CAMPYLOBACTER AND *HELICOBACTER PULLORUM* COINFECTION IN POULTRY

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Abstract – *Helicobacter pullorum*, a bacterium initially isolated from the feces and livers of poultry, has been associated with human diseases. Infections by this pathogen are underestimated due to phenotypic concordance between *Helicobacter* and *Campylobacter* genres.

This study aims to assess the presence of *H. pullorum* and *Campylobacter* spp. in broilers at slaughterhouse level. Samples from intestines and neck skins from extensive indoor (n=five) and intensive production (n=four) were collected, totaling 15 ceca pools, and 18 neck skin pools, representing 29.245 birds. Samples were tested for *H. pullorum* and *Campylobacter spp*. by using Polymerase Chain Reaction (PCR).

The results of this experiment showed a high frequency of both *H. pullorum* and *Campylobacter* spp. in flocks. Broiler intestines and neck skin samples from slaughterhouse presented coinfection for these pathogens. Given that *H. pullorum* is an emergent foodborne pathogen, the study becomes relevant in the public health scope .

Key Words – Poultry, safety, Helicobacter, Campylobacter, food-borne pathogens.

I. INTRODUCTION

Helicobacter pullorum, a urease negative organism, was classified as a new bacterial species by Stanley *et al* [1]. It is an enterohepatic helicobacter that colonizes the lower segments of the intestinal tract – mainly the cecum, the liver and the bile ducts of avian species, mice and humans [2]. This microorganism is associated with hepatitis and enteritis in chicken, but can appear in asymptomatic bird cecum [3].

H. pullorum has been increasingly recognized as a food-borne zoonotic pathogen, and avian species appear to be a relevant reservoir of this organism [4]. It was isolated and identified in humans, being associated with several inflammatory bowel diseases, such as Chron's disease [5], and hepatobiliary diseases, such as hepatitis, acute and chronic cholecystitis, cirrhosis, and hepatocellular carcinoma [6]. In addition, it may cause bacteremia and systemic disease in immunocompromised and immunocompetent patients [7]. It has been also demonstrated its relevance in the progression of chronic hepatitis C to cirrhosis and hepatocellular carcinoma [8]. *H. pullorum* only develops in microaerobic conditions [9] and can be distinguished from other helicobacters by the lack of sheathed flagella [10].

Campylobacteriosis is the most commonly reported foodborne disease in the last years by EFSA and ECDC (2015). Poultry is pointed out as the main source of infection with *Campylobacter* in humans.

This work aims to (i) evaluate the frequency and the contamination level of *H. pullorum* on poultry at slaughterhouse level (ii) reporting the detection of co-infection of *H. pullorum* and *Campylobacter spp*. in broiler flocks

II. MATERIALS AND METHODS

Samples collection

Sampling was performed on different working days at a poultry slaughterhouse from a total population of 29245 chickens from flocks extensive indoor (9330, n = 5), and intensive production (19915, n=4), (identified and recorded according to producer origin, carcass weight, resting time before slaughter and electrical stunning conditions).

All birds from the same producing system were slaughtered in identical conditions (electrically stunned, bled, scalded, defeathered, eviscerated and rinsed). For each chicken flock, five ceca were randomly sampled twice, and the neck skins of five carcasses, after the inside-outside shower and before rapid cooling, were collected twice constituting also two pool samples. All samples were transported to the laboratory in an isothermal box in less than one hour for microbial analysis. A total of 18 ceca pools and 18 neck skin samples pools were analyzed for *Helicobacter* and *Campylobacter* isolation and detection.

At laboratory, under aseptic conditions, a pool of cecal content was prepared collecting the material of each of the five broiler ceca pool samples that were used in subsequent analysis.

Direct identification of Helicobacter and Campylobacter from cecal samples by PCR

DNA extraction was performed from approximately 200 mg of cecal content using a commercial stool kit (QIAamp DNA stool mini kit; QIAGEN) [11]. DNA samples were tested by PCR for *Helicobacter* and *Campylobacter* detection.

Detection and isolation of Helicobacter and Campylobacter from cecal samples

Initially 5g of cecal content pool were diluted in 5 ml of a sterile solution of 0,9% of NaCl. From this preparation, 100µl were diluted in 400 µl of a sterile mixture containing Bacto-Brain Heart Infusion, inactivated horse serum and glucose [11]. The 300 µl of caecal diluted sample was applied on a 0.65 µm cellulose filter membrane (Advantec MFS, CA, USA) directly placed on Columbia agar with 5% horse blood [16]. This plate was incubated aerobically at 37±1ºC, for 1 h; the membrane was then removed and the plate incubated under H_2-enriched microaerophilic atmosphere for 44 ±4 h at 41.5 ± 1 ºC. Eight presumptive colonies of *Helicobacter* or *Campylobacter* were picked from each plate and submitted to confirmation by genetic tests.

Detection of Helicobacter and Campylobacter from neck skin samples

Each sample pool of neck skin was added to 75 ml of Bolton broth (selective pre-enrichment) and incubated under H_2-enriched microaerophilic atmosphere at (37±1) ºC for 4 to 6 h, followed by 44±4 h at 41.5±1 ºC. Then, 100µl of the culture were applied on a 0.65 µm cellulose filter membrane (Advantec MFS, CA, USA) directly placed on Columbia agar with 5% horse blood [12]. This plate was incubated aerobically at 37±1ºC for 1 h; the membrane was then removed and the plate incubated under H_2-enriched microaerophilic atmosphere for 44 ±4 h at 41.5 ± 1 ºC. All the procedure was followed as described in previous section.

DNA extraction from isolates

Presumptive colonies of *Campylobacter* or *Helicobacter* were cultured on blood agar plates. DNA was extracted by using the boiling extraction technique.

PCR methods for Helicobacter and Campylobacter

A PCR assay amplifying a 140 bp fragment of the *cdtB* gene of *H. pullorum* was performed, using the F1-cdtB-pullorum, 5´ GTCTTTTGAGTGGATTGGATTCT 3´, and R2-cdtB-pullorum 5´CACTCCGGGTGCTTGTGTAT 3´ primers.

The reference strain MIT 98-5489 (H436), isolated in Canada from the stools of a patient suffering from gastroenteritis, was used as a positive control for *Helicobacter pulorum* [13].

A PCR for thermophilic *Campylobacter* was performed using the primer pair THERM 1 (5′-TATTCCAATACCAACATTAGT-3′) and THERM 4 (5′-CTTCGCTAATGCTAACCC-3′). These primers are based on the most variable part of the 23S rRNA gene and amplify a 491 bp sequence from all four thermophilic species, *C. jejuni, C. coli, C. lari* and *C. upsaliensis*.

Following PCR amplification, products were cut by AluI and Tsp509I to identify the thermophilic Campylobacter species [14].

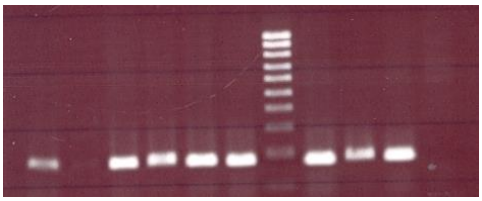
Campylobacter jejuni DSM 4688 and *Campylobacter coli* DSM 4689 were used as positive controls.

III. RESULTS AND DISCUSSION

The results related to the frequency of *Helicobacter* and thermophylic *Campylobacter* detection in the nine flocks analyzed are summarized in Table 1. Six of the nine flocks were positive for *H. pullorum* and six for *Campylobacter*; five of them were positive for both pathogens. The prevalence of *H. pullorum* in flocks from intensive and extensive indoor production was 22% and 44%, respectively. These results suggest that *H. pullorum* is present in flocks without signs of disease, and that co-detection with *Campylobacter* is frequent. Also, the presence of these pathogens in the neck skin, and their absence in cecal samples, suggest cross contamination of poultry carcasses during slaughtering practices.

There was a high frequency of *H. pullorum* detection from cecal samples by direct PCR compared with culture from those samples, particularly from neck skin samples, mostly due to *Proteus* contamination.

Figure 1. Detection of *Helicobacter pullorum* in caecal content samples of broilers carcasses by PCR assay, amplifying 140bp segment of *cdtB* gene.



Lane 1: *Helicobacter pullorum* MIT 98-5489; lane 2: negative control; lane 3-6 and lane 8-11: *Helicobacter pullorum* broiler strains to four broiler flocks, lane7: marker 100bp ladder.

The direct detection method by PCR, using a commercial stool kit to extract DNA, was the easiest and fastest way to confirm the presence or absence of *Campylobacter* or *Helicobacter* (Figure 1) on caecal samples. This method can be used to easily screen these pathogens followed by bacterial culture. On the other hand, the use of the membrane-based culture method without selective plating agar hampers emergent *Helicobacter* species detection and isolationfrom samples with complex microbiota, such as neck skin, although for caecal samples the isolation method was suitable.

The isolation of *Helicobacter* and *Campylobacer* from flock samples will allow the genetic and phenotypic characterization of the isolates.

Table 1 Frequency of positive *Helicobacter* and thermophilic *Campylobacter* flocks in a Portuguese slaughterhouse.

Flocks/production system; and producers N=9	Total number of birds/flock	Direct identification in cecal samples by PCR N=18	Detection and isolation from cecal samples N=18	Detection from neck skin N=18	Total number of isolates obtained Ratio Helicobacter: Campylobacter
1/E; A	1636	Negative	Negative	Positive (*C. jejuni*, *C. coli* and *H. pullorum*)	0* : 7
2/E; B	2050	Negative	Negative	Positive (*C. jejuni* and *C. coli*)	0* : 5
3/I; C	8850	Negative	Negative	Negative	-
4/E; D	2650	Positive (*C. jejuni*, *C. coli* and *H. pullorum*)	Positive (*C. jejuni*, *C. coli* and *H. pullorum*)	Positive (*C. coli* and *C. jejuni*)	8 : 2
5/I; E	2995	Negative	Negative	Negative	-
6/E; F	1627	Positive (*C. coli* and *H. pullorum*)	Positive (*C. coli* and *H. pullorum*)	Positive (*H. pullorum*)	1 : 9
7/E; G	1367	Positive (*C. jejuni*, *C. coli* and *H. pullorum*)	Positive (*C. jejuni*, *C. coli* and *H. pullorum*)	Positive (*H. pullorum*)	11 : 5
8/I, H	3320	Positive (*C. jejuni* and *H. pullorum*)	Positive (*C. jejuni* and *H. pullorum*)	Positive (*H. pullorum*)	8 : 10
9/I; I	4750	Positive (*H. pullorum* and *Campylobacter*)	Positive (*H. pullorum* and *Campylobacter*))	Negative	2 : 1
Detection Helicobacter : Campylobacter at flock level (%)		**44,4%:50%**	**33,3%;50%**	**27,8.%:16,7%**	**30 : 37**

1-9: different numbers correspond to different flocks producers;
Intensive production =I and extensive indoor production =E
A-I: different letters correspond to different producers
* In these cases, *H. pullorum* isolates could not be recovered probably due to Proteus contamination.

IV. CONCLUSION

The results of this study are important to define the frequency of *H. pullorum* in poultry meat, a topic not yet explored in Portugal. Since this bacterium is transmitted to humans through undercooked poultry meat, it is necessary to draw the attention to this fact. Indeed, the under-detection of *H. pullorum* is a public health problem associated with digestive diseases that should be controlled by the implementation of preventive measures. This microorganism´s high frequency in the intestinal tract of poultry is accordance with other studies, and reflects the serious risk of crossed contamination of chicken carcasses, confirming the need to address this issue as a public health problem.

Finally, it is interesting to report the co-detection of *H. pullorum* and *Campylobacter* in broiler flocks for human consumption.

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