et al., 2011) and water (Patriarchi et al., 2011), which may indicate that the potential sources are actually becoming contaminated by the broiler flock when it is colonised. Catching crews and independent operators (Young et al., 2010) were identified as a potential source of introducing new genotypes in the flock during late grow-out when *Campylobacter* shedding in the contaminated flocks

peaks, increasing the chances of within-farm and industry-wide *Campylobacter* dissemination. Our study revealed that broiler flock No. 7 had a huge genetic diversity of campylobacters as 10 different genotypes were found in broilers and in the environment of the broiler house. The broiler flock was raised in April−May and the temperature was within the range of 7−16ºC. Meanwhile, in flocks No. 2, 4, and 5, we found fewer different genotypes. All these flocks were raised in winter and the temperature was below 0ºC. From this, we can state that the temperature has effects on the *Campylobacter* survival mechanism. Many studies have shown that *Campylobacter* are sensitive to low temperatures (Silva et al., 2011; Vashin and Stoyanchev, 2011) and, therefore, have fewer chances to survive in the environment and spread to flocks. A similar pattern with high diversity of types in a warm season and lower diversity of types in a cold season has been reported in other studies (Hiett et al., 2002; Kudirkienė et al., 2009). The most dominant *fla*A genotype was XIV, which was detected in 6 flocks out of 7. These flocks were raised in different seasons (spring, autumn and winter). We could speculate that bacteria assigned to this *fla*A genotype are more tolerant to environmental stresses. According to Duffy et al. (2015) and Allen et al. (2007), some genotypes could be more tolerant to environmental stresses in poultry processing.

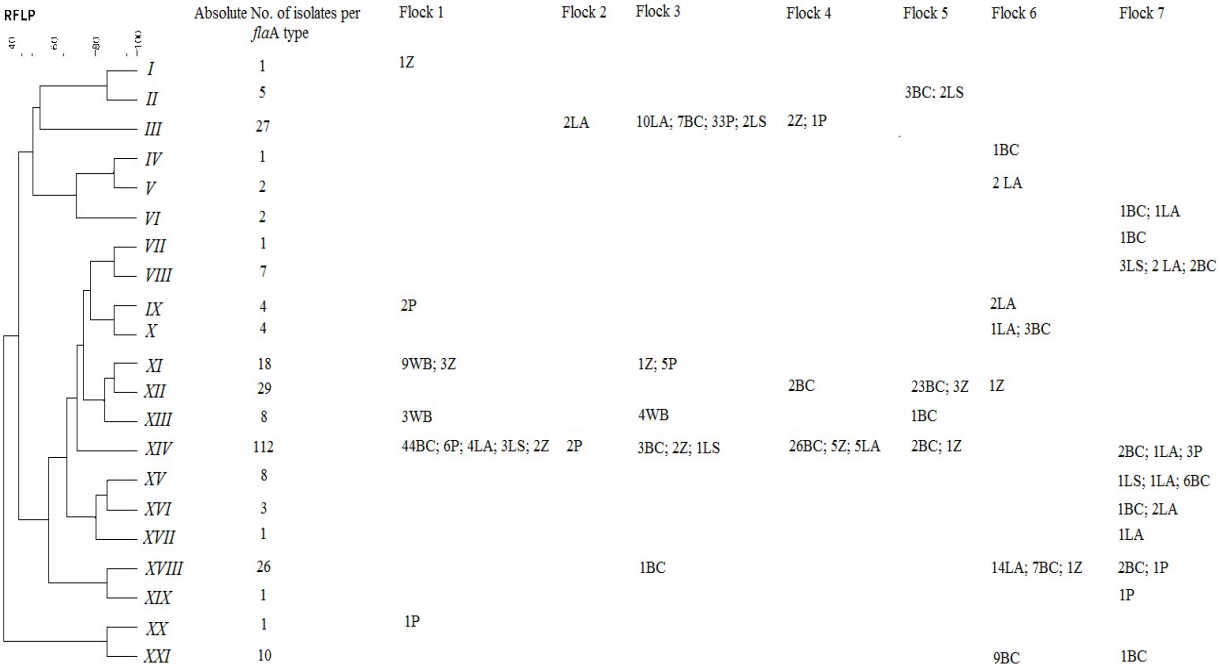

Figure 4. **Prevalence and diversity of *C. jejuni fla*A genotypes assigned to different sources**
*Source of isolate: LS – litter of broiler house No. 3; LA – litter of broiler houses No. 2, 4, 10; A – anteroom; Z – zones; P – puddles; WB – wild birds; BC – broiler cloacal.

The results of our study indicate that broilers and the environment around broiler houses are very often contaminated with *Campylobacter spp*. High genetic diversity of *C. jejuni* isolates reveals contamination links between broilers and the environment of broiler houses. The barriers between broilers and the environment are the most important in preventing introduction of *Campylobacter jejuni* from the environment into broiler houses. Further studies are needed to analyse all possible sources of *Campylobacter* spread including whole genome sequencing analysis to improve our knowledge about Campylobacter epidemiology.

Conclusions

1. Most of the broiler flocks were colonised with *Campylobacter* spp. (85.7%) within 1 year period.

2. Broiler cloacae samples were the most often contaminated with *Campylobacter* spp. bacteria, followed by puddle water samples taken near the broiler house and litter of broiler houses (44.6%, 53.6% and 30.9%, respectively).

3. Eleven out of the 21 identified *C. jejuni fl*aA genotypes were found in broilers and the surrounding environment of the broiler houses, and these findings highlight the potential contamination links between the environment and broilers flocks.

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