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

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26 **ABSTRACT**

27 *Campylobacter* is the most common bacterial cause of human gastrointestinal disease in
28 most developed countries. It is generally accepted that poultry products are a significant
29 source of foodborne *Campylobacter* infections in humans. Assessing the effectiveness
30 of any potential intervention at farm level requires monitoring of the *Campylobacter*
31 status of broiler flocks, using appropriate sampling methods. The aim of this study was
32 to assess the influence of the sample type across the rearing period for the detection of
33 *Campylobacter* spp. at farm level. During this study, 21 commercial broiler farms were
34 intensively sampled. Each farm was visited and sampled at different times during the
35 rearing period (d 1, 7, 14, 21, 28, 35 and 42). On the first day of rearing, the status of
36 the house and the day-old flock was evaluated, collecting environmental samples and
37 caecal samples, respectively. During rearing, four different sample types were collected,
38 including faeces with sock swabs (sock swabs), faeces directly from the litter (faeces),
39 cloacal swabs and caecal content. All samples were analysed according to ISO 10272-
40 1:2006 (Annex E) and also by direct culture. The results of this study showed that
41 *Campylobacter* spp. was detected in all of the sample types on day 14 of rearing. From
42 this point on, the detection increased significantly during rearing, with a maximum
43 detection rate by the end of rearing, regardless of the sample type. All samples that were
44 negative for direct culture were also negative after pre-enrichment. At the end of
45 rearing, the percentage of *Campylobacter* spp. positive samples was 71.4% for caecal
46 samples, 61.9% for cloacal swabs, 45.2% for sock swabs and 69.1% for faecal samples.
47 *C. jejuni* was detected in all the sample types, with positive rates ranging from 67.1% to
48 76.0% for caecal samples and cloacal content, respectively. Caecal, cloacal swabs and
49 faecal samples cultured by direct plating onto mCCDA without pre-enrichment have the
50 same sensitivity for detection of *Campylobacter* spp. in broiler flocks independently of

51 the day of rearing.

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

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72 **INTRODUCTION**

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

79 It is generally accepted that poultry products are a significant source of foodborne
80 *Campylobacter* infections in humans. In the European context, broiler meat may
81 account for 20-30% of the human campylobacteriosis, while 50-80% may be attributed
82 to the chicken reservoir as a whole (EFSA, 2014). The control of *Campylobacter* in
83 primary broiler production is therefore a key element in public health strategies to
84 reduce the number of human campylobacteriosis cases (EFSA, 2011). However,
85 although several control options are available, there is no gold standard measure which
86 could be successfully implemented across Europe, and control strategies are still being
87 evaluated (Vidal et al., 2013). Assessing the effectiveness of any potential intervention
88 at farm level calls for monitoring of the *Campylobacter* status of broiler flocks using
89 appropriate sampling methods (Bronzwaer et al., 2009). However, current intervention
90 strategies are based on risk factors identified in field surveys (Van de Giessen et al.,
91 1998; Evans and Sayers, 2000; Bouwknegt et al., 2004). An important disadvantage of
92 these field surveys is that they used associative static models to determine an
93 association between risk factors and the presence of *Campylobacter* in a flock and were
94 based on qualitative data on the infection status of the flocks at the end of the
95 production period (Van Gerwe et al., 2005). However, these studies did not take the
96 dynamic aspects of a *Campylobacter* infection in a flock into account (Van Gerwe et al.,

2005). 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.

122 Only one flock was studied on each farm. These farms belong to 2 companies, which
123 handle the majority of the poultry slaughtered in Spain. To participate in the study,
124 farms had to be commercial broiler farms with chickens reared on the floor. All the
125 farm owners were willing to cooperate during the lifespan of the flock.

126 ***Sample collection and processing***

127 Each farm was visited and sampled at different times during the rearing period. The first
128 visit occurred just before placing day-old chicks (d 1) and then each farm was visited at
129 weekly intervals until the slaughter day (d 7, 14, 21, 28, 35 and 42).
130 Before the arrival of day-old chicks, to assess the status of the house for *Campylobacter*
131 contamination, surface samples, water samples (one from the tank and another from
132 final dispenser lines), feed and farmers' boot samples were taken. House surfaces and
133 farmer boot samples were taken with sterile wet gauze pads with disinfectant neutraliser
134 (AES laboratories, Bruz Cedex, France). Water samples (500mL) were homogenised at
135 the laboratory and 25 mL was analysed from each source. When the feed arrived at the
136 farm, one sample was collected directly from feeders (500 g). Then, the feed sample
137 was homogenised in the laboratory and 25 g was analysed. Broiler houses were declared
138 contaminated and discarded from the study only if one or more samples tested positive
139 for *Campylobacter*. When the chickens arrived, 10 chicks per batch were slaughtered
140 and caecal contents removed to assess the *Campylobacter* status of the batch.
141 During the rearing period (days 7, 14, 21, 28, 35 and 42), four different sample types
142 were collected, including faeces with sock swabs, faeces directly from the litter, cloacal
143 swabs and caecal content. To collect faeces with sock swabs, first, the floor area of the
144 houses was divided into two equal sectors and one pair of sock swabs was used in each
145 sector for sampling. Samples were taken by walking over the chosen sector and each
146 pair of sock swabs with faecal material fixed was analysed as an individual sample

147 (Vidal et al., 2013). Samples of faeces were taken aseptically from the bedding with
148 sterile gloves (two sterile pots with 500 g of faeces, approximately, Sandberg et al.,
149 2006). Cloacal samples were taken using sterile swabs from 10 individuals in each
150 house (Cary Blair sterile transport swabs, DELTALAB, Rubí, Spain). Finally, these
151 chickens were slaughtered and each pair of caeca and obtained and placed into an
152 individual sterile plastic pot.
153 All samples collected, with the exception of caeca, were placed in a pot with semi-solid
154 Cary-Blair transport medium (CM0519; OXOID, Dardilly, France), then refrigerated at
155 5ºC and analysed within 24h of collection.
156 ***Isolation of Campylobacter and biochemical confirmation***
157 Bacteriological culture was performed according to the ISO 10272-1:2006 (Annex E)
158 for the detection of *Campylobacter* spp (ISO, 2006). Moreover, all samples were tested
159 by direct culture. Only if direct culture was negative, pre-enriched samples were
160 cultured. Water samples were processed mixing 25 mL with 225 mL of PBS and
161 homogenised. Feed samples were processed mixing 25 g with 225 mL of PBS and
162 homogenised for 60 s using a filter stomacher bag (Separator 400; Seward, West
163 Sussex, United Kingdom) and a stomacher (Stomacher 400; Seward, West Sussex,
164 United Kingdom). Surfaces and boot samples were processed mixing the sterile wet
165 gauze pad with 50 mL of PBS and homogenised. Sock swabs were mixed with 100 mL
166 of PBS and homogenised. The faeces samples were processed mixing 25 g from each
167 pot with 225 mL of PBS and homogenised. The caecal samples were processed and
168 cultured as described by Rodgers et al. (2010). Briefly, the whole content of ten
169 individual pairs of caeca was harvested into a Petri dish and mixed thoroughly. A
170 pooled caecal sample was created by homogenising 0.02 g of caecal content from one
171 caecum from each of the ten birds collected from the house into 2 mL of PBS. From all

172 sample types, 10 μL aliquots of each suspension were plated onto Modified Charcoal
173 Cefoperazone Deoxycholate Agar (mCCDA, CM0739 and SR0155, OXOID, Dardilly,
174 France) and Preston Agar (CM0689, SR0117 and SR0048, OXOID, Dardilly, France).
175 Then the samples were incubated at 41.5 ± 1°C, in a microaerobic atmosphere (84% N_2,
176 10% CO_2, 6% O_2) for 48 h, except for cloacal swabs, which were directly plated onto
177 mCCDA and Preston and incubated as previously described. Moreover, samples were
178 pre-enriched in 1:10 vol/vol Bolton Broth (CM0983, OXOID, Dardilly, France) and
179 then pre-incubated at 37±1ºC for 5±1hours. Finally, the pre-enriched broth was
180 incubated at 41.5 ± 1°C for 43±1hours. Afterwards, 100 μL of the sample was cultured
181 on the two selective agar plates (mCCDA and Preston agar) and incubated as described
182 above. *Campylobacter*-like colonies were purified on blood agar and identified to
183 species level on the basis of standard procedures comprising tests for hippurate and
184 indoxyl acetate hydrolysis, catalase production, and susceptibility to cephalotin and
185 nalidixic acid.

186 ***Statistical analyses***

187 A generalised linear model, which assumed a binomial distribution for *Campylobacter*
188 colonising, was fitted to the data to determine whether there was an association with
189 sample type (sock swabs, faeces, cloacal swabs and caecal content) and dynamic
190 aspects (7, 14, 21, 28, 35 and 42 d of rearing period). For this analysis, the error was
191 designated as having a binomial distribution and the probit link function was used.
192 Binomial data for each sample was assigned a 1 if it had *Campylobacter* prevalence or a
193 0 if it had not. A *P value* of less than 0.05 was considered to indicate a statistically
194 significant difference. Data are presented as least squares means ± standard error of the
195 least squares means. All statistical analyses were carried out using a commercially
196 available software program (SPSS 16.0 software package; SPSS Inc., Chicago, Illinois,

197 USA, 2002).

198

199 RESULTS

200 On the first day of rearing, none of the day-old chick flocks or environmental, feed and
201 water samples were positive for *Campylobacter*. Therefore, all houses were included in
202 the study. The number of positive flocks by each sample type during rearing is given in
203 Table 1. In total, 20 flocks were positive for *Campylobacter* in at least one of the
204 samples tested. The number of positive flocks detected varied between sample types and
205 the day of rearing (Table 1). The culture of cloacal swabs allowed the detection of all
206 the positive flocks. Caecal and faecal samples allowed the detection of 17 and 16 of the
207 positive flocks, respectively. Nevertheless, sock swab samples failed to detect nine
208 positive flocks. In *Campylobacter* detection, the bacteria were first detected in one of
209 these flocks after 7 days, but *Campylobacter* spp. was detected in all of the sample types
210 on day 14 of rearing. From this moment on, the detection increased significantly during
211 rearing, with a maximum detection rate at the end of rearing, regardless of the sample
212 type.
213 At sample level, the number of positive samples and the species recovered are
214 summarised in Table 2. All samples that were negative for direct culture were also
215 negative after pre-enrichment. At the end of rearing (d 42), the percentage of
216 *Campylobacter* spp. positive samples was 71.4% for caecal samples, 61.9% for cloacal
217 swabs, 45.2% for sock swabs and 69.1% for faecal samples. *C. jejuni* was detected in all
218 the sample types, with positive rates ranging from 67.1% to 76.0% for caecal samples
219 and cloacal content, respectively. *Campylobacter* detection was significantly different
220 between sample types collected and the day of rearing (d 7, 14, 21, 28, 35, and 42).
221 However, the interaction was not significant, so it was removed from the analysis.

222 As shown in the Figure 1, *Campylobacter* could not be detected in all of the sample
223 types until day 14. The positive results for *Campylobacter* among the analysed samples
224 were similar until day 21, yielding 19.0% for caecal content, 15.2% for cloacal swabs,
225 16.7% for sock swabs and 19.0% for faeces. Moreover, isolation rates depend
226 significantly on the rearing period time. There was also a significant effect of the
227 sample types on *Campylobacter* isolation. After day 28, a significant decrease of
228 *Campylobacter* isolation on sock swabs was detected (14.3%) compared with the
229 detection in the other sample types (28.6%, 30.9% and 33.3% for caecal, cloacal swabs
230 and faeces, respectively). These results were consistent with those for the rest of the
231 rearing period (Figure 1). *Campylobacter jejuni* was the most commonly isolated
232 species (73.6%) found in all sample types.
233

234 **DISCUSSION**

235 The present study was conducted to compare the effect of sample types across the
236 rearing period for detection of *Campylobacter* in broiler flocks. Control of
237 *Campylobacter* in primary broiler production is a key element of public health strategies
238 to reduce the number of human campylobacteriosis cases (EFSA, 2011). Assessing the
239 effectiveness of any potential intervention at farm level requires monitoring of the
240 *Campylobacter* status of broiler flocks using appropriately structured sampling methods
241 (Bronzwaer et al., 2009). To this end, the development of a harmonised protocol for the
242 detection of *Campylobacter* at the farm level will require careful consideration of the
243 optimum sample type, sample collection method, transport conditions and laboratory
244 protocols (Vidal et al., 2013).
245 Horizontal transmission after chicks are placed on a farm appears to be the normal route
246 of infection for intensively reared flocks (Newell et al., 2011). In this study, although all

247 environmental and one-day old chick samples were negative, *Campylobacter* was first
248 detected in one of these flocks after 7 days in caecal, cloacal and faecal samples.
249 However, *Campylobacter* was detected in all of the samples types on day 14. These
250 results concur with those of related studies, where *Campylobacter* is rarely recovered
251 from intensively reared broiler chicks until 14 to 21 days of age (Evans and Sayers,
252 2000; Shreeve et al., 2000; Stern et al., 2001; Hiett et al., 2002; Bull et al., 2006). It is
253 known that broilers are free of *Campylobacter* at day of hatch, although intensively
254 reared broiler flocks become *Campylobacter*-positive at 2 to 3 weeks of age (Ridley et
255 al., 2011). From this moment on, infection spreads rapidly to most of the broilers in a
256 flock and at 36-42 days of age, over 60% of the flocks might be colonised by
257 thermophilic *Campylobacter* (Evans and Sayers, 2000). These findings coincide with
258 our results (59.3% at the end of the rearing). There is currently no agreement on the
259 reasons for the delay in colonisation, but it is unlikely to be due the lack of exposure to
260 *Campylobacter* (Bull et al., 2006). Broilers are probably not free of *Campylobacter* at
261 day of hatch, but the classical culture methods are out of the detection limit. Rodgers et
262 al. (2010) showed that direct culture of caecal contents on mCCDA on day of hatch
263 could detect *Campylobacter* in samples containing as low as 10^1 CFU g^{-1} of caecal
264 content with 10^2 CFU g^{-1} being the lowest level detected in most batches. Nevertheless,
265 further studies should be performed to investigate this hypothesis.
266 Caecal sampling is the standard method for sampling at abattoir level (EC, 2007), while
267 several sampling methods are in use to detect *Campylobacter* in broiler houses,
268 including cloacal swabs (Hansson et al., 2004; OIE, 2008), faecal samples (Sandberg et
269 al., 2006) and sock swabs (Vidal et al., 2013). In our study, all sample types tested
270 resulted in the same detection rate until 21 days of rearing. However, the sock swab
271 samples taken between 28 to 42 days of rearing failed to detect positive samples, whilst

272 the use of caecal, faecal and cloacal samples isolated significantly more samples. Vidal
273 et al. (2013) reported that sock swabs, moistened in Cary-Blair medium, are a sensitive
274 sampling method for detection of *Campylobacter* spp. in broiler flocks. Our
275 methodology, although the samples were moistened in Cary-Blair medium, was based
276 on the direct culture of all sampling types onto mCCDA medium without an enrichment
277 step. Using an enrichment step prior to plating usually provides better recovery when
278 target cells are either low in number, injured, or stressed (Richardson et al. 2009;
279 Williams et al. 2009). Specifically, Vidal et al. (2013) reported that enrichment
280 increased the sensitivity of the sock swabs. Moreover, when analysing large numbers of
281 samples, the workload should be minimised and avoidance of duplication of selective
282 agar, or omission of an enrichment step, might be an attractive choice, even accepting a
283 possible consequential lesser sensitivity (Ugarte-Ruiz et al., 2012). Our results showed
284 that pre-enrichment does not increase the sensitivity for *Campylobacter* detection
285 because all samples that were negative by direct culture were also negative by pre-
286 enrichment. Therefore, in the present study, the fast, simple and cheap method of direct
287 plating was shown to yield similar isolation efficiency for detection of *Campylobacter*
288 in caecal, faecal and cloacal samples. However, some authors have suggested that using
289 both methods in parallel (direct and enrichment) could enhance the sensitivity (Hald et
290 al. 2000; Maher et al. 2003; Habib et al. 2008; Rodgers et al., 2010). In our study, all
291 samples that were negative for direct culture were also negative after pre-enrichment. In
292 summary, caecal, cloacal swab and faecal samples cultured by direct plating onto
293 mCCDA without pre-enrichment have the same sensitivity for detection of
294 *Campylobacter* spp. in broiler flocks independently of the day of rearing. Nevertheless,
295 further research into improvement of culture procedures seems necessary to detect
296 *Campylobacter* spp. from broilers, especially at the onset of rearing.

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303

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Table 1. Results from 21 broiler flocks tested for *Campylobacter* recovered by different sample types across rearing.

Sample type	Total positive		Number of positive samples during rearing											
			7 d		14 d		21 d		28 d		35 d		42 d	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Flocks[a]	20	95.2	1	4.8	3	14.3	7	33.3	11	52.4	16	76.2	20	95.2
Caecal content	18	85.7	1	5.5	3	16.7	5	27.8	8	44.4	12	66.7	17	94.4
Cloacal swab	20	95.2	0	0.0	2	10.0	5	25.0	10	50.0	16	80.0	20	100.0
Sock swab	14	66.7	1	7.1	1	7.1	5	35.7	3	21.4	7	50.0	11	78.6
Faeces	19	90.5	1	5.3	1	5.3	6	31.6	7	36.8	9	47.4	16	84.2

n: number of positive flocks.

[a] A flock was positive if at least one of the samples was positive by any of the culture methods.