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FOOD MICROBIOLOGY

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

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

IMPORTANCE *Campylobacter* bacteria are the cause of the vast majority of registered cases of foodborne illness in the industrialized world. In fact, the bacteria caused 246,571 registered cases of foodborne illness in 2018, which equates to 70% of all registered cases in Europe that year. An important tool to prevent campylobacters from making people sick is good data on where in the food chain the bacterium is present. The present study reports a new test method that quadruples the likelihood of identifying campylobacter-positive chicken flocks. It is important to identify campylobacter-positive flocks before they arrive at the slaughterhouse, because negative flocks can be slaughtered first in order to avoid cross-contamination along the production line.

KEYWORDS campylobacteriosis, PCR, rapid diagnosis, zoonoses, air sampling, pathogen testing, risk assessment

Campylobacter infection is one of the most widespread foodborne infectious diseases of the last century (1, 2). The incidence and prevalence of campylobacteriosis have increased in both developed and developing countries over the last 10 years, and

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campylobacteriosis is the leading cause of foodborne illness in the United States (3) and the European Union (4). The dramatic increase in the industrialized world is alarming, and data from parts of Africa, Asia, and the Middle East indicate that campylobacteriosis is endemic in these areas, especially in children (2).

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

The United States has published new performance standards for *Campylobacter* in not ready-to-eat comminuted chicken and turkey products, in addition to raw chicken parts (7). In the European Union, the control of *Campylobacter* in food production chain is regulated in the industrialized food production systems (1, 8). An amendment to EC regulation 2073/2005 brings *Campylobacter* sampling processes, limits, and corrective actions in line with those for *Salmonella* and *Listeria*, which have been regulated since 2005 (9). The main change for poultry producers is a requirement to comply with a limit of 1,000 CFU/g in at least 60% of chickens tested. It is important to note that this requirement sets an indicative contamination value above which corrective actions are required in order to maintain the hygiene of the process in compliance with the legislation.

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

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

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

RESULTS

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

All samples examined in Norway were negative. In contrast, in Italy all boot swabs were *Campylobacter* positive (Table 1), as were the corresponding air filters examined using real-time PCR (Table 2).

Cultivation method. (i) Boot swabs. Using the direct plating approach, 33.8% ($n = 21$) and 32.2% ($n = 20$) of boot swabs homogenized in Preston and Bolton broth, respectively, were *Campylobacter* positive (Table 1). Enrichment in Preston broth,

TABLE 1 Results of cultivation (ISO 10272-1:2017) to detect *Campylobacter* from boot swabs

Country	No. of flocks	No. of positive boot swabs			
		Direct plating		Enrichment	
		Preston	Bolton	Preston	Bolton
Czech Republic	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

however, yielded 37.1% ($n = 23$) positive boot swabs, whereas enrichment in Bolton yielded only 16.1% ($n = 10$) positive samples (Table 1).

Comparison of the two cultivation approaches (direct plating and enrichment) showed that direct plating and Preston enrichment were not statistically different, while the Bolton enrichment showed a frequency of positive findings (16.1%) significantly lower than that of direct plating ($P < 0.05$; McNemar's test) but also of enrichment in Preston broth ($P < 0.001$; chi-square test).

(ii) Air filters. By cultivation, *Campylobacter* was detected only in four (6.5%) and six (9.7%) air filters after enrichment in Preston and Bolton broth, respectively (Table 2). Direct plating did not yield positive results from any of the air filters. Real-time PCR gave positive results for DNA specific for *Campylobacter* in 58% ($n = 36$) of all the tested air filters ($n = 62$) (Table 2 and Table S1). The frequency of positive findings in air filters using PCR was statistically significantly higher than that obtained with cultivation methods ($P < 0.01$; standardized residuals). Comparison between microbiological and molecular methods showed that molecular methods had a statistically significant higher frequency of detection of *Campylobacter* ($P < 0.05$; Fisher´s exact test).

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

DISCUSSION

Campylobacter is one of the most important pathogens in relation to chicken meat production, and it has been the most frequent etiological agent of zoonosis in the industrialized world for more than a decade. Chicken flocks represent the main reservoir, and current strategies to control *Campylobacter* at the farm level, mainly based on farm biosecurity, seem to be insufficient. Healthy but cecal carrier chickens shed campylobacters in the farm environment, and they are considered the most important source of contamination of carcasses during their processing at the slaughterhouse

TABLE 2 Results of cultivation and real-time PCR to detect *Campylobacter* from air filters

Country	No. of flocks	No. of positive findings from air filters				
		Direct plating		Enrichment		
		Preston	Bolton	Preston	Bolton	Real-time PCR
Czech Republic	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

level (21). Proper management on farms, including welfare practices together with efficient sampling methods, can improve the food safety of the poultry meat chain at the slaughterhouse and retail levels.

The usual critique of more sensitive methods is that they are too sensitive and detect contaminated flocks where they should not, which is why it was important to compare the new sampling approach with conventional swab methods in geographical regions with different prevalences of *Campylobacter*. The air sampling protocol in combination with real-time PCR found no contaminated flocks in Norway, while this figure was 100% in Italy. Such differences might be due to climate conditions, environmental pressure, and different infection rates in chickens identified in the countries participating in the study (5, 22, 23).

Comparison of the results of real-time PCR directly on the filters with the method utilizing cultivation of air filters in two different broths revealed that the PCR-based approach had an advantage over the culture method, which identified only 9.7% positive samples (enrichment in Bolton broth). However, detection of *Campylobacter* in filters using direct plating identified no positive samples. The direct PCR results obtained were then compared to results of a commonly used method of feces collection by boot swabs, utilized for, e.g., *Salmonella* monitoring in poultry flocks (24). The filter and boot swab samples were collected in parallel in the five participating countries using the standardized protocols. Among 62 samples of boot swabs, 37.1% flocks were positive by this method (enrichment in Preston broth). The cultivation from boot swabs indicated that direct plating and Preston enrichment worked better than direct plating and enrichment in Bolton, highlighting the idea that these particular samples had a high level of background flora together with the campylobacters present. Boot swab cultivation yielded more positive *Campylobacter* results than 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 than when cultivation of boot swabs was used. 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 campylobacters, including live, dead, and viable but nonculturable 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 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 much earlier detection of flock contamination (up to 2 weeks) than the currently used boot swabs (11, 17). However, this should be further investigated by conducting studies in geographical regions with different prevalences of *Campylobacter* in chicken flocks, monitoring farms over time. As real-time PCR is a more sensitive method than traditional cultivation of *Campylobacter*, the boot swabs could also be analyzed using the same real-time PCR as for the air filters for a more direct comparison of methods in future studies. Such early results may be important for a subsequent investigation of the flock contamination level and epidemiological analyses. In a follow-up, shotgun metagenomics will also be applied on the air filter samples investigated in this study for investigating the microbiome and the resistome of the air samples from the chicken houses as well as for comparison of detection of *Campylobacter* by a different technology.

The results obtained, based on the harmonized air sampling protocol established

during the present study and tested in different parts of Europe, indicate that the method can be useful in different countries and under different chicken production conditions. This approach, utilizing the same air sampling conditions with regard to air volume and airflow, makes the results comparable between poultry farms and chicken breeding systems. Application of a molecular approach on air filters allows reduced influence of inhibitors present in the matrix (boot swabs), since air is assumed to contain lower levels of inhibitors than fecal material. Additionally, during extraction of DNA, any inhibitors are more easily removed. Furthermore, an internal amplification control is included in the real-time PCR to provide reliable results. Using the present real-time PCR method, the sensitivity was 96.4%. In only one case, *Campylobacter* was detected solely by cultivation of boot swabs. Failure of the air sample-PCR approach could be explained by low relative humidity in the house. Schroeder et al. (25) described low positivity of air samples in poultry houses with relative humidity below 44%. The sensitivity observed in the present study is higher than the values from the validation studies shown in ISO 10272-1:2017, i.e., from 28% (chicken cecal material) to 64% (chicken skin) (20). However, the validation studies were carried out on other types of matrices with known levels of contamination, and the sensitivities cannot be compared directly, as the levels of contamination in the air samples are not known. It must also be taken into account that real-time PCR detects DNA from living cells, dead cells, and VBNC, while the ISO method detects only living bacteria. As reported previously, the same air sampling and direct real-time PCR method used for *Campylobacter* detection gave a detection limit of approximately 100 cell equivalents per cubic meter of air in chicken houses (11).

Due to the high sensitivity of the approach presented, this air sampling approach might be optional in countries and farms with flocks with low *Campylobacter*-positive rates, as culture-based methods might give more false-negative results; however, this needs further investigation. Moreover, analysis of air filters with real-time PCR has an advantage over the reference ISO method, giving the result in a much shorter time for *Campylobacter* detection in chicken flocks, i.e., 1 working day compared to 4 days, respectively.

Efficient measures for pathogen control in poultry environments are of primary importance to reduce direct or indirect food contamination. *Campylobacter*, *Salmonella*, *Escherichia coli*, *Listeria*, *Clostridium*, *Staphylococcus*, and others are associated with increased veterinary costs, animal health and welfare, and ultimately public health. The diagnostics is a primary preventive control measure, and molecular methods potentially identify various animal pathogens and avoid the limitations of traditional microbiology. The potential use of air sampling combined with PCR could be a valuable tool in such a scenario for rapid and cost-effective diagnostics of target microbes. Finally, the method described here could be also applied in the field by using portable PCR systems, making point-of-care diagnostics possible, even if further validation studies are necessary.

In conclusion, air sampling combined with real-time PCR is proposed as a multipurpose, low-cost, and convenient screening method that can be up to four times faster and four times more sensitive than the current boot-swab testing scheme used for screening biosecure chicken production environments. However, to obtain both isolates and PCR results, enriched swab samples should be preferred.

MATERIALS AND METHODS

Study design. The sampling was carried out in the Czech Republic, Denmark, Italy, Norway, and Poland to include various European regions with different climates as well as different levels of *Campylobacter* prevalence in chicken flocks. Each laboratory collected samples from at least 10 chicken flocks from at least two separate biosecure chicken farms as described by Johannessen et al. (18), with some modifications as described below. Three samples were taken from each flock, a pair of boot swabs and two air filters, giving a total number of 186 samples.

Sample collection. The samples were collected as described by Johannessen et al. (18). Briefly, before the sampling, boot swabs were moistened in a sterile diluent or premoistened boot swabs were used. The person performing the sampling wore the swabs over boots and walked around the chicken house, covering as much area as possible (26).

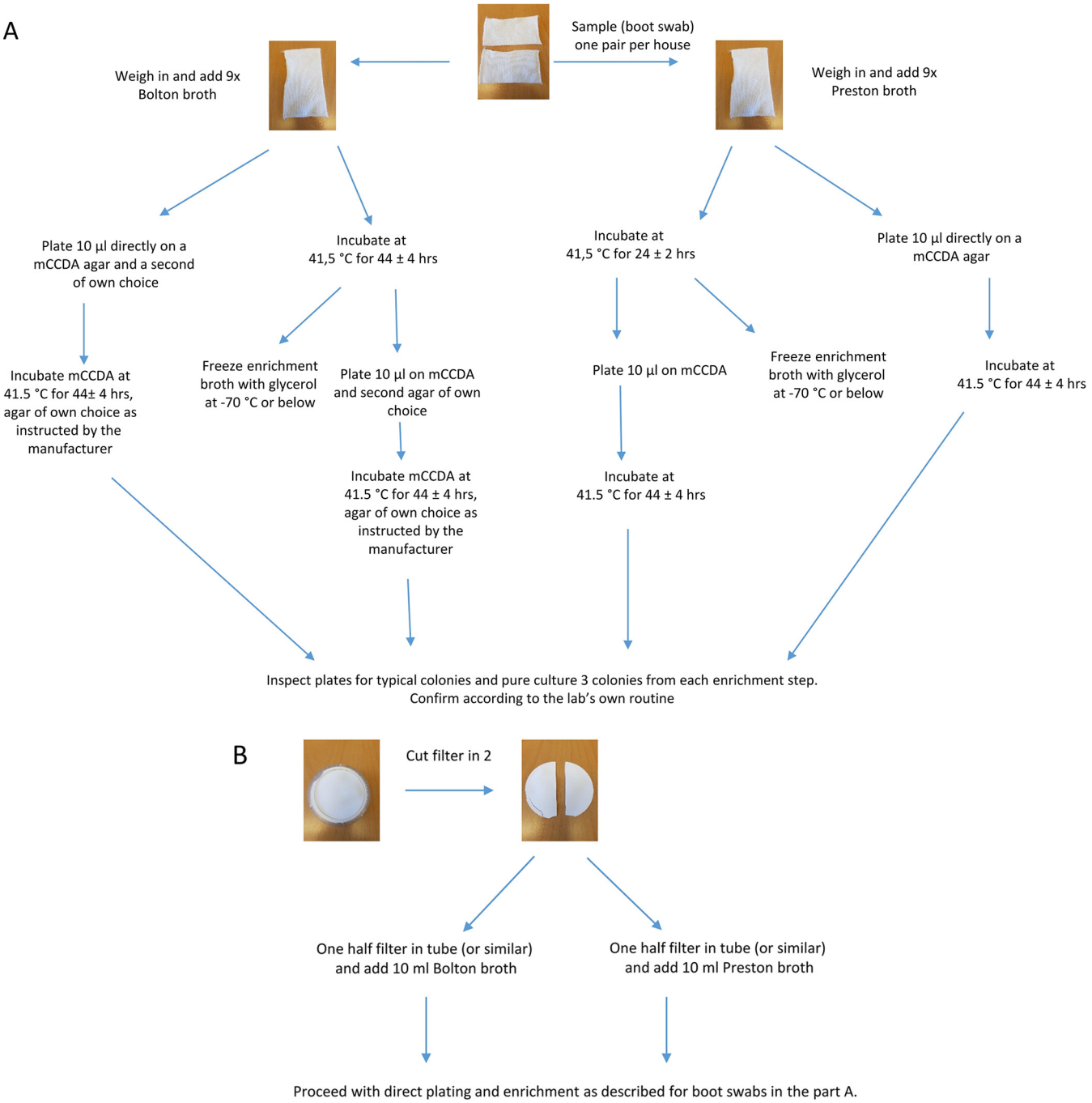

FIG 1 Flow diagram showing detection of *Campylobacter* spp. from boot swab samples (A) and air filters (B) using ISO 10272-1:2017.

Air samples were taken using an AirPort MD8 device (Sartorius Stedim Biotech, France) with disposable gelatin membrane filters (80 mm in diameter; Sartorius no. 17528-80ACD) as described by Johannessen et al. (18). Briefly, the samples were taken using an airflow rate of 50 liters/min for 15 min, giving a total air volume of 750 liters. The samples were transported at 4°C to the laboratory and processed within 48 h for cultivation or within 5 days for DNA extraction and subsequent real-time PCR. The details of sampling periods and flocks in each country are shown in Table S1.

Analyses. (i) Cultivation method. ISO 10272-1:2017 was used for detection of *Campylobacter* (20). Direct plating from homogenates prior to enrichment was done as described below. The steps of cultivation of boot swabs and air filters are presented in Fig. 1A and B.

(a) Boot socks and swabs. A total of 62 boot swab samples were collected. Briefly, the boot swab samples were divided into two groups (Fig. 1A). Each boot swab was weighed separately, and Bolton broth or Preston broth was added to obtain a ratio 1:10 (wt/vol). Prior to incubation of the Bolton and

Preston broths, a 10-μl aliquot of Bolton broth was plated directly on a modified charcoal cefoperazone deoxycholate agar (mCCDA) plate and a second agar plate as determined by the individual laboratory, while 10 μl of Preston broth was plated on mCCDA only. The plates were incubated under microaerobic conditions at 41.5°C for 44 $\pm$ 4 h. The Bolton and Preston broths were incubated at 41.5°C for 44 $\pm$ 4 h and 41.5°C for 24 $\pm$ 2 h, respectively, followed by subsequent plating as described for direct plating above. After incubation, the plates were checked for the presence of typical *Campylobacter* colonies, and a total of three presumptive *Campylobacter* colonies from each enrichment procedure were confirmed using the routine confirmation test used in the respective laboratories. Additionally, aliquots of the enrichment broths from every step were frozen at −70°C after addition of glycerol to a final concentration of 15%.

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

(ii) Real-time PCR. The second air filter was used for DNA extraction and real-time PCR for the detection of *Campylobacter*. The air filter was split in half, and DNA was extracted separately from each half using the same protocol. Briefly, the filter was pretreated by dissolving one half in 3.5 ml molecular-grade water tempered to room temperature with 100 μl of alkaline protease (Protex GL; Genencor International, Leiden, The Netherlands). The suspension was vortexed and split into equal volumes, which were placed in two tubes. Then, the tubes were incubated in a thermal shaker for 6 min at 37°C at 1,000 rpm and subsequently centrifuged at 8,000 $\times$ *g* for 5 min at 4°C. The supernatant was discarded, and the pellet was used for DNA extraction with a DNeasy blood and tissue kit (Qiagen GmbH, Germany) according to the manufacturer's instructions with modifications as described below. After incubation at 56°C for 1 h with shaking, the samples were cooled to 40°C, 4 μl of RNase A (100 mg/ml; Qiagen) was added, and the mixture was vortexed and incubated at room temperature for 5 min. DNA was eluted from the spin column with 100 μl Tris-EDTA (TE) buffer containing 0.1 mM EDTA prewarmed to 70°C and incubated at room temperature for 1 min before centrifugation. DNA was stored at −20°C for brief storage and at −80°C for longer storage.

Real-time PCR was done as described by Josefsen et al. (19) using a master mix and reagents 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 $MgCl_2$, 7% glycerol, 0.6 mM deoxynucleoside triphosphate (dNTP), 250 μg/ml bovine serum albumin, and 1 U of *Tth* DNA polymerase, with 500 nM forward primer (5′-CTGCTTAACACAAGTTGAGTAGG-3′), 500 nM reverse primer (5′-TTCCTTAGGTACCGTC AGAA-3′), and 75 nM LNA (locked nucleic acid) probe (5′-FAM [6-carboxyfluorescein]-CA[+T]CC[+T]CC ACGCGGCG[+T]TGC-BHQ1 [black hole quencher 1]-3′) (where plus signs indicate labeled sites). This assay detects *Campylobacter jejuni*, *C. coli*, and *C. lari*. An internal amplification control (IAC) was included in the same PCR, using the same primers and 50 nM 5′-JOE (6-carboxy-4′,5′-dichloro-2′,7′-dimethoxyfluorescein)-[+T]TCATGAGGACACCTGAGTTGA-TAMRA (6-carboxytetramethylrhodamine)-3′ probe in a duplex reaction. Templates for the IAC reaction were 5 $\times$ 10^3 copies of IAC (124 bp) with the sequence CTGCTIAACACAAGTTGAGTAGGCAACTCAGGTGTCCTCATGAATTIGAAGACATAAACAAGGGACTG GTCTCCGTCCCAACCAAGATCATCCATCTCCCGCTATTCTGACGGTACCTAAGGAA. 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 cycle threshold (C_T) value of $<$36 was used to score positive samples as recommended by Josefsen et al. (19).

(iii) Statistical analysis. Statistical analysis was performed using Fisher's exact test, McNemar's test, and the chi-square test (Statistica 13.2; Dell, USA). A *P* value of less than 0.05 was considered statistically significant. Relative sensitivity, specificity, and diagnostic accuracy were calculated according to Malorny et al. (27). Relative sensitivity was calculated as 100 $\times$ {(positive agreement between culture and PCR)/[(positive agreement between culture and PCR)+(false-negatives by PCR)]}. Relative specificity was calculated as 100 $\times$ {(negative agreement between culture and PCR)/[(negative agreement between culture and PCR)+(false-positives by PCR)]}. Diagnostic accuracy was calculated as 100 $\times$ [(positive and negative agreement between culture and PCR)/(total number of samples tested)].

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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