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Occurrence and determination of *Helicobacter pullorum* in conventional broiler-chicken farms in the Czech Republic between 2013 and 2014

Das Auftreten und Identifikation des Helicobacter pullorum in den konventionellen Broilerfarmen in der Tschechischen Republik zwischen den Jahren 2013 und 2014

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Summary

The aim of this study was to monitor occurrence of *Helicobacter* spp. in 29 conventional broiler-chicken farms, in the Czech Republic, of which 6 were sampled repeatedly. In the period February 2013–March 2014, a total of 615 samples of caecum content of slaughtered broilers were examined. Detection was performed by optimized cultivation techniques using Brucella Agar with 5 % sheep blood and by PCR method. Identification at genus and species level was performed using PCR-RFLP (restriction fragment length polymorphism). Presence of *Helicobacter* spp. was confirmed in 248 broilers (40.32 %). Using PCR-RFLP method with the restriction enzyme *Apa*LI, these 248 positive isolates were identified as *Helicobacter pullorum*. Occurrence of this new potential foodborne pathogen was confirmed in 17 conventional farms, the within-farm prevalence ranged from 40–100 %. Seasonality was not statistically significant. The results show that *Helicobacter pullorum* is another potential foodborne pathogen in the order *Campylobacterales* with a high incidence in the digestive tract of broiler chickens and may present a potential risk for human consumers. In order to ensure the safety of food of animal origin, it appears to be essential to pay attention to monitoring and further characterization of this pathogen in poultry farms in Central Europe.

Keywords: foodborne pathogen, broilers, enterohepatic *Helicobacter,* PCR-RFLP

Zusammenfassung

Ziel dieser Studie war es das Vorkommen von *Helicobacter* spp. Bei Broilern aus konventionellen Broilerbetrieben in der Tschechischen Republik zu bestimmen. Das Vorkommen von *Helicobacter* spp. wurde in 29 Geflügelhaltungen erfasst, in sechs davon wiederholt. Im Zeitraum von Februar 2013 bis März 2014 wurden insgesamt 615 Proben Darminhalt (Caecum) geschlachteter Broiler untersucht. Die Untersuchungen wurden mittels optimierter Kultivierungstechniken mit Brucella-Agar mit 5 % Hammelblut und PCR-Methode (Polymerase-Kettenreaktion) durchgeführt. Die Gattungs- und Artenidentifizierung und -typisierung erfolgte mittels der PCR-RFLP-Methode (Restriktionsfragmentlängenpolymorphismus). Das Vorkommen von *Helicobacter* spp. wurde bei 248 Broilern bestätigt (40,32 %). Mittels der PCR-RFLP-Methode und des Restriktionsenzyms Apa LI wurden alle 248 Isolate als *Helicobacter pullorum* typisiert. Das Auftreten des neuen potenziell Lebensmittel-assoziierten Pathogens wurde in 17 Broilerbetrieben bestätigt; die Innerherden-Prävalenz der einzelnen Betriebe betrug 40–100 %. Saisonale Unterschiede waren statistisch nicht ersichtlich. Aus den Ergebnissen der Studie geht hervor, dass *Helicobacter pullorum* ein weiteres potenziell Lebensmittel-assoziiertes Pathogen mit hohem Vorkommen im Verdauungstrakt von Broilern ist und damit ein mögliches Risiko für den Konsumenten darstellt. Zur Gewährleistung von Sicherheit und gesundheitlicher Unbedenklichkeit von Lebensmitteln tierischen Ursprungs, erscheint es von grundlegender Bedeutung, Monitoring und weitere Charakterisierungen dieses Pathogens in Geflügelzuchten in Mitteleuropa durchzuführen.

Schlüsselwörter: Lebensmittel-assozierte Zoonose, Broiler, enterohepatische *Helicobacter,* PCR-RFLP

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Introduction

The genus *Helicobacter* is a group of taxonomically related Gram-negative, microaerobic bacteria. Some species are pathogenic and it is known that they are able to colonize the gastrointestinal tract and hepatobiliary system of many animal species (Solnick et Schauer, 2001). The whole genus is divided into two groups generally related to their natural ecological niche, to gastric and enterohepatic (EHS) species (Solnick et Schauer, 2001). The clinical significance of *Helicobacter pylori* (the best-known type of gastric species) is well known (Fox et al., 2002). It is responsible for the formation of gastric and duodenal ulcers and gastric cancer in humans (Bohr et al., 2004). During the last decade some enterohepatic species obtained a status of potentially new pathogens whose clinical significance has not been determined yet (Fox et al., 2002).

Genus *Helicobacter*

Bacterial species of the genus *Helicobacter* were initially incorrectly classified in the genus *Campylobacter. Helicobacter* spp. was recognized as a separate genus in 1989, based on sequence analysis of 16S rRNA (Goodwin et al., 1989) *Helicobacter* spp. together with the genera *Campylobacter* spp., *Arcobacter* spp. and *Anaerobiospirillium* spp. belong to the order *Campylobacteriales* in the class *Epsilonproteobacteria,* which is characterized as curved or spiral shaped bacteria (Stanley et al., 1994). One common factor of these organisms is the fact that the environment where they occur is often in an extremely geographically different environment – from high temperature and press of deep-sea hydrothermal vents, through the highly acidic stomach environment to sulfidic caves (Porter et Engel, 2008). These environments clearly require a unique repertoire of processes of cell survival, which makes *Epsilonproteobacteria* unique (Gilbreath et al., 2011). Morphologically they are nonsporulating and slightly curved to spiralic rods of 0.3–0.6 µm x 1–5 µm. They can pass into coccoid forms depending on the culture conditions, particularly the content of atmospheric oxygen and the age of the culture (Dewhirst et al., 2000). Mobility of bacteria is ensured by polar located flagellum or flagella. Movement is mostly corkscrew or slower wave (Garrity, 2005) They are microaerobic, they do not grow under aerobic conditions. In anaerobic conditions they are able to grow only slightly. *Helicobacter* spp. is not dependent on the presence of hydrogen in the atmosphere, although its use would promote growth. Growth occurs at 37 °C to 42 °C (Melito et al., 2000, Ceelen et al., 2006).

Helicobacter spp. in poultry

It was found that among the enterohepatic species only *Helicobacter pullorum* (Stanley et al., 1994) and *Helicobacter canadensis* (Fox et al., 2000) are specifically present in poultry. *Helicobacter pullorum* is a new pathogen whose DNA was originally found in the liver and intestinal contents of laying hens with hepatitis and in ceacal content of clinically healthy chickens (Stanley et al., 1994). The occurrence of *H. pullorum* is the most associated with farmed birds, especially with chickens, turkeys and guinea fowls (Nebbia et al, 2007). This pathogen is potentially zoonotic as associated with human gastritis, chronic cholecystitis, cholelithiasis, liver diseases, tumor diseases of the liver and gallbladder and with diseases of immune system (Hansen et al., 2011). Recent studies have also stated that *H. pullorum* together with *H. canadensis,* both that of various genotypes are repeatedly identified as the most common species in patients with Crohn's disease, but their role in pathogenesis of this disease has not been elucidated yet (Ceelen et al., 2006, Laharie et al., 2009, Hansen et al., 2011 Zanoni et al., 2011). Asymptomatic poultry is considered to be the main reservoir hosts and source for human infection with these two species due to their presence in gastrointestinal tract from where they are able to move to the external environment. This assumption was discussed by Ceelen et al. (2007). They found that *H. pullorum* was present in feces during the whole growing period (42 days) of experimentally infected chickens and high numbers of bacteria were found in the intestinal contents of the chickens. Throughout the period of fattening, concentration of *H. pullorum* in jejunum ranged from 1.7 to 5.1 log cfu g^{-1}. *Helicobacter* is closely related to *Campylobacter* with minimal infectious dose could be as low as 800 cells. (Kothary et Babu, 2001). Infected chickens had no apparent clinical signs of infection. Their study showed that *H. pullorum* persists in broilers at the age of slaughter and there is a chance for the contamination of carcasses during slaughter processing due to carcass contamination of broilers at the slaughterhouse (Allen et al., 2007, Reich et al., 2008). Atabay et al. (1998) investigated qualitatively the degree of contamination of carcasses taken from the line in a poultry factory immediately after evisceration. Out of total 15 samples 9 (60 %) were positive for *Helicobacter pullorum.* Strains were isolated directly from carcass washings. Therefore, contaminated raw poultry meat and poultry products might be a source of human infection by *H. pullorum,* same as it is described for the bacterial species of *Arcobacter* and *Campylobacter* (Ceelen et al., 2006). Tenacity on meat surface is low because of its sensitivity to oxygen but *H. pullorum* can survive in moist broiler skin after spray or water chilling (Corry et Atabay, 2001). Information about the incidence caused by *H. pullorum* are significantly underestimated due to difficult cultivation of this pathogen and the phenotypic similarity between species *Helicobacter* and *Campylobacter* (Ceelen et al., 2006). The occurrence of *H. pullorum* in chickens was studied in a number of countries: Switzerland (Burnens et al, 1994), Denmark (Atabay et al, 1998), Belgium (Ceelen et al, 2006), Italy (Zanoni et al., 2007), Czech Republic (Svobodova et Steinhauserova, 2011), Turkey (Kahraman et Ak, 2013), Egypt (Hassan et al., 2014). Gold standard method for isolation is the modified method for *Campylobacter* detection with filter technique (Manfreda et al., 2006). However, the studies are hardly comparable to each other due to the use of different cultivation methods and PCR protocols.

Isolation and identification of *Helicobacter* spp.

Cultivation of *Helicobacter* spp. is very difficult. Faster-growing bacteria than *Helicobacter* spp. represent the biggest issue for the cultivation of this pathogen. In particular, the phenotypically similar genus *Campylobacter* spp. overgrows *Helicobacter* spp. during cultivation on media and it often leads to false-negative incorrect identification (Ceelen et al., 2006). The cultivation of *Helicobacter* spp. requires resuscitation of sublethally damaged cells and the use of special culture media. However, these techniques are based on the methods for the isolation of *Campylobacter*

spp., so their specificity and selectivity may be limited, but are not still defined (González et al., 2008). Suitable culture media are particularly Brucella agar and Columbia agar, always with the addition of blood. *H. pullorum* requires a microaerophilic environment (Burnens et al., 1994, Atabay et al., 1998). In order to increase selectivity of the cultivation method, filter technique is frequently used, taking advantage of the ability of *Helicobacter* spp. to penetrate through pores of membrane unlike other intestinal bacteria with exception of *Campylobacter* spp. Polymerase chain reaction (PCR) method in combination with the determination of restriction fragment length polymorphism (RFLP) is a frequently used method for the bacterial species identification. Polymerase chain reaction has the advantage that it is capable to detect hard-to-culture bacteria and nonculturable bacteria. By the use of this method we can examine bacterial species not only from the intestinal tissue, but also from intestinal contents (Ceelen et al., 2006). PCR method in combination with RFLP according to Fox et al. (2000) is the most commonly used method for the specification of *H. pullorum.* With this published method we are able to differentiate it even from the closely related species *H. canadensis.* One of the main advantages of these molecular genetics methods is that they are highly specific and efficient.

Material and Methods

The occurrence of *Helicobacter* spp. in farmed broiler chikkens was studied during the period February 2013– March 2014. Broilers sampled at the slaughterhouse (region South Moravia) originated from 29 conventional broiler rearing farms from 7 regions in the Czech Republic. The number of animals on individuals farms ranged from 29 000 to 275 000 pieces. Minimal number of cycles per year was 5 and maximum 8, with an average 36.44 days of feeding before slaughtering. The average feed conversion ratio was 1.77 kg of feed mixtures and average daily gain was reaching up to 0.06 kg. Fattening on farms was conducted with two hybrid combinations Ross 308 and Cobb 500. The digestive tract (caecum) (n = 615) of broiler chickens were taken from the processing line. From each farm 15–25 samples of digestive tract of randomly selected broilers were taken. The samples were immediately transported to the laboratory. The caecal content was separated in a sterile way from the gastrointestinal tract. The end of caecum was cut off by the sterile scissors and caecal content was squeezed into a sterile plastic bag. Saline solution was added to the sample, forming a thick suspension. Resuscitation of sublethally damaged cells of *Helicobacter* spp. in obtained suspension was carried out in a mixture of 25 ml brain hearth infusion, 7.5 g glucose and 75 ml horse serum and in the next step of cultivation was used a modified Steele and McDermott membrane filter technique as described by Zanoni et al. (2007). Cultivation was carried out on Brucella agar (Oxoid, UK) supplemented with 5 % sheep blood under microaerobic conditions (5 % O_2, 10 % CO_2 and 85 % N_2) in incubator at 37 °C ± 0.5 °C for 72h ± h. Grown cultures were visually checked every 24 hours and presumptive colonies were subcultivated on Brucella agar with 5 % sheep blood under same conditions as previously. The DNA from suspected colonies was isolated (Qiagen Tissue Kit, Germany) according to the manufacturer's instructions and species identification was carried out using PCR method (polymerase chain reaction) and PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) by Fox et al. (2000) with the restriction enzyme *Apa*LI. This PCR protocol is able to amplify the 16S rDNA gene, using *Apa*LI site at position 1040, resulting digestion into two fragments (250 bp, 950 bp) of *H. canadensis,* while it didn't effected *H. pullorum.* Mixed DNA from caecal content isolated by the QIAamp DNA Stool Mini Kit (Qiagen, Germany) was used for direct detection and isolation of *Helicobacter* spp., followed by PCR identification by Fox et al. (2000). *H. pullorum* CIP 104787T and *H. canadensis* CCUG 471/63 were used as a positive control for PCR.

Results

The results of monitoring confirmed the presence of *Helicobacter* spp. at the Czech poultry farms. During the period February 2013 to March 2014, a total of 615 samples of caecal contents of chickens originated from 29 conventional broiler farms were examined. From the processing line of the poultry abattoir were taken 15–25 samples of digestive tract of randomly selected broilers from each farm. According to the PCR results there was a positive detection in 248 samples (40.32 %). This microorganism was found at 17 of 29 farms, 12 farms were negative. Prevalence of only 7.64 % was detected by cultivation technique. The results are presented in Table 1.

TABLE 1: *Prevalence of Helicobacter spp., determined by cultivation and PCR methods, in caecal contents of broiler chickens.*

Year	Number of farms	Number of samples		Number of positive samples	
		Cultivation	PCR	Cultivation (%)	PCR (%)
2013	23	375	375	45 (12.0)	223 (59.5)
2014	6	240	240	2 (0.8)	25 (10.4)
Total	**29**	**615**	**615**	**47 (7.64)**	**248 (40.32)**

Using the PCR-RFLP method with the restriction enzyme *Apa*LI, all 248 isolates were identified as *Helicobacter pullorum.* This pathogen was confirmed to be present at 17 broiler farms. The within-farm prevalence ranged from 40 to 100 %. In case of 11 farms all the investigated samples were positive. A total of 6 farms were investigated repeatedly to include both cold (December, January, February, March) and warm months (May to August). Seasonality in occurrence of *Helicobacter* spp. in the digestive tract of broiler chickens was not statistically conclusive as can be seen in Figure 1 and Figure 2. *Helicobacter canadensis* was not present in the tested samples at all.

Discussion

Helicobacter pullorum is another potential foodborne pathogen in the order *Campylobacterales* with a high incidence in the digestive tract of broiler chickens and may present a potential zoonotic risk for consumers. In our study, the total prevalence of *Helicobacter pullorum* in farms destined for fattening of broiler chickens in the Czech Republic was 40.32 %. Cultivation was successful in detection of *Helicobacter* spp. in only 7.64 % of all analyzed

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samples due to high sensitivity of *Helicobacter* spp. and difficulty to differentiate closely related bacterial species (particularly thermophilic *Campylobacter*) during isolation. All samples were simultaneously examined by molecular genetics methods. Several scientific studies were published about the occurrence of *H. pullorum* in broiler chickens especially in Italy, Belgium and Turkey. It is difficult to compare their studies due to different methods used for the detection of the pathogen and the different processing of samples. A high occurrence of *H. pullorum* in the caecum of broiler chickens was shown in an Italian study by Manfreda et al. (2011), who examined 34 flocks from 30 conventional broiler farms. The samples of caecal content (n = 169) were examined only by cultivation technique and 142 samples (84 %) were found as positive. The same technique was used for the examination of broilers caecal contents in another Italian study, Zanoni et al. (2007). This study confirmed presence of *H. pullorum* in all of collected samples (n = 60). But the amount of 60 samples doesn t comprise an adequate and representative cross-section. In an Egyptian study conducted by Hassan et al. (2014), a total of 900 samples of cloacal swabs, caecal swabs and livers from chickens were examined using conventional phenotypic methods for isolation and identification. *H. pullorum* was found in 39.33 % of the samples. Ceelen et al. (2006) determined the occurrence of pathogens using specific PCR and detected *H. pullorum* in 33.6 % of broilers at 11 Belgian broilers conventional farms. Similarly, Kahraman et Ak (2013) reported the incidence of 55.21 % in Turkish broilers. In the Czech Republic, there was a study about the occurrence of *H. pullorum* in the period 2006–2010, when a total of 36 farms were investigated (Svobodova et Steinhauserova, 2011). A total of 500 samples were examined by cultivation on Brucella agar supplemented with sheep blood and only 7 % of the samples were found positive. The authors stated that the low level of detection was probably caused by high cultivation requirements of this pathogen and by overgrowth of the cultures by competitive *Campylobacter* spp. during cultivation. In this study the samples were also tested using molecular genetics techniques. There were 372 samples examined by PCR method and *H. pullorum* was detected in 161 samples (43.3 %). A comparison of our results with the results of this study shows that the current prevalence of *H. pullorum* in Czech broiler farms is at a comparable level as in 2006–2010. It appears that the molecular genetics method used in these studies is more sensitive for the detection and direct identification of this pathogen and standard culture techniques should be combined with it. Molecular genetics methods have already been used as standard methods for species identification in all the published studies.

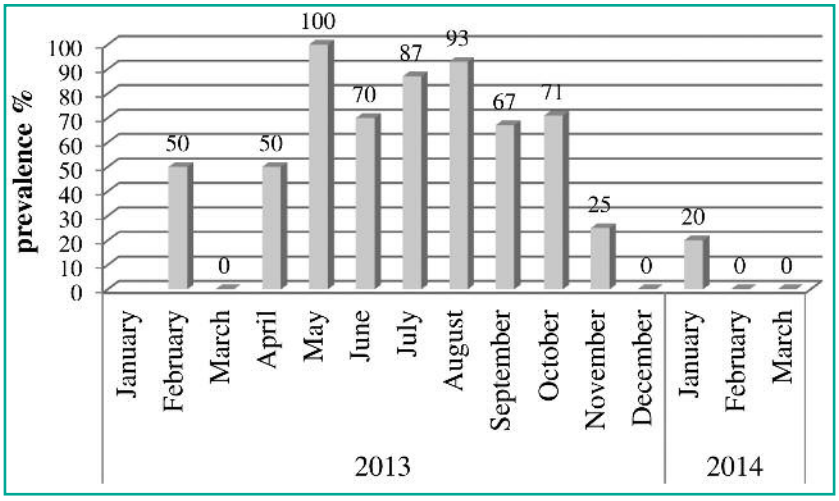

FIGURE 1: *Monthly prevalence of Helicobacter spp. in all the farms (n = 29).*

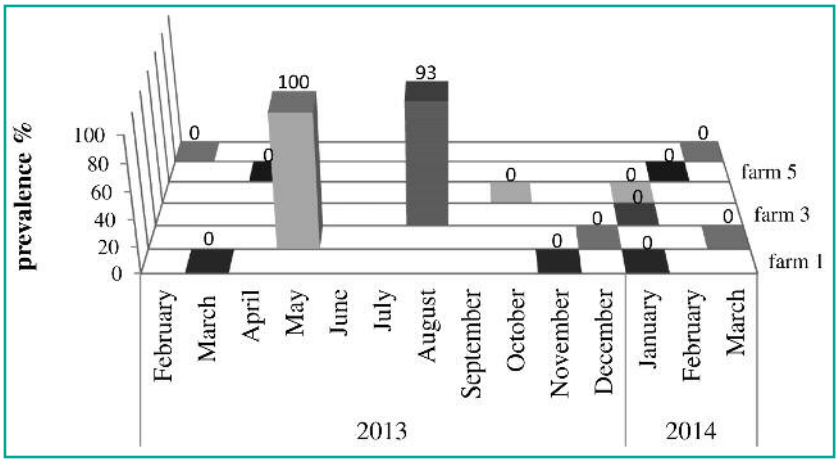

FIGURE 2: *Prevalence of Helicobacter spp. in farms 1–6 in repeated sampling.*

Conclusion

The aim of our study was to evaluate the data on the current prevalence of *Helicobacter* spp. in broiler chicken in the Czech Republic. The results confirm the occurrence of EHS *Helicobacter pullorum* in Czech conventional broiler farms. The prevalence of this pathogen as determined in this study (40.32 %) shows that its occurrence in the digestive tract of poultry is very common. In the case of contamination of carcasses during slaughter processing, raw poultry meat could be a source of infection for humans. Species of *Helicobacter* spp. which colonize the digestive tract of poultry could be responsible this way in the development of inflammatory bowel disease including Crohn's disease. However, this assumption requires more detailed research involving mainly investigation of the pathogenicity of these species and the study of relatedness of human and animal isolates.

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Conflict of interest

To whom it may concern, we certify that we have no affiliations with or involvement in any organization/entity with any financial or non-financial interest in the subject matter or materials included in this manuscript.

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