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Campylobacter in chicken – critical parameters for international, multicentre evaluation of air sampling and detection methods

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Abstract

The present pilot study aimed at evaluating air sampling as a novel method for monitoring *Campylobacter* in poultry farms. We compared the bacteriological isolation of *Campylobacter* from boot swabs and air filter samples using ISO 10272-1:2017. A secondary aim was to evaluate the use of molecular methods, i.e. real time PCR, on the same sample set. Samples from 44 flocks from five European countries were collected, and included air samples, in parallel with boot swabs.

Campylobacter spp. was isolated from seven of 44 boot swabs from three of five partners using the enrichment method. Two of these positive boot swab samples had corresponding positive air samples. Using enrichment, one positive air sample was negative in the corresponding boot swabs, but *Campylobacter* spp. was isolated from direct plating of the boot swab sample. One partner isolated *Campylobacter* spp. from six of 10 boot swabs using direct plating. Overall, 33 air filter samples were screened directly with PCR, returning 14 positive results. In conclusion, there was a lack of correspondence between results from analysis of boot swabs and air filters using ISO 10272-1:2017. In contrast, the combination of air filters and direct real-time PCR might be a way forward. Despite the use of the detailed ISO protocols, there were still sections that could be interpreted differently among laboratories. Air sampling may turn into a multi-purpose and low-cost sampling method that may be integrated into self-monitoring programs.

1. Introduction

Since 2005, campylobacteriosis is the most frequently reported gastrointestinal infection of bacterial origin in humans (EFSA and ECDC, 2018). Thermophilic *Campylobacter* species, such as *C. jejuni* and *C. coli*, are most commonly associated with human infections and are also often present in poultry. *Campylobacter* has been isolated from the environment and a range of wild and domesticated animals but poultry, especially broilers and laying hens, has been considered as the main reservoir (EFSA, 2012, Bailey *et al.*, 2018). In the EU, monitoring of *Campylobacter* is mandatory according to Directive 2003/99/EC (Anonymous, 2003), however, the stages in the food chain where the monitoring is applied, may vary among the member states. Since 2018, Commission Regulation (EC) 2017/1495 amending EC No 2073/2005 on *Campylobacter* in broiler carcasses has been implemented in the member states (Anonymous, 2017). It is a process hygiene criterion for *Campylobacter* in broiler carcasses, which aims at keeping under control the contamination of carcases during the slaughtering. According to the Commission Regulation 2017/1495 (Anonymous, 2017), a process hygiene criterion "set indicative contamination values above which are required in order to maintain the hygiene and the process in compliance with food law". In addition, to ensure a whole chain approach as recommended by the EFSA opinion on control options for *Campylobacter*, control measures should also be considered at the farm level (BIOHAZ, 2011). Multi-pathogen testing is not yet easily applicable to current sampling techniques, because improvements are necessary for adopting molecular methods, reduce the cost of handling and transport, and provide faster results. At present, on-farm sampling of poultry is done by taking fecal droppings or boot swabs, which are also widely used for *Salmonella* monitoring (Mueller-Doblies *et al.*, 2009). Swabs of feeding pans or drinkers (Schroeder *et al.*, 2014) are used. At the slaughterhouse, samples consist of cloacae swabbing, faeces, and caeca or their contents (Hansson *et al.*, 2004, Sandberg *et al.*, 2006, Rosenquist *et al.*, 2009). The epidemiology of *Campylobacter* in primary production can be studied by sampling poultry and the surrounding environment. A composite sample approach is often applied, pooling multiple swabs. Ventilation shafts, dust on surfaces, floors, transport crates, etc. can be sampled with moist gauze swabs.

Recent research indicated air sampling as an alternative strategy for determining the *Campylobacter* status of broiler flocks (Olsen *et al.*, 2009). Studies have suggested that air becomes contaminated only after the flock in the house has been colonized (Pearson *et al.*, 1993). Nevertheless, both the construction and dynamics of ventilation systems and humidity can influence the risk of flock positivity (Refregier-Petton *et al.*, 2001, Schroeder et al. 2014). Preliminary studies demonstrated the presence of *Campylobacter* in air samples of broiler flocks houses, in some cases significantly earlier than the current conventional methods (Olsen *et al.*, 2009, Sondergaard *et al.*, 2014). No technical

77 skills are required to perform air sampling in poultry flocks, and the sample is assumed to be suitable
78 for molecular and immunological detection methods thus can be shipped for analysis by ordinary
79 mail, rendering the approach suitable in self-monitoring programs (Woodward *et al.*, 2004).

80 *Campylobacter* is generally accepted as a commensal of avian intestine and is isolated in high
81 numbers from caecum (10^6 to 10^8 CFU/g). Ingestion of few colonies (approx. 35 CFU) may be
82 sufficient to colonize chicks and within a few days the majority of the flock is colonized and stay so
83 until slaughter (Stern *et al.*, 1988, van Gerwe *et al.*, 2009, Hermans *et al.*, 2011). *Campylobacter*
84 typically does not invoke any clinical signs in poultry, although some studies reported alteration of
85 gut morphology and impairment of weight gain (Awad *et al.*, 2014, Awad *et al.*, 2015). Several of the
86 *Campylobacter* colonization determinants have been identified in chicken in the last few years, e.g.
87 iron metabolism or oxidative stress defence seems to be crucial for colonization, but still there is no
88 effective strategy to eliminate this pathogen from farms and chickens (Hermans *et al.*, 2011).

89 Here we present the results from a multi-country pilot study where the aim was to evaluate air
90 sampling in broiler houses as an alternative to sampling by boot swabs. The study comprised boot
91 swabs as the golden standard and air sampling as an alternative method analysed by the use of ISO
92 10272-1:2017 (ISO, 2017). Secondly, on a voluntary basis, the samples were analysed also the using
93 molecular methods, i.e. PCR to assess if this might be a way forward.

94

2. Materials and methods

2. 1 Study design

Sampling was carried out in the Czech Republic, Denmark, Italy, Norway, and Poland to include European regions with different geographical origin, climate and with known negative, low, medium or high *Campylobacter* flock prevalence. In each country, up to 10 samples were collected from at least two separate biosecured broiler farms. One flock was represented by one chicken house with at least 1000 chickens. Each flock was sampled with a pair of boot socks (boot swab sample) and a corresponding air filter. The analysis of boot sock samples was treated as a reference and air filter samples as an alternative method.

2.2 Sample collection

One boot swab sample consisted of two socks, worn over the boots of the person performing the sampling. Before the sampling, the boot swabs were moistened with a sterile diluent (phosphate buffered saline, PBS or water). The sampling covered as much area of the chicken house as possible (at least 100 steps) by walking around the chicken house (ISO, 2013a).

For air sampling, the AirPort MD8 device (Sartorius Stedim Biotech, France) was used in the poultry houses with disposable gelatine membrane filters (80 mm diameter; Sartorius, 17528-80ACD). Each air sample was taken at 50 – 80 cm above the floor level, with a total air volume of 750 litres using an airflow rate of 50 litres/min.

After the sampling, the filter cassette and boot swab samples were put into separate plastic bags, tagged and transported in chilled conditions to the laboratory within max 6-8 hrs. In the laboratory, the samples were immediately processed or stored in a refrigerator for no longer than 48 h before culturing, or 4-5 days for direct PCR.

2.3 Analyses

2.3.1 Cultivation method

ISO 10272-1:2017 (ISO, 2017) was used as the standard method. Briefly, Bolton broth was added to boot swab samples to obtain a 1:10 dilution and homogenized followed by incubation in micro-aerobic conditions for 44 ± 4 h at 41.5 °C (ISO, 2017). After incubation, one loopful of the enriched culture was plated onto a modified Charcoal Cefoperazone Deoxycholate agar (mCCDA) and a second agar of choice and incubated as described above. Additionally, aliquots (1-5 ml) of the enriched samples with a final concentration of 15% (vol/vol) of glycerol were stored at -70°C. A total of three

colonies from one sample were picked and pure-cultured onto a non-selective blood agar for *Campylobacter* confirmation. Each partner confirmed presumptive *Campylobacter* spp. according to their own routine methods.

The air sample (gelatine membrane filter) was divided into two equal parts and analysed separately (replicate samples). To each half of the filter, 10 ml of Bolton broth was added and incubated as described for the boot swab samples. After enrichment, plating, confirmation and freezing of the enrichment broth was carried out as described above.

2.3.2 Real-time PCR

As an optional method, the partners could also use real-time PCR for detection of *Campylobacter* both in boot swabs and air samples (gelatine filters), either before and/or after the enrichment step. Additional air filters from the same poultry houses had to be sampled for performing the real time PCR. If DNA was extracted directly from air sample (gelatine filter), the following protocol was used: A quarter of the filter was dissolved in 1.75 ml of double distilled water with 50 µl of alkaline protease (Protex GL, Genencor International, Leiden, The Netherlands) added. The suspension was vortexed, incubated in a thermal shaker for 6 min at 37 °C at 1000 x *g* and then centrifuged at 8 000 x *g* for 5 min at 4 °C. The supernatant was discarded and the pellet was used for DNA extraction, followed by qualitative real time PCR for the detection of *Campylobacter* bacteria according to the participant's own protocols. The cycle threshold (Ct) was recommended as previously established where Ct <36 was considered positive (Josefsen *et al.*, 2010).

2.3.3 Statistical analysis

Statistical analysis was performed using Fisher's exact test (Statistica 13.2, Dell, USA). *p*-value of less than 0.05 was considered as statistically significant.

3. Results

A total of 44 broiler houses/flocks in five countries were visited and sampled in the period from May to September 2018. The Czech Republic, Italy and Norway sampled from 10 houses/flocks each, while Poland and Denmark sampled eight and six houses/flocks, respectively (Supplementary material).

3.1 Cultivation method

All participating partners used the ISO 10272-1:2017 for analysing both boot swabs and air filters. After enrichment of boot swab samples, *Campylobacter* spp. was isolated from seven of 44 samples from three of five partners. An overview of the results is listed in Table 1, while information of the results for single samples are detailed in Table 2. Only two of these positive boot swab samples had corresponding positive air samples. One positive air sample, after enrichment, had negative corresponding boot swab sample (after enrichment), but *Campylobacter* spp. was isolated from direct plating of this boot swab sample. *Campylobacter* spp. was isolated from six of 10 boot swab samples after direct plating (one partner), but the corresponding direct plating from the air samples was negative. The replicated air samples showed concordant results.

Although all partners used ISO 10272-1:2017 with incubation of the samples at 41.5 °C for 44 – 48 hrs, there were variation between the labs in how the method was carried out. This could particularly be observed for the volume of Bolton broth that was added to the boot swabs for the initial dilution. Three of the labs used a fixed volume of broth, but this varied between 90, 100 and 250 ml depending on the lab. The last two labs used 1:10 (1+9) weight/volume dilution. One of the labs added an extra plating step of the samples, with plating directly after the initial dilution, both from the boot swabs and air filters.

All partners plated out after enrichment as described in the ISO 10272-1:2017using mCCDA as the first plating medium and a second medium of choice, which included Brilliance Campy Count agar, Karmali agar and Campy Food Agar. All microbiological media and reagents were in concordance with the ISO standard, even when provided by different producers. Typical colonies were confirmed as *Campylobacter* spp. using either MALDI TOF-MS (one partner) or PCR.

PCR method

Four out of five partners reported PCR results (Table 1 and 2). All partners used the primer-probe combination described by Josefsen *et al.* (2010), with different DNA extraction methods and PCR

platforms (Table 3). Of the 33 air filter samples that were screened directly with PCR, 14 returned positive results. Two partners screened a total of 14 air filters directly and after enrichment, resulting in five positive samples both directly and after enrichment, while two were only positive directly. Interestingly, the two samples that were only positive directly, were negative by PCR on the corresponding enriched boot swab samples, and also negative by the cultivation method. The five samples that were positive on all PCRs (boot swabs and air filters, both directly and after enrichment), were all positive on direct plating from boot swabs, but negative after enrichment of boot swabs and direct plating from air filters. Only one of these samples (Italy 2_2A) was positive after enrichment of the air filter.

Discussion

The findings from the present study raises several issues, with regard to the alternative sampling method, and also on how to carry out such multi-centre evaluation studies. First, air sampling mostly correlated with the conventional boot swab when the air filters were tested by PCR. Boot swabs were more often positive by culture than the air filters ($p<0.05$, Fisher's exact test). Secondly, the air filters were found positive more often when tested directly (no enrichment) with real time PCR in comparison to the cultivation method described in ISO 10272-1:2017 ($p<0.01$, Fisher's exact test), suggesting *Campylobacter* cells do not survive well on the air filters due to the dry environment or the oxygen stress. It is worth to note that air samples might provide a "cleaner" analytical material for testing than boot swabs, particularly considering the well-studied sensitive nature of PCR enzyme activity to the inhibitory material present in sample matrix. Third, although the ISO method for the detection of *Campylobacter* is detailed, there are still sections that can be interpreted differently among laboratories, e.g. the procedure for producing the initial suspension of boot swabs is not clearly defined neither in ISO 10272-1:2017 nor in ISO 6887-6:2013 (ISO, 2013b). In ISO 6887-6:2013 it is stated for boot socks swabs that all parts of the swabs should be submerged and an example is given for *Salmonella* where at least 225 ml of the diluent should be added. In the specific *Campylobacter* standard (ISO, 2017) it is stated that in general, the amount of test portion is mixed with a quantity of enrichment medium to yield a tenfold dilution, while for some sample types (e.g. boot sock swabs) it might be necessary to use another ratio. Despite the variations in initial suspension, the enrichment step with Bolton broth apparently worked well for four partners. For the fifth partner, however, isolation was only achieved by direct plating. These findings suggest that there are variations in different farms or countries with respect to number of *Campylobacter* and background microbiota in the boot swabs. Moreover, the background microbiota might have different composition with e.g. different proportion of ESBL bacteria that may hamper the enrichment step and subsequent isolation step or the direct plating. Nevertheless further studies are necessary for a clearer understanding of these issues. The initial dilution may influence the final result and it have to be clear among the participants in similar studies, which dilution ratio should be used for samples. Observations such as the ones obtained from this study, with respect to interpretation of standards, are important input for revisions of the appropriate ISO standards. Finally, in a study where the sampling method is evaluated, the use of different PCR methods may affect the conclusion of the final result. Thus, in such situations, it should be agreed upon in advance which methods to use in order to minimize the variation of the methods of analyses.

In order to facilitate comparison of diagnostic data for epidemiological surveillance, food screening studies, or herd surveillance, we have systematically pursued international validation studies for new

testing approaches, in particular enrichment PCR for detection of zoonotic foodborne pathogens (Reviewed in Hoorfar, 2011). In addition, rapid monitoring of *Campylobacter* on farm and at slaughterhouse level is important to prevent contaminated meat from entering the food market. Improved monitoring tools are important for the control of *Campylobacter* bacteria in primary poultry production. Standardized reference culture methods issued by national and international standardization organisations are useful but need to be combined with rapid and low-cost methods for screening of large numbers of samples. Currently, real-time PCR is fulfilling above mentioned criteria to a certain extent. Further development of real-time PCR, in combination with low-cost sampling may improve the monitoring capacity. Combined with innovative sampling and sample treatment, these techniques could become realistic options for on-farm or on-site monitoring at slaughterhouses (Josefsen *et al.*, 2015). Olsen et al. (2009) compared the sensitivity of PCR-detection of *Campylobacter* in fecal samples, dust, and air in poultry flocks (Olsen *et al.*, 2009). The sensitivity of detection in air was found comparable to that in the other sample matrices. *Campylobacter* could also be detected in the air at the hanging stage during the slaughter process, but not at other stages in the slaughterhouse. The authors proposed that the exploitation of dust in poultry houses as a sample matrix for detection of *Campylobacter* and other pathogens as a new possibility, especially in conjunction with new detection technologies, for allowing continuous or semi-continuous monitoring of colonization status. Findings in the present study is in agreement with the previous report (Sondergaard *et al.*, 2014) that showed air sampling to be sensitive and user-friendly approach under various poultry farming conditions.

Using PCR or other molecular detection methods puts great demand on the sample preparation step. Regardless of the downstream analysis, the target bacteria itself, or its DNA, has to be concentrated, separated from the inhibitory compounds of the sample matrix, as well as a sample-size-reduction is required for detection. Sampling for *Campylobacter* in the primary poultry production often includes complex matrices, e.g. fecal material, posing even higher demands on sample preparation. The components of a sample directly affect the sensitivity and amplification efficiency of a PCR assay, and for this reason, no universal sample preparation procedure exists. As sub-project to a large EU project (https://onehealthejp.eu/), our ultimate goal is to prepare a draft standard protocol that harmonizes and validates these tools to propose as the next-generation *Campylobacter* screening to the European Food Safety Authority (EFSA).

In conclusion, there was a lack of correspondence between results from analysis of boot swabs and air filters when a traditional culturing method was employed. In contrast, the results obtained by use of real-time PCR suggest that the combination of air filters and direct real-time PCR might be a way forward. However, this approach needs further evaluation. Furthermore, it was observed that despite the use of the detailed ISO protocols, there were still sections that could be interpreted

differently among laboratories. This underlines the importance of mutual agreement and understanding of the protocols employed in international multi-centre studies. Air sampling may turn into a multi-purpose and low-cost sampling and integrated into self-monitoring programs, provided the diagnostic sensitivity, specificity and robustness of the method are documented in the next phase of the project, which is in progress.

Perspectives:

The project may provide the European community with a harmonized tool for interventions and codes of best practices.

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354 Table 1. Overview of total results from culturing method and optional PCR of boot swabs and air
355 filters. Number of positive results are reported.

		Samples positive by cultivation method				Samples positive by real-time PCR			
		Boot swabs		Air filters		Boot swabs		Air filters	
Country	No. of flocks	Direct	Enrichment	Direct	Enrichment	Direct	Enrichment	Direct	Enrichment
Italy	10	6	0	0	1	7	5[1]	8	5[1]
Czech Rep.	10	-[2]	0	-	0	-	0	2[3]	0
Norway	10	-	3	-	0	-	-	-	-
Poland	8	-	3	-	1	-	-	3	-
Denmark	6	-	1	-	1	-	-	1	-

356 [1)] Five samples of ten tested after enrichment.
357 [2)] – Not done.
358 [3)] Nine of ten samples tested directly with PCR.

359 Table 2. Detailed results from cultivation and PCR screening from boot swabs and air filters.

		Cultivation method				Real-time PCR			
		Boot swabs		Air filters		Boot swabs		Air filters	
Country	Broiler house/flock	Direct	Enrichment	Direct	Enrichment	Direct	Enrichment	Direct	Enrichment
Italy	1_3A	Neg	Neg	Neg	Neg	Neg	-*	**Pos**	-
Italy	1_3B	Neg	Neg	Neg	Neg	Neg	-	Neg	-
Italy	1_3C	Neg	Neg	Neg	Neg	Neg	-	Neg	-
Italy	1_4A	Neg	Neg	Neg	Neg	**Pos**	-	**Pos**	-
Italy	1_4B	**Pos**	Neg	Neg	Neg	**Pos**	-	**Pos**	-
Italy	2_1A	**Pos**	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**	**Pos**
Italy	2_1B	**Pos**	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**	**Pos**
Italy	2_1C	**Pos**	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**	**Pos**
Italy	2_2A	**Pos**	Neg	Neg	**Pos**	**Pos**	**Pos**	**Pos**	**Pos**
Italy	2_2B	**Pos**	Neg	Neg	Neg	**Pos**	**Pos**	**Pos**	**Pos**
Czech Rep.	A1	-	Neg	-	Neg	-	Neg	Neg	Neg
Czech Rep.	A4	-	Neg	-	Neg	-	Neg	Neg	Neg
Czech Rep.	A3	-	Neg	-	Neg	-	Neg	Neg	Neg
Czech Rep.	B1	-	Neg	-	Neg	-	Neg	Neg	Neg
Czech Rep.	B2	-	Neg	-	Neg	-	Neg	Neg	Neg
Czech Rep.	B6	-	Neg	-	Neg	-	Neg	**Pos**	Neg
Czech Rep.	C1	-	Neg	-	Neg	-	Neg	**Pos**	Neg
Czech Rep.	C2	-	Neg	-	Neg	-	Neg	Neg	Neg
Czech Rep.	D1	-	Neg	-	Neg	-	Neg	Neg	Neg
Czech Rep.	D2	-	Neg	-	Neg	-	Neg	-	Neg
Norway	1_1	-	Neg	-	Neg	-	-	-	-
Norway	2_1	-	Neg	-	Neg	-	-	-	-
Norway	3_1	-	Neg	-	Neg	-	-	-	-
Norway	4_1	-	Neg	-	Neg	-	-	-	-
Norway	5_1	-	Neg	-	Neg	-	-	-	-
Norway	6_1	-	Neg	-	Neg	-	-	-	-
Norway	7_1	-	**Pos**	-	Neg	-	-	-	-
Norway	7_2	-	Neg	-	Neg	-	-	-	-
Norway	8_1	-	**Pos**	-	Neg	-	-	-	-
Norway	8_2	-	**Pos**	-	Neg	-	-	-	-
Poland	1_1	-	Neg	-	Neg	-	-	**Pos**	-
Poland	2_1	-	**Pos**	-	Neg	-	-	**Pos**	-

Poland	2_2	-	**Pos**	-	**Pos**	-	-	**Pos**	-
Poland	1_2	-	Neg	-	Neg	-	-	Neg	-
Poland	1_3	-	Neg	-	Neg	-	-	Neg	-
Poland	2_3	-	**Pos**	-	Neg	-	-	Neg	-
Poland	1_4	-	Neg	-	Neg	-	-	Neg	-
Poland	1_5	-	Neg	-	Neg	-	-	Neg	-
Denmark	1_1	-	Neg	-	Neg	-	-	Neg	-
Denmark	1_2	-	Neg	-	Neg	-	-	Neg	-
Denmark	1_3	-	Neg	-	Neg	-	-	Neg	-
Denmark	2_1	-	Neg	-	Neg	-	-	Neg	-
Denmark	2_2	-	Neg	-	Neg	-	-	Neg	-
Denmark	3_1	-	**Pos**	-	**Pos**	-	-	**Pos**	-

360 * - Not done

361 Table 3. Details of PCR used by the four partners that carried out PCR using the primers and probe
362 described by Josefsen at al. (2010).

Partner	DNA extraction Kit	PCR platform	PCR reagents	Internal control
Italy	Maxwell® 16 Tissue DNA Purification Kit	CFX96 Touch™ Real-Time PCR Detection System	TaqMan® Fast Universal PCR Master mix (2X)	Applied Biosystems® TaqMan® Exogenous Internal Positive Control Reagents contain a pre-optimized internal positive control (IPC)
Czech Rep.	Qiagen Blood and tissue kit	Lightcycler 480 (Roche)	LightCycler 480 Probes master (Roche)	TaqMan Exogenous Internal Positive Control Reagents (Fisher Scientific)
Poland	Qiagen, Blood and tissue kit	CFX96 real-time, C1000 Thermal Cycler BioRad	LightCycler® TaqMan®Master (Roche)	1.5 pmol IAC probe labelled with Texas Red
Denmark	Qiagen Blood and tissue kit	Mx3005P (Agilent Technologies)	According to Josefsen et al., 2010.	1.5 pmol IAC probe labelled with JOE reporter dye and TAMRA quencher dye

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