A Multicenter Proposal for a Fast Tool To Screen Biosecure Chicken Flocks for the Foodborne Pathogen *Campylobacter*

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1 Foodborne *Campylobacter*: A multi-center proposal for a fast screening tool in biosecured
2 chicken flocks

3
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19 **Keywords:**

20 zoonoses; air sampling; PCR, campylobacteriosis; risk assessment.

21

22 Abstract

23 The present multi-centre study aimed at assessing the performance of air sampling as a novel
24 method for monitoring *Campylobacter* in biosecured poultry farms. We compared, using a
25 harmonized procedure, the bacteriological isolation protocol (ISO 10272-1:2017), and a real-
26 time PCR method on air filter samples. Air samples and boot swabs were collected from 62
27 biosecured flocks from five European countries during the summer of 2019. For air filters, the
28 frequency of PCR-positive findings was significantly higher (n=36; 58%) compared to the
29 cultivation methods ($p<0.01$; Standardized Residuals). The cultivation protocols (one with
30 Bolton enrichment and one with Preston enrichment) were comparable to each other, but
31 returned fewer positive samples (0-8%). The association between type of sample and
32 frequency of PCR-positive findings was statistically confirmed ($p<0.01$; Fisher's exact test),
33 although no culture-positive air filters were detected using direct plating. For the boot swabs,
34 the highest number of positive samples were detected after enrichment in Preston broth
35 (n=23; 37%), followed by direct plating after homogenization in Preston (n=21; 34%) or
36 Bolton (n=20; 32%). It is noteworthy that the Norwegian flocks, a country known to have low
37 *Campylobacter* prevalence in biosecured chicken flocks, tested negative for *Campylobacter*
38 using the new sensitive approach. In conclusion, air sampling combined with real-time PCR is
39 proposed as a multi-purpose, low-cost and convenient screening method that can be up to four
40 times faster and four times more sensitive than the current boot-swab testing scheme used for
41 screening biosecured chicken production.

42

43 Importance

44 *Campylobacter* bacteria are the cause of the vast majority of registered cases of foodborne
45 illness in the industrialized world. In fact, the bacteria caused 246,571 registered cases of
46 foodborne illness in 2018, which equates to 70% of all registered cases in Europe that year.

47 An important tool to prevent campylobacter from making people sick is good data on where
48 in the food chain the bacterium is present. The present study reports a new test method that
49 quadruples the likelihood of identifying campylobacter-positive chicken flocks. It is important
50 to identify campylobacter-positive flocks before they arrive at the slaughterhouse. This is
51 because negative flocks may be slaughtered first in the morning in order to avoid cross-
52 contamination along the production line.

53 1. Introduction

54 *Campylobacter* spp. infection is one of the most widespread foodborne infectious diseases of
55 the last century (1, 2). The incidence and prevalence of *Campylobacteriosis* have increased in
56 both developed and developing countries over the last 10 years and is the leading cause of
57 foodborne illness in the United States (3) and the EU (4). The dramatic increase in the
58 industrialized world is alarming, and data from parts of Africa, Asia, and the Middle East
59 indicate that *Campylobacteriosis* is endemic in these areas, especially in children (2).

60 *Campylobacter* species, specifically *C. jejuni* and *C. coli*, are most often isolated from the
61 intestinal tract of poultry as well as in poultry products (5), which are the most frequently
62 reported cause of foodborne illness. *Campylobacter* contamination of raw poultry products
63 occurs during slaughter operations, as well as during the live-animal rearing process and can
64 coat the exterior of the bird and remain attached to the skin (6).

65 The US has published new performance standards for *Campylobacter* in not ready-to-eat
66 comminuted chicken and turkey products, in addition to raw chicken parts (7). In the EU, the
67 control of *Campylobacter* in food production chain is regulated in the industrialized food
68 production systems (1, 8). An amendment to EC Regulation No 2073/2005
69 brings *Campylobacter* sampling processes, limits and corrective actions, in line with
70 *Salmonella* and *Listeria*, which have been regulated since 2005 (9). The main change

71 for poultry producers is a requirement to comply with a limit of 1000 CFU/g in at least
72 60 percent of chickens tested. It is important to note that they set an indicative
73 contamination value above which corrective actions are required in order to maintain
74 the hygiene of the process in compliance with the legislation.

75 Proper sampling can facilitate the monitoring of *Campylobacter* on farm and before dispatch
76 for slaughtering. However, current sampling techniques are mostly a century-old and need
77 modernization in order to adapt to automated and molecular detection techniques, reduce the
78 cost of handling and transport, and provide faster laboratory results close to, or in real-time
79 for multi-pathogen testing (10). At present, on-farm sampling of poultry is done by taking
80 faecal droppings or boot swabs (11). Additionally, upon collecting or hanging chickens for
81 slaughter, cloacae swabbing or caecum sampling is performed.

82 The occurrence of *Campylobacter* in the primary production can be studied by sampling
83 poultry and the surrounding environment (12, 13, 14, 15). A composite sample approach is
84 often applied, pooling multiple swabs (5-10). Ventilation shafts, dust on surfaces, floors,
85 transport crates, etc. can be sampled with moist gauze swabs (16). Drinking water can be
86 taken directly from the tap in the poultry house or outdoor water supplies and collected in
87 sterile containers.

88 A novel method to screen for *Campylobacter* is sampling of air on gelatine filters (11, 17),
89 which has been previously demonstrated to detect the presence of *Campylobacter* in ambient
90 air from chicken flocks in production houses, in some cases even earlier than the current
91 conventional and costly methods (14, 17, 18). We report here a harmonized multi-center study
92 to assess the diagnostic sensitivity of air sampling protocol in biosecured farms in five
93 different climatic and geographical regions of Europe with different epidemiology; Norway
94 has a low reported prevalence of *Campylobacter* in chickens, while Italy has a high
95 prevalence of *Campylobacter*-contaminated chicken flocks.

96

97 **2. Materials and methods**98 **2.1 Study design**

99 The sampling was carried out in the Czech Republic, Denmark, Italy, Norway, and Poland to
100 include European regions with different geographical origin, climate, as well as different level
101 of *Campylobacter* prevalence in chicken flocks. Each laboratory collected samples from at
102 least 10 chicken flocks from at least two separate biosecured chicken farms as described by
103 Johannessen et al. (18), with some modifications as presented below. Three samples were
104 taken from each flock, a pair of boot swabs and two air filters giving a total number of 186
105 samples.

106 **2.2 Sample collection**

107 The samples were collected as described by Johannessen et al. (18). Briefly, before the
108 sampling, boot swabs were moistened in a sterile diluent or pre-moistened boot swabs were
109 used. The person performing the sampling wore the swabs over boots and walked around the
110 chicken house covering as much area as possible (19).

111 The air samples were taken using the AirPort MD8 device (Sartorius Stedim Biotech, France)
112 with disposable gelatine membrane filters (80 mm in diameter; Sartorius 17528-80ACD) as
113 described by Johannessen et al (2020). Briefly, the samples were taken using an airflow rate
114 of 50 L/min for 15 min giving a total air volume of 750 L. The samples were transported at
115 4°C conditions to the laboratory and processed within 48 hrs for cultivation, or within five
116 days for DNA extraction and the following real-time PCR. The details about sampling periods
117 and flocks in each country are shown in Supplementary data, S1.

118

119 2.3. Analyses

120 2.3.1 Cultivation method

121 ISO 10272-1:2017 was used for detection of *Campylobacter* (20). Direct plating from
122 homogenates prior to enrichment were included as described below. The steps of cultivation
123 of boot swabs and air filters are presented in Fig. 1a and Fig 1b.

124

125 2.3.1.1 Boot socks/swabs

126 A total of 62 boot swab samples were collected. Briefly, the boot swab sample were split in
127 two parts (Figure 1a). Each boot swab was weighted separately and Bolton broth or Preston
128 broth was added to obtain a ratio 1:10 (w/v). Prior to incubation of the Bolton and Preston
129 broths, a 10 µl aliquot of Bolton broth was plated directly on modified Charcoal
130 Cefoperazone Deoxycholate (mCCDA) plate and a second agar plate of own choice, while 10
131 µl of Preston broth was plated on mCCDA, only. The plates were incubated in micro-aerobic
132 conditions at 41.5 °C for 44 ± 4 hrs. The Bolton and Preston broths were incubated at 41.5 °C
133 for 44 ± 4 hrs and 41.5 °C for 24 ± 2 hrs, respectively, followed by subsequent plating as
134 described for direct plating above. After incubation, the plates were checked for the presence
135 of typical *Campylobacter* colonies, and a total of three presumptive *Campylobacter* colonies
136 from each enrichment procedure were confirmed using the routine confirmation test used in
137 the respective laboratories. Additionally, aliquots of the enrichment broths from every step
138 were frozen at ≤70°C after adding glycerol to a final concentration of 15%.

139

140 2.3.1.2 Air filters

141 A total of 124 air filters were collected (two filters per chicken house). One of two air filters
142 was used for cultivation as shown in Fig. 1b. The filter was split in two halves, to which 10 ml
143 of Bolton broth was added to one of the halves, and 10 ml of Preston broth was added to the
144 other half. The plating and incubation steps were carried out as described for boot sock
145 samples.

146

147 2.3.2. Real-time PCR

148 The second air filter was used for DNA extraction and real-time PCR for the detection of
149 *Campylobacter*. The air filter was split in two halves and DNA was extracted separately from
150 each half using the same protocol. Briefly, the filter was pre-treated by dissolving a half in 3.5
151 ml molecular grade water tempered to room temperature with 100 µl of alkaline protease
152 (Protex GL, Genencor International, Leiden, The Netherlands). The suspension was vortexed
153 and split into two tubes with equal volumes. Then, the tubes were incubated in a thermal
154 shaker for 6 min at 37°C at 1000 RPM and subsequently centrifuged at 8000 x g for 5 min at
155 4°C. The supernatant was discarded and the pellet was used for DNA extraction with DNeasy
156 Blood & Tissue Kit (Qiagen GmbH, Germany) according to manufacturer's instructions with
157 modifications as described below. After incubation at 56°C for one hour with shaking the
158 samples were cooled down to 40°C and 4 µl of RNase A (100 mg/ml; Qiagen) was added,
159 vortexed and incubated at room temperature for 5 min. DNA was eluted from the spin column
160 with 100 µl TE buffer with 0.1 mM EDTA pre-warmed to 70°C and incubated at room
161 temperature for 1 min before centrifugation. DNA was stored at -20°C for short time storage,
162 and at - 80°C for longer storage.

163 Real-time PCR was done as described by Josefsen et al. (21) using master mix and reagents
164 prepared centrally by the Danish partner and distributed to all participants in advance. PCR

were performed in 10 mM Tris-HCl pH 8.9, 0.1 M KCl, 4 mM MgCl₂, 7% glycerol, 0.6 mM dNTP, 250 µg/ml bovine serum albumin and 1 U of Tth DNA polymerase, with 500 nM of forward primer (5'-CTGCTTAACACAAGTTGAGTAGG-3'), 500 nM of reverse primer (5'-TTCCTTAGGTACCGTCAGAA-3') and 75 nM of LNA probe (5'-FAM-CA[+T]CC[+T]CCACGCGGCG[+T]TGC-BHQ1-3'). This assay will detect *Campylobacter jejuni*, *C. coli* and *C. lari*. An Internal Amplification Control (IAC) was included in the same PCR reaction, using the same primers and 50 nM of 5'-Joe-+TTCATGAGGACACCTGAGTTGA-tamra3' probe in a duplex reaction. Template for the IAC were 5 x 10³ copies of IAC (124 bp) with the sequence,
CTGCTIAACACAAGTTGAGTAGGCAACTCAGGTGTCCTCATGAATTIGAAGA
CATAAACAAGGGACTGGTCTCCGTCCCAACCAAGATCATCCATCTCCCGCTATTC
TGACGGTACCTAAG GAA. The cycle profile was as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 58°C for 60 s. A cutoff value of Ct < 36 was used to score positive samples as recommended by Josefsen et al. (21).

179

2.3.3 Statistical analysis

Statistical analysis was performed using Fisher's exact test, McNemar test and Chi-square test (Statistica 13.2, Dell, USA). A *p*-value of less than 0.05 was considered as statistically significant. Relative sensitivity, specificity and diagnostic accuracy were calculated according to Malorny et al. (22). The formulas used for the analysis were:

Relative sensitivity (%) = 100 × (positive agreement between culture and PCR)/(positive agreement between culture and PCR)+(false-negatives by PCR);

Relative specificity (%) = 100 × (negative agreement between culture and PCR)/(negative agreement between culture and PCR)+(false-positives by PCR);

189 Diagnostic accuracy (%) = $100 \times (\text{positive and negative agreement between culture and}$
190 PCR)/total number of samples tested.

191

192 3. Results

193 A total of 62 chicken houses/flocks in five countries were visited and sampled from March to
194 August 2019. Italy and Norway sampled 10 houses/flocks each, the Czech Republic and
195 Poland 12 houses/flocks, while Denmark sampled 18 houses/flocks.

196 All samples examined in Norway were negative. On the contrary, in Italy all boot swabs were
197 *Campylobacter* positive (Table 1), the same as the corresponding air filters examined using
198 real-time PCR (Table 2).

199

200 3.1 Cultivation method

201 3.1.1 Boot swabs

202 Using the direct plating approach, 33.8% (21) and 32.2% (20) of boot swabs homogenized in
203 Preston and Bolton broth, respectively, were *Campylobacter* positive (Table 1). Enrichment in
204 Preston, however, yielded 37.1% (23) positive boot swabs compared to enrichment in Bolton,
205 where only 16.1% (10) of positive samples was detected (Table 1).

206 The comparison between the two types of cultivation approaches (direct plating and
207 enrichment) showed that direct plating and enrichment in Preston were not statistically
208 different while the Bolton enrichment showed a frequency of positive finding (16.1%)
209 significantly lower compared to direct plating ($p < 0.05$; McNemar test) but also versus
210 enrichment in Preston broth ($p < 0.001$; Chi-square test).

211

212 3.1.2 Air filters

213 By cultivation, *Campylobacter* was detected only in four (6.5%) and six (9.7%) air filters after
214 enrichment in Preston and Bolton broth, respectively (Table 2). Direct plating did not yield
215 positive results from any of the air filters. Real-time PCR gave positive results for DNA
216 specific for *Campylobacter* in 58% (n=36) of all the tested air filters (n=62). (Table 2 and the
217 supplementary data, S1). The frequency of positive findings in air filters using PCR was
218 statistically significantly higher compared to cultivation methods ($p<0.01$; standardized
219 residuals). Comparison between microbiological and molecular methods used showed that
220 molecular methods had a statistically significant higher frequency of detection of
221 *Campylobacter* ($p<0.05$; Fisher's exact test).

222

223 3.2 *Campylobacter* spp. findings by flocks

224 Out of 62 tested flocks, 25 were negative by all methods. In 27 flocks out of 36 positive by
225 PCR analysis of air filters, presence of *Campylobacter* was confirmed in corresponding boot
226 swabs using cultivation method (direct plating or enrichment approach). Only one flock was
227 positive exclusively from boot swab samples, using enrichment in Preston (Poland - 2/4; see
228 supplementary data S1). The sensitivity of the detection approach, which employs air filter
229 combined with real-time PCR detection, compared to boot swabs examined by cultivation
230 method, was determined to be 96.4 %, its specificity 73.5 % and diagnostic accuracy 83.8%
231 (Table 3).

232 4. Discussion

233 *Campylobacter* spp. is one of the most important pathogens in relation to the chicken meat
234 production and it is the aetiological agent of the most frequent zoonosis in the industrialized
235 world for more than a decade. Chicken flocks represent the main reservoir, and current

236 strategies to control *Campylobacters* on farm level, mainly based on the farm biosecurity,
237 seem to be insufficient. Healthy but caecal carrier chickens are shedding *Campylobacters* in
238 the farm environment and they are considered to be the most important source of
239 contamination of carcasses during their processing at the slaughterhouse level (23). Proper
240 management on farms including welfare practices together with efficient sampling method
241 can improve the food safety of poultry meat chain at slaughterhouse and retail level.
242 The usual critique of more sensitive methods is that they are too sensitive and detect
243 contaminated flocks where they should not, which is why it was important to compare the
244 new sampling approach with the conventional swab methods in geographical regions with
245 different prevalence of *Campylobacter*. The air sampling protocol in combination with real-
246 time PCR, found no contaminated flocks in Norway, while this figure was 100% in Italy.
247 Such differences might be due to climate conditions, environmental pressure and various
248 infection rates in chickens identified in the countries participating in the present study (5, 24,
249 25).

250 Comparison of the result from real-time PCR directly on the filters with the method utilizing
251 cultivation of air filters in two different broths revealed that the PCR-based approach had an
252 advantage over the culture method, which identified only 9.7% of positive samples
253 (enrichment in Bolton broth). However, detection of *Campylobacter* in filters using direct
254 plating identified no positive samples. The direct PCR results obtained, were then related to a
255 commonly used method of faeces collection by boot swabs, utilized for e.g. *Salmonella*
256 monitoring in poultry flocks (26). The filter and boot swab samples were collected in parallel
257 in the five participating countries using the standardized protocols. Among 62 samples of boot
258 swabs, 37.1% flocks were positive in this method (enrichment in Preston broth). The
259 cultivation from boot swabs indicated that direct plating and Preston enrichment worked
260 better than compared to direct plating and enrichment in Bolton, highlighting that these

particular samples a high background flora together with the *Campylobacters* present. Comparing the cultivation results from boot swabs and air filters indicated that more positive *Campylobacter* boot swabs were identified as compared to cultivation of air filters. However, *Campylobacter* was identified in more of the tested chicken flocks when air filters and direct real-time PCR were applied as compared to cultivation of boot swabs. Taking into account all above mentioned findings it can be suggested that the most efficient approach to detect *Campylobacter* in chicken flocks was the use of filters together with real-time PCR. The low number of positive flocks obtained from the cultivation of air samples in the study was probably the result of adverse environmental conditions present on filters. These observations are in agreement with our earlier results on the comparison of filter and culture methods for detection of *Campylobacter* in chickens (18).

The high detection rate in the direct air filter real-time PCR approach obtained in the present study was probably due to identification of all *Campylobacter*, including live, dead, and viable but not culturable cells (VBNC) as well as extracellular DNA of these bacteria. However, since the molecular method is intended for use as a screening tool, differentiation between dead and live cells is not so crucial.

With regard to the sampling place, poultry houses with all-in/ all-out management and regular disinfection, it can be assumed that the PCR positive findings reflect the reality of flock's positivity more closely than cultivation method. Furthermore, previous studies have also indicated that the air filter sampling method combined with real-time PCR allowed for a much earlier detection of flock contamination (up to two weeks) compared to the currently used boot swabs (11, 17). However, this should be further investigated with studies in geographical regions with different prevalence of *Campylobacter* in chicken flocks following farms over time. As real-time PCR is a more sensitive method than traditional cultivation of *Campylobacter*, the boot swabs could also be analysed using the same real-time PCR as for

286 the air filters for a more direct comparison of methods in future studies. Such early results
287 may be important for a subsequent investigation of the flock contamination level and
288 epidemiological analyses. In a follow-up, shotgun metagenomics will also be applied on the
289 same air filter samples investigated in this study for investigating the microbiome and the
290 resistome of the air samples from the chicken houses as well as for comparison of detection of
291 *Campylobacter* by a different technology.

292 The results obtained, based on the harmonized air sampling protocol established during the
293 present study and tested in different parts of Europe indicate that the method can be useful in
294 various countries and chicken production conditions. This approach, utilizing the same air
295 sampling condition with the regards of air volume and airflow, makes the results comparable
296 between poultry farms and chicken breeding systems. Application of a molecular approach on
297 air filters allows reduced influence of inhibitors present in the matrix (boot swabs) since air is
298 assumed to contain less inhibitors than faecal material. Additionally, during extraction of
299 DNA, any inhibitors are more easily removed. Furthermore, an internal amplification control
300 is included in the real-time PCR to provide reliable results. Using the present real-time PCR
301 method, the sensitivity was 96.4%. In only one case, *Campylobacter* was detected solely by
302 cultivation of boot swab. Failure of air sample/PCR approach could be explained by low
303 relative humidity in the house. Schroeder et al. (27) described low positivity of air samples in
304 poultry houses with relative humidity below 44%. The sensitivity observed in the present
305 study is higher than the values from the validation studies shown in ISO 10272-1:2017, i.e.
306 from 28% (chicken caecal material) to 64% (chicken skin) (20). However, the validation
307 studies were carried out on other types of matrices with known level of contamination, and
308 the sensitivities cannot be compared directly as the levels of contamination in the air samples
309 are not known. It must also be taken into account that real-time PCR detects DNA from
310 living, dead and VNBC bacteria, while the ISO method detects only living bacteria. As

311 reported previously, the same air sampling and direct real-time PCR method applied for
312 *Campylobacter* detection gave a detection limit of approximately 100 cell equivalents per 1
313 m³ of air in chicken house (11).
314
315 Due to the high sensitivity of the approach presented this might be optional in countries and
316 farms with flocks with low *Campylobacter*-positive rates as culture-based methods might give
317 more false-negative results, however this needs further investigation. Moreover, analysis of
318 air filters with real-time PCR has an advantage over the reference ISO method giving the
319 result in a much shorter time for *Campylobacter* detection in chicken flocks, i.e. one working
320 day compared to four days, respectively.
321 Efficient measures for pathogen control in poultry environments are of primary importance to
322 reduce direct or indirect food contamination. *Campylobacter*, *Salmonella*, *E.coli*, *Listeria*,
323 *Clostridium*, *Staphylococcus*, and more are associated with increased veterinary costs and
324 animal health, welfare and ultimately public health. The diagnostics is a primary preventive
325 control measure, and molecular methods potentially identify various animal pathogens and
326 prevents the limitations of traditional microbiology. The potential use of air sampling
327 combined with PCR could be a valuable tool in such scenario for rapid and cost-effective
328 diagnostics of target microbes. Finally, the method herein described could be also applied in
329 the field by using portable PCR systems making point of care diagnostics possible, even if
330 further validation studies are necessary.
331
332 In conclusion, air sampling combined with real-time PCR is proposed as a multi-purpose,
333 low-cost and convenient screening method that can be up to four times faster and four times
334 more sensitive than the current boot-swab testing scheme used for screening biosecured

335 chicken production. However, to obtain both isolates and PCR results, enriched swab samples
336 should be preferred.

337 Supplemental Material

338 Supplemental Table S1

339

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349

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431 *Campylobacter* spp. levels between chicken grow-out environmental samples and processed
432 carcasses. Poultry science 93:734-741.

433 Table 1. Results from the use of cultivation (ISO 10272-1:2017) to detect *Campylobacter*
434 from boot swabs.

Country	No. of flocks	No. of positive boot swabs			
		Direct plating		Enrichment	
		Preston	Bolton	Preston	Bolton
Czech Rep.	12	5	4	2	0
Denmark	18	0	0	6	0
Italy	10	10	10	10	10
Norway	10	0	0	0	0
Poland	12	6	6	5	0

435

436

437 Table 2. Results from the use of cultivation and real-time PCR to detect *Campylobacter* from
438 air filters.

Country	No. of flocks	No. of positive findings from air filters					
		Direct plating		Enrichment		Real-time PCR	
		Preston	Bolton	Preston	Bolton		
Czech Rep.	12	0	0	1	2	5	
Denmark	18	0	0	0	0	15	
Italy	10	0	0	0	0	0	
Norway	10	0	0	0	0	0	
Poland	12	0	0	1	1	6	

439

440 Table 3. *Campylobacter* detection in flocks according to used approaches

No. of flocks				Diagnostic method		
Positive		Negative		Air filter combined with real-time PCR detection		
By both approaches	Detection by boot swabs and cultivation only	Detection by air filter and real- time PCR only	by both approaches	Sensitivity	Specifity	Diagnostic accuracy
27	1	9	25	96.4%	73.5 %	83.8%

441

442 Fig. 1. Flow diagram showing detection of *Campylobacter* spp. from boot swabs samples (A)
443 and air filters (B) using ISO 10272-1:2017.

444

B

Proceed with direct plating and enrichment as described for boot swabs in the part A.