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Molecular Detection of *Campylobacter spp* in Day-Old Chick Demonstrate Vertical Transmission in Poultry Production

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Abstract

Campylobacteriosis is the most common cause of foodborne gastrointestinal illness in the industrialized world, and poultry is considered the main source. While horizontal transmission is a route clearly linked to the spread of *Campylobacter* at farm level, few studies support the notion of vertical transmission. Currently, epidemiological research indicates that newly hatched chicks appear to be free of *Campylobacter*. Thus, we carried out the present study to investigate the occurrence of *Campylobacter* in day-old chicks using molecular methods to examine vertical transmission in poultry production. A total of 12 broiler flocks were monitored from the time of housing day-old chicks (day 1) and at the end of the rearing period (day 42). Samples were culture according with official method ISO 10272:2006 and analyzed using reverse transcription quantitative real-time PCR method. Our results revealed that no evidence of *Campylobacter* was found in the day-old chicks by bacterial culture method. Nevertheless, 4 flocks out of 12 were found to be positive by the molecular method. Real-time PCR identification revealed that *C. coli* was detected in all 4 flocks, while *C. jejuni* was identified in 3 flocks. No presence of *Campylobacter spp.* was observed in the environmental samples. These results reflect the evidence for vertical transmission of *Campylobacter spp.* While studies do not definitively rule out the detection problems and an accepted standard method will be developed for the detection and isolation of *Campylobacter spp.* at farm level, no standard measure may be successfully implemented in broiler production and therefore, from a public health point of view, strategies to reduce the number of human campylobacteriosis cases will not be efficient.

Keywords

Poultry, Food Safety, Vertical Transmission, qPCR

1. Introduction

Campylobacteriosis is the most frequently reported zoonosis in the EU and one of the most common causes of diarrheal illness in the United States, and the incidence appears to be increasing [1], [2]. The European Food Safety Authority (EFSA) reported a total of 214,268 cases of human campylobacteriosis in 2012, and the Center for Disease Control (CDC) estimates that each year 845,024 cases of human campylobacteriosis occur in the United States [3]. Poultry and poultry products are considered the main source of human campylobacteriosis [2], and the majority of infections result from consumption of undercooked poultry or other foods [4], [5]. Specifically, in the European context broiler meat may account for 20-30% of human campylobacteriosis, while 50-80% can be attributed to the chicken reservoir as a whole [2].

The epidemiology of *Campylobacter* in poultry production is still incompletely understood [6]. For more than a decade, there has been a major debate on whether vertical or horizontal transmissions are responsible for the introduction of *Campylobacter* into flocks [6], [7]. Clearly, horizontal transmission has been identified through different sources,

while the vertical transmission from parent flocks and their progeny still remains unclear [6], [8]. Nevertheless, there is not yet an acceptable standard method for the detection and isolation of *Campylobacter* spp at farm level [9]. For food legislation purposes, the ISO method 10272-2 is the official method for detection and enumeration of *Campylobacter* spp, while the molecular methods are not considered "confirmatory" tests [10]. A culture-independent approach based on DNA amplification (qPCR) has several advantages over classical bacteriology for *Campylobacter* detection, notably a faster performance combined with a lower detection limit [11]. Real-time PCR yields highly sensitive and specific results while avoiding manipulation of PCR products after amplification, thereby reducing the risk of cross-contamination; it can be used for rapid quantitative screening of samples [12], [13] and [14].

This study aimed to investigate the occurrence of *Campylobacter* in day-old chicks using real-time PCR to examine indications of vertical transmission in poultry production.

2. Materials and Methods

2.1. Animals and Study Sample

The Ethics and Animal Welfare Committee of the Universidad CEU Cardenal Herrera approved this study. All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette). During the period between January 2012 and August 2013, 12 flocks were monitored from the time of housing day-old chicks and at the end of rearing period. Each flock was located on one farm. The experiment was carried out in the Valencian Region (Eastern Spain).

2.2. Sample Collection

Broiler flocks samples were collected at the beginning and end of the rearing period (days 1 and 42). The first visit occurred just before placing the day-old chicks in the houses and the last just before broilers were transported to the slaughterhouse. Then, ten animals were randomly selected and cecals were obtained and processed according with Vidal et al. [9]. A pooled cecal sample was created by homogenizing 0.02 g of cecal content from each of ten individual ceca into 2 mL of phosphate-buffered saline (PBS), 0·1 mol l^{-1}, pH 7.2. All samples were kept refrigerated during transport to the laboratory. Samples were immediately cultured or frozen with liquid nitrogen and stored at −80 °C until molecular analysis after reception. Environmental samples were collected from the cleaned and disinfected broiler houses prior to chick placement. Samples from water, dust, surfaces, feed, and farming boots were taken. Each sample was taken using different strategies. First, the boots that farmers use to work during rearing period were tested. Farming boots were swabbed with sterile wet gauze pads with disinfectant neutralizer (AES Laboratories®, Bruz Cedex, France). Feed samples were collected from the truck and feeders (about 500 g) and water was sampled from the tank and final dispenser lines (500 mL). Then, dust samples were also collected in different parts (25 to 30 g) of the breeder house and placed into individual sterile plastic pots. Finally, samples of surfaces from each broiler house were collected with sterile wet gauze pads (AES Laboratories®, Bruz Cedex, France).

2.3. Detection of *Campylobacter spp.* by Bacterial Culture Method

The samples collected were tested by direct culture [9] and by enrichment culture based on ISO 10272:2006 recommendations (Annex E). First, cecal and environmental samples were directly streaked onto the two selective agar plates (mCCDA and Preston, AES laboratories®, Bruz Cedex, France) and incubated at 41.5±1°C for 44±4 hours. At the same time, samples were pre-enriched in 1: 10 vol/vol Bolton Broth (OXOID, Dardilly, France) and then pre-incubated at 37±1°C for 5±1hours. Afterwards, 100 µL of each sample was cultured on the two selective agar plates as described above and incubated at 41.5±1°C for 44±4 hours. Pre-enriched samples of positive *Campylobacter* direct culture plates were also stored at -18ºC for further molecular studies. All plates and broths were incubated in a micro-aerobic atmosphere (84% N2, 10% CO_2 and 6% O_2) generated in a gas charged incubator (CampyGen, Oxoid). Plates were examined for grey, flat, irregular and spreading colonies typical of *Campylobacter*. One putative colony was subcultured from each plate onto sheep blood agar for confirmation as *Campylobacter* spp. *Campylobacter* confirmation was performed by a mobility test using a dark field microscope, by oxidase and catalase biochemical test and by streaking at different temperatures and atmospheres on Columbia blood agar (AES Laboratories ®, Bruz Cedex, France). Finally, characterization of the bacterial species was performed with a hippurate hydrolysis test.

2.4. Detection and Quantification of *Campylobacter spp* by qPCR Method

After thawing the old-day chick cecal samples, 0.1 g of cecal contents was diluted in 1 ml of PBS, mixed vigorously by pipetting and centrifuged 10 min at 10.000 g. The supernatant was removed and the pellet re-suspended with 300 µL Buffer Lysis. Thereafter, the total DNA isolation followed the manufacturer's instructions for Genomic DNA from the tissue Kit (Macherey-Nagel). DNA concentration, quality, and integrity were evaluated by using a NanoDrop 2000C Spectrophotometer (Fisher Scientific SL, Spain). The extracted DNA was diluted with nuclease-free water (Ambion, USA) until 100 ng DNA μL^{-1} and used as a template. The results were expressed as copies per mg of DNA.

The primers used to quantify *C. jejuni* (accession number: NC_002163) and *C. coli* (accession number: X88849.1) were developed by Bui et al. [15] and [16], respectively. Oligonucleotide sequences were: 16S rRNA (forward 5′-GCGTAGGCGGATTATCAAGT-3′ and Rev 5′-

CGGATTTTACCCCTACACCA-3′) for *C. jejuni*, and *ceuE* (forward 5′-AAATTTCCGCTTTTGGACCT-3′ and Rev 5′-CCTTGTGCGCGTTCTTTATT-3′) *C. coli*.

The specificity was confirmed by melting curve analysis, gel electrophoresis, and sequencing of the qPCR products. To quantify and detect *Campylobacter spp*, qPCR assays were carried out and expression analyses performed using a model 7500 unit (Applied Biosystems) with Maxima SYBR Green/ROX qPCR Master Mix (Fermentas GMBH). The PCR protocol included an initial step of 50°C for 2 min, followed by 95°C for 10 min, and 40 cycles of 95°C for 1 sec, annealing at 56°C for 20 sec and extended at 72º for 15s. To evaluate assay specificity, the machine performed a melting curve analysis directly following PCR by slowly (0.1 ºC/s) increasing the temperature from 68 to 95 ºC, with continuous recording of changes in fluorescent emission intensity. The DNA extracts of 10-fold dilutions from 10^8 to 10^1 number of plasmid copies/µl were used for qPCR assays to establish the standard curve and to quantify *Campylobacter spp.* in cecal samples. The total volume for every PCR reaction was 20 µL, performed from diluted (1:10) DNA template (5 µL), forward and reverse primers (250 nM each), and SYBR Green/ROX Master Mix (12 µL). The samples were run in duplicate PCR reactions, and a standard curve was included in each run. Non-template control (DNA was replaced by water) for each primer pair were run on all plates.

2.5. Molecular Cloning of *C. jejuni* and *C. coli* PCR Products

PCR amplification was performed in an ABI GeneAmpTM system 2700 thermo cycler. The reaction mixture of 25 µL contained 1x PCR buffer (Invitrogen), 200 µM dNTPs (Invitrogen), 0.1 IU of Taq DNA polymerase (Invitrogen), 500 nM of each primer and 1 µL of DNA template. The first PCR amplification was run as follows: denaturation at 94 ºC for 3 min, followed by 40 cycles at 94 ºC for 30 s, 56 ºC for 20 s, and 72 ºC for 30 s, and finally an extension step at 72 ºC for 10 min. The PCR products were visualized in 2 % agarose gel stained with ethidium bromide and bands of expected size were purified using a Qiaquick Gel Extraction kit (Qiagen) and ligated into the pGEM-T easy vector (Promega, WI, USA). Cloning was performed in competent E. coli JM109 cells (Promega). Positive colonies were isolated and plasmids extracted by a Qiagen Plasmid Mini Kit (Qiagen). Plasmids with inserts were sequenced using an ABI 3730 DNA analyzer (Applied Biosystems, Universitat Politècnica de València sequencing service, Valencia, Spain).

3. Results

No presence of Campylobacter spp. was verified in the day-old chick by bacterial culture method, while 4 flocks out of 12 were found to be positive for Campylobacter by molecular method and also tested positive at the end of the rearing period. Real-time PCR identification revealed that C. coli was detected in the 4 flocks, while C. jejuni was identified in 3 flocks. Quantitative data on C. coli were in a range of 1.3 x 10^2 to 4.9 x 10^3 CFU/ml, whereas data on C. jejuni were in a range of 7.7 x 10^4 to 3.4 × 10^5 CFU/ml. No presence of Campylobacter spp. was verified in the environmental samples. All 12 broiler flocks were found to be contaminated with Campylobacter by the end of the rearing period.

4. Discussion

The control of *Campylobacter* in primary broiler production is a key element of the public health strategies to reduce the number of human campylobacteriosis cases [17]. For food legislation purposes, ISO standard 10272-2 is the official method for detection and enumeration of *Campylobacter spp.* The development of molecular methods constitutes an especially important breakthrough in reducing the time required and specific for the identification of *Campylobacter spp.* combined with a lower detection limit [10], [11]. Nevertheless, the two regulatory agencies in charge of food safety in the USA, the Food and Drug Administration of the Department of Health and Human Services, and the Food Safety and Inspection Services of the U.S. Department of Agriculture, do not consider these molecular tests "confirmatory" and so the actual culture has to be obtained from presumptive positive samples for confirmation purposes [10]. The vast majority of the studies reported that the *Campylobacter* colonization started from day 14 and reached its peak on day 42 [6], [18], [19], [20].

In this study, qPCR successfully demonstrated the presence of DNA from *Campylobacter* in cecal content of day-old chicks, suggesting that vertical transmission could be involved in the colonization. Epidemiological investigations of commercial flocks indicate that newly hatched chicks appear to be free of *Campylobacter* [21]. This negativity persists until at least 10 days of age (the so-called lag phase), and most flocks become infected only 2 to 3 weeks after placement of chicks into a broiler house [22], [23], [24], [25], and once infected, prevalence in a flock can often be close to 100% [26]. Few studies currently support the possibility of vertical transmission [27]. So far, horizontal transmission is the only route clearly linked to the spread of *Campylobacter* [28], [29]. However, recently Agunos et al. [8] suggest that the inability to culture *Campylobacter* from birds less than 2 weeks old presents a major barrier when researching *Campylobacter* in broilers. Our results clearly suggest that the inability to culture *Campylobacter* from day-old chicks can be considered a constraint to knowing the ecology of *Campylobacter* and therefore the exact routes of transmission. Interestingly, in this work *Campylobacter* was detected from these broiler flocks at the end of the rearing cycle.

The major limitation of the DNA-based qPCR method is the potential detection of both live and dead, or non-culturable cells [30], [31]. Moreover, according to ISO 20838, these can be regarded as true positive due to the target-specific DNA probe-based PCR response [15]. In our study, four flocks were found to be *Campylobacter* positive by Real-time PCR but not by culture. Although the 4 flocks started out *Campylobacter*

negative, all flocks were *Campylobacter* positive at the end of the rearing. According to these data, the results highlight our lack of knowledge of the ecology for transmission of *Campylobacter* in poultry, in agreement with Cox et al. [6]. Several hypotheses have been put forward to explain researchers' difficulty to isolate *Campylobacter* during the first two weeks of placement. First, protective maternal antibody effects delay *Campylobacter* colonization [7], [32]. Second, *Campylobacter* may be in a non-culturable form as there were several studies that successfully detected *Campylobacter* DNA, but failed to culture [7], [33], [34]. Thus, there is a need to explore the use of a more reliable molecular technique for detecting viable or "potentially infectious units" of *Campylobacter* [35] from hatchery and chick samples [8]. Third, different isolation techniques have highly variable sensitivity that may affect results if *Campylobacter* concentration is below the detection limits [36]. Because of the inherently low number of cells in eggs/eggshells, embryos, yolk sac, and neonatal intestines, enhanced recovery techniques (e.g., combining membrane filtration and enrichment) [37] need to be explored to improve our detection limits in these samples. Fourth, the type of sample may be important, for example, *Campylobacter* may not be present in the cecal or faecal samples during early rearing because it is still colonizing the small intestine [38], [39].

5. Conclusion

In this study, *Campylobacter* were only identified using molecular methods from day-old chicks. These results could reflect the evidence for vertical transmission of *Campylobacter spp.* While studies do not definitively rule out the detection problems and an accepted standard method will be developed for the detection and isolation of *Campylobacter spp.* at farm level, no standard measure may be successfully implemented in broiler production and therefore, from a public health point of view, strategies to reduce the number of human campylobacteriosis cases will not be efficient.

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