Comparison of different sampling types across the rearing period in broiler flocks for isolation of Campylobacter spp.

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ABSTRACT

Campylobacter is the most common bacterial cause of human gastrointestinal disease in most developed countries. It is generally accepted that poultry products are a significant source of foodborne Campylobacter infections in humans. Assessing the effectiveness of any potential intervention at farm level requires monitoring of the Campylobacter status of broiler flocks, using appropriate sampling methods. The aim of this study was to assess the influence of the sample type across the rearing period for the detection of Campylobacter spp. at farm level. During this study, 21 commercial broiler farms were intensively sampled. Each farm was visited and sampled at different times during the rearing period (d 1, 7, 14, 21, 28, 35 and 42). On the first day of rearing, the status of the house and the day-old flock was evaluated, collecting environmental samples and caecal samples, respectively. During rearing, four different sample types were collected, including faeces with sock swabs (sock swabs), faeces directly from the litter (faeces), cloacal swabs and caecal content. All samples were analysed according to ISO 10272-1:2006 (Annex E) and also by direct culture. The results of this study showed that Campylobacter spp. was detected in all of the sample types on day 14 of rearing. From this point on, the detection increased significantly during rearing, with a maximum detection rate by the end of rearing, regardless of the sample type. All samples that were negative for direct culture were also negative after pre-enrichment. At the end of rearing, the percentage of Campylobacter spp. positive samples was 71.4% for caecal samples, 61.9% for cloacal swabs, 45.2% for sock swabs and 69.1% for faecal samples. C. jejuni was detected in all the sample types, with positive rates ranging from 67.1% to 76.0% for caecal samples and cloacal content, respectively. Caecal, cloacal swabs and faecal samples cultured by direct plating onto mCCDA without pre-enrichment have the same sensitivity for detection of Campylobacter spp. in broiler flocks independently of
the day of rearing.

**Key words:** *Campylobacter*, sample type, broiler.
INTRODUCTION

*Campylobacter* is the most common bacterial cause of human gastrointestinal disease in most developed countries (Olson et al., 2008; EFSA, 2014). It is estimated that there are approximately nine million cases of human campylobacteriosis per year in the EU (EFSA, 2014). *C. jejuni* accounts for the majority of the human cases, followed by *C. coli*, which has also been reported as a significant cause of human disease (Gillespie et al., 2002; Tam et al., 2003; Sopwith et al., 2010).

It is generally accepted that poultry products are a significant source of foodborne *Campylobacter* infections in humans. In the European context, broiler meat may account for 20-30% of the human campylobacteriosis, while 50-80% may be attributed to the chicken reservoir as a whole (EFSA, 2014). The control of *Campylobacter* in primary broiler production is therefore a key element in public health strategies to reduce the number of human campylobacteriosis cases (EFSA, 2011). However, although several control options are available, there is no gold standard measure which could be successfully implemented across Europe, and control strategies are still being evaluated (Vidal et al., 2013). Assessing the effectiveness of any potential intervention at farm level calls for monitoring of the *Campylobacter* status of broiler flocks using appropriate sampling methods (Bronzwaer et al., 2009). However, current intervention strategies are based on risk factors identified in field surveys (Van de Giessen et al., 1998; Evans and Sayers, 2000; Bouwknecht et al., 2004). An important disadvantage of these field surveys is that they used associative static models to determine an association between risk factors and the presence of *Campylobacter* in a flock and were based on qualitative data on the infection status of the flocks at the end of the production period (Van Gerwe et al., 2005). However, these studies did not take the dynamic aspects of a *Campylobacter* infection in a flock into account (Van Gerwe et al.,
Quantitative knowledge of the transmission of Campylobacter is important for the development of control programmes (Cawthraw et al., 1996) and may help to determine the moment of introduction of Campylobacter in commercial broiler flocks under field conditions (Shanker et al., 1990; Harrington et al., 2003; Heres et al., 2004).

Moreover, there is not yet an accepted standard method for the detection and isolation of Campylobacter spp. at farm level (Vidal et al., 2013). A harmonised protocol for the detection of Campylobacter at the farm level will require careful consideration of the optimal sample type, sample collection method, transport conditions and laboratory protocols (Vidal et al., 2013). Several sampling methods are in use to detect Campylobacter in broiler houses, including cloacal swabs (Hansson et al., 2004), faecal samples (Sandberg et al., 2006), caecal contents (Allen et al., 2007; Rosenquist et al., 2007) and sock swabs or the equivalent boot sock model (Bull et al., 2006; Ellis-Iversen et al., 2011; Ridley et al., 2011; Vidal et al., 2013). However, to our best knowledge, the interaction between sampling methods and the shedding detection of Campylobacter spp. during rearing has not been estimated.

This study assessed the influence of the sample type across the rearing period in detection of Campylobacter spp. at farm level.

MATERIAL AND METHODS

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).

Study sample

From March to August 2013, 21 commercial broiler farms were intensively sampled.
Only one flock was studied on each farm. These farms belong to 2 companies, which handle the majority of the poultry slaughtered in Spain. To participate in the study, farms had to be commercial broiler farms with chickens reared on the floor. All the farm owners were willing to cooperate during the lifespan of the flock.

**Sample collection and processing**

Each farm was visited and sampled at different times during the rearing period. The first visit occurred just before placing day-old chicks (d 1) and then each farm was visited at weekly intervals until the slaughter day (d 7, 14, 21, 28, 35 and 42).

Before the arrival of day-old chicks, to assess the status of the house for *Campylobacter* contamination, surface samples, water samples (one from the tank and another from final dispenser lines), feed and farmers’ boot samples were taken. House surfaces and farmer boot samples were taken with sterile wet gauze pads with disinfectant neutraliser (AES laboratories, Bruz Cedex, France). Water samples (500mL) were homogenised at the laboratory and 25 mL was analysed from each source. When the feed arrived at the farm, one sample was collected directly from feeders (500 g). Then, the feed sample was homogenised in the laboratory and 25 g was analysed. Broiler houses were declared contaminated and discarded from the study only if one or more samples tested positive for *Campylobacter*. When the chickens arrived, 10 chicks per batch were slaughtered and caecal contents removed to assess the *Campylobacter* status of the batch.

During the rearing period (days 7, 14, 21, 28, 35 and 42), four different sample types were collected, including faeces with sock swabs, faeces directly from the litter, cloacal swabs and caecal content. To collect faeces with sock swabs, first, the floor area of the houses was divided into two equal sectors and one pair of sock swabs was used in each sector for sampling. Samples were taken by walking over the chosen sector and each pair of sock swabs with faecal material fixed was analysed as an individual sample.
Samples of faeces were taken aseptically from the bedding with sterile gloves (two sterile pots with 500 g of faeces, approximately, Sandberg et al., 2006). Cloacal samples were taken using sterile swabs from 10 individuals in each house (Cary Blair sterile transport swabs, DELTALAB, Rubí, Spain). Finally, these chickens were slaughtered and each pair of caeca and obtained and placed into an individual sterile plastic pot.

All samples collected, with the exception of caeca, were placed in a pot with semi-solid Cary-Blair transport medium (CM0519; OXOID, Dardilly, France), then refrigerated at 5°C and analysed within 24h of collection.

Isolation of Campylobacter and biochemical confirmation

Bacteriological culture was performed according to the ISO 10272-1:2006 (Annex E) for the detection of Campylobacter spp (ISO, 2006). Moreover, all samples were tested by direct culture. Only if direct culture was negative, pre-enriched samples were cultured. Water samples were processed mixing 25 mL with 225 mL of PBS and homogenised. Feed samples were processed mixing 25 g with 225 mL of PBS and homogenised for 60 s using a filter stomacher bag (Separator 400; Seward, West Sussex, United Kingdom) and a stomacher (Stomacher 400; Seward, West Sussex, United Kingdom). Surfaces and boot samples were processed mixing the sterile wet gauze pad with 50 mL of PBS and homogenised. Sock swabs were mixed with 100 mL of PBS and homogenised. The faeces samples were processed mixing 25 g from each pot with 225 mL of PBS and homogenised. The caecal samples were processed and cultured as described by Rodgers et al. (2010). Briefly, the whole content of ten individual pairs of caeca was harvested into a Petri dish and mixed thoroughly. A pooled caecal sample was created by homogenising 0.02 g of caecal content from one caecum from each of the ten birds collected from the house into 2 mL of PBS. From all
sample types, 10 µL aliquots of each suspension were plated onto Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, CM0739 and SR0155, OXOID, Dardilly, France) and Preston Agar (CM0689, SR0117 and SR0048, OXOID, Dardilly, France). Then the samples were incubated at 41.5 ± 1°C, in a microaerobic atmosphere (84% N₂, 10% CO₂, 6% O₂) for 48 h, except for cloacal swabs, which were directly plated onto mCCDA and Preston and incubated as previously described. Moreover, samples were pre-enriched in 1:10 vol/vol Bolton Broth (CM0983, OXOID, Dardilly, France) and then pre-incubated at 37±1°C for 5±1 hours. Finally, the pre-enriched broth was incubated at 41.5 ± 1°C for 43±1 hours. Afterwards, 100 µL of the sample was cultured on the two selective agar plates (mCCDA and Preston agar) and incubated as described above. Campylobacter-like colonies were purified on blood agar and identified to species level on the basis of standard procedures comprising tests for hippurate and indoxyl acetate hydrolysis, catalase production, and susceptibility to cephalotin and nalidixic acid.

**Statistical analyses**

A generalised linear model, which assumed a binomial distribution for Campylobacter colonising, was fitted to the data to determine whether there was an association with sample type (sock swabs, faeces, cloacal swabs and caecal content) and dynamic aspects (7, 14, 21, 28, 35 and 42 d of rearing period). For this analysis, the error was designated as having a binomial distribution and the probit link function was used. Binomial data for each sample was assigned a 1 if it had Campylobacter prevalence or a 0 if it had not. A P value of less than 0.05 was considered to indicate a statistically significant difference. Data are presented as least squares means ± standard error of the least squares means. All statistical analyses were carried out using a commercially available software program (SPSS 16.0 software package; SPSS Inc., Chicago, Illinois,
USA, 2002).

**RESULTS**

On the first day of rearing, none of the day-old chick flocks or environmental, feed and water samples were positive for *Campylobacter*. Therefore, all houses were included in the study. The number of positive flocks by each sample type during rearing is given in Table 1. In total, 20 flocks were positive for *Campylobacter* in at least one of the samples tested. The number of positive flocks detected varied between sample types and the day of rearing (Table 1). The culture of cloacal swabs allowed the detection of all the positive flocks. Caecal and faecal samples allowed the detection of 17 and 16 of the positive flocks, respectively. Nevertheless, sock swab samples failed to detect nine positive flocks. In *Campylobacter* detection, the bacteria were first detected in one of these flocks after 7 days, but *Campylobacter* spp. was detected in all of the sample types on day 14 of rearing. From this moment on, the detection increased significantly during rearing, with a maximum detection rate at the end of rearing, regardless of the sample type.

At sample level, the number of positive samples and the species recovered are summarised in Table 2. All samples that were negative for direct culture were also negative after pre-enrichment. At the end of rearing (d 42), the percentage of *Campylobacter* spp. positive samples was 71.4% for caecal samples, 61.9% for cloacal swabs, 45.2% for sock swabs and 69.1% for faecal samples. *C. jejuni* was detected in all the sample types, with positive rates ranging from 67.1% to 76.0% for caecal samples and cloacal content, respectively. *Campylobacter* detection was significantly different between sample types collected and the day of rearing (d 7, 14, 21, 28, 35, and 42). However, the interaction was not significant, so it was removed from the analysis.
As shown in the Figure 1, *Campylobacter* could not be detected in all of the sample types until day 14. The positive results for *Campylobacter* among the analysed samples were similar until day 21, yielding 19.0% for caecal content, 15.2% for cloacal swabs, 16.7% for sock swabs and 19.0% for faeces. Moreover, isolation rates depend significantly on the rearing period time. There was also a significant effect of the sample types on *Campylobacter* isolation. After day 28, a significant decrease of *Campylobacter* isolation on sock swabs was detected (14.3%) compared with the detection in the other sample types (28.6%, 30.9% and 33.3% for caecal, cloacal swabs and faeces, respectively). These results were consistent with those for the rest of the rearing period (Figure 1). *Campylobacter jejuni* was the most commonly isolated species (73.6%) found in all sample types.

**DISCUSSION**

The present study was conducted to compare the effect of sample types across the rearing period for detection of *Campylobacter* in broiler flocks. Control of *Campylobacter* in primary broiler production is a key element of public health strategies to reduce the number of human campylobacteriosis cases (EFSA, 2011). Assessing the effectiveness of any potential intervention at farm level requires monitoring of the *Campylobacter* status of broiler flocks using appropriately structured sampling methods (Bronzwaer et al., 2009). To this end, the development of a harmonised protocol for the detection of *Campylobacter* at the farm level will require careful consideration of the optimum sample type, sample collection method, transport conditions and laboratory protocols (Vidal et al., 2013). Horizontal transmission after chicks are placed on a farm appears to be the normal route of infection for intensively reared flocks (Newell et al., 2011). In this study, although all
environmental and one-day old chick samples were negative, *Campylobacter* was first detected in one of these flocks after 7 days in caecal, cloacal and faecal samples. However, *Campylobacter* was detected in all of the samples types on day 14. These results concur with those of related studies, where *Campylobacter* is rarely recovered from intensively reared broiler chicks until 14 to 21 days of age (Evans and Sayers, 2000; Shreeve et al., 2000; Stern et al., 2001; Hiett et al., 2002; Bull et al., 2006). It is known that broilers are free of *Campylobacter* at day of hatch, although intensively reared broiler flocks become *Campylobacter*-positive at 2 to 3 weeks of age (Ridley et al., 2011). From this moment on, infection spreads rapidly to most of the broilers in a flock and at 36-42 days of age, over 60% of the flocks might be colonised by thermophilic *Campylobacter* (Evans and Sayers, 2000). These findings coincide with our results (59.3% at the end of the rearing). There is currently no agreement on the reasons for the delay in colonisation, but it is unlikely to be due the lack of exposure to *Campylobacter* (Bull et al., 2006). Broilers are probably not free of *Campylobacter* at day of hatch, but the classical culture methods are out of the detection limit. Rodgers et al. (2010) showed that direct culture of caecal contents on mCCDA on day of hatch could detect *Campylobacter* in samples containing as low as $10^1$ CFU g$^{-1}$ of caecal content with $10^2$ CFU g$^{-1}$ being the lowest level detected in most batches. Nevertheless, further studies should be performed to investigate this hypothesis. Caecal sampling is the standard method for sampling at abattoir level (EC, 2007), while several sampling methods are in use to detect *Campylobacter* in broiler houses, including cloacal swabs (Hansson et al., 2004; OIE, 2008), faecal samples (Sandberg et al., 2006) and sock swabs (Vidal et al., 2013). In our study, all sample types tested resulted in the same detection rate until 21 days of rearing. However, the sock swab samples taken between 28 to 42 days of rearing failed to detect positive samples, whilst
the use of caecal, faecal and cloacal samples isolated significantly more samples. Vidal et al. (2013) reported that sock swabs, moistened in Cary-Blair medium, are a sensitive sampling method for detection of *Campylobacter* spp. in broiler flocks. Our methodology, although the samples were moistened in Cary-Blair medium, was based on the direct culture of all sampling types onto mCCDA medium without an enrichment step. Using an enrichment step prior to plating usually provides better recovery when target cells are either low in number, injured, or stressed (Richardson et al. 2009; Williams et al. 2009). Specifically, Vidal et al. (2013) reported that enrichment increased the sensitivity of the sock swabs. Moreover, when analysing large numbers of samples, the workload should be minimised and avoidance of duplication of selective agar, or omission of an enrichment step, might be an attractive choice, even accepting a possible consequential lesser sensitivity (Ugarte-Ruiz et al., 2012). Our results showed that pre-enrichment does not increase the sensitivity for *Campylobacter* detection because all samples that were negative by direct culture were also negative by pre-enrichment. Therefore, in the present study, the fast, simple and cheap method of direct plating was shown to yield similar isolation efficiency for detection of *Campylobacter* in caecal, faecal and cloacal samples. However, some authors have suggested that using both methods in parallel (direct and enrichment) could enhance the sensitivity (Hald et al. 2000; Maher et al. 2003; Habib et al. 2008; Rodgers et al., 2010). In our study, all samples that were negative for direct culture were also negative after pre-enrichment. In summary, caecal, cloacal swab and faecal samples cultured by direct plating onto mCCDA without pre-enrichment have the same sensitivity for detection of *Campylobacter* spp. in broiler flocks independently of the day of rearing. Nevertheless, further research into improvement of culture procedures seems necessary to detect *Campylobacter* spp. from broilers, especially at the onset of rearing.
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Table 1. Results from 21 broiler flocks tested for *Campylobacter* recovered by different sample types across rearing.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Total positive</th>
<th>Number of positive samples during rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Flocks&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
<td>95.2</td>
</tr>
<tr>
<td>Caecal content</td>
<td>18</td>
<td>85.7</td>
</tr>
<tr>
<td>Cloacal swab</td>
<td>20</td>
<td>95.2</td>
</tr>
<tr>
<td>Sock swab</td>
<td>14</td>
<td>66.7</td>
</tr>
<tr>
<td>Faeces</td>
<td>19</td>
<td>90.5</td>
</tr>
</tbody>
</table>

n: number of positive flocks.

<sup>a</sup> A flock was positive if at least one of the samples was positive by any of the culture methods.