Research Note

Low-Cost Monitoring of *Campylobacter* in Poultry Houses by Air Sampling and Quantitative PCR

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ABSTRACT

The present study describes the evaluation of a method for the quantification of *Campylobacter* by air sampling in poultry houses. Sampling was carried out in conventional chicken houses in Poland, in addition to a preliminary sampling in Denmark. Each measurement consisted of three air samples, two standard boot swab fecal samples, and one airborne particle count. Sampling was conducted over an 8-week period in three flocks, assessing the presence and levels of *Campylobacter* in boot swabs and air samples using quantitative real-time PCR. The detection limit for air sampling was approximately 100 *Campylobacter* cell equivalents (CCE)/m³. Airborne particle counts were used to analyze the size distribution of airborne particles (0.3 to 10 μm) in the chicken houses in relation to the level of airborne *Campylobacter*. No correlation was found. Using air sampling, *Campylobacter* was detected in the flocks right away, while boot swab samples were positive after 2 weeks. All samples collected were positive for *Campylobacter* from week 2 through the rest of the rearing period for both sampling methods. This will presumably underestimate the total number of *Campylobacter* in chicken meat in the European Union, making it relevant to monitor the prevalence in primary poultry production (9).

There are several different methods for sampling and detection of *Campylobacter* in poultry production, using either culture-based methods (15) or molecular methods like quantitative real-time PCR (qPCR) (18). Culturing is time consuming and only determines the number of culturable bacteria. This will presumably underestimate the total number of viable campylobacters (10, 27). Viable but nonculturable (VBNC) cells can be detected with PCR, but so can DNA from dead cells, unless appropriate pretreatment steps are introduced (14, 18).

Sampling can be done early in the production chain by collecting fecal samples, e.g., on boot swabs, or later by analyzing raw poultry products (8, 18, 26). Sampling of air is a noninvasive technique, although levels 1- to 2-log CCE higher were found with air sampling. At week 8, the levels were approximately 10⁴ and 10⁵ CCE per sample for boot swabs and air, respectively. In conclusion, using air samples combined with quantitative real-time PCR, *Campylobacter* contamination could be detected earlier than by boot swabs and was found to be a more convenient technique for monitoring and/or to obtain enumeration data useful for quantitative risk assessment of *Campylobacter*.

*Campylobacter* is one of the leading causes of human acute gastroenteritis worldwide, and in 2010, it was the most commonly reported zoonosis in the European Union, often associated with chicken meat (9, 12, 25). The number of confirmed human cases has been increasing from 2006 to 2010, and there is a continuing high occurrence of *Campylobacter* in chicken meat in the European Union, making it relevant to monitor the prevalence in primary poultry production (9).

Cecal content or droppings provide more accurate results but require the animals to be sacrificed or the droppings to be manually collected, which is labor intensive (8, 28). Sampling of air is a noninvasive method with little hands-on time (23, 24) and is a cost-effective alternative to produce data for quantitative microbiological risk assessment.

Air sampling is being widely used, e.g., in animal production facilities (1, 29; for a review, see reference 27). Air sampling has usually been followed by culture enrichment to detect the collected bacteria, but recently, qPCR has also been used (20, 23, 24). For *Campylobacter*, air sampling has been used to monitor cross-contamination at slaughterhouses (3, 23) and the prevalence in poultry houses (2, 23, 30). It has been shown that *Campylobacter* could be detected in chicken houses by air sampling before it could be detected by boot swab sampling followed by qPCR (23). However, to our knowledge, the reproducibility has not been assessed, and this is needed to provide documentation for implementation of this approach in pan-European settings.

The objective of this study was to assess the use of filter-based air sampling coupled with qPCR, using nonproprietary, open-formula methods and devices (1, 6, 20), for quantitative monitoring of *Campylobacter* in poultry houses as an alternative to collecting and analyzing...
boot swab samples. Three chicken (Gallic gallus) flocks in Poland were included, and air and boot swabs were sampled in parallel throughout an 8-week period. Samples were analyzed by the standard culture-based method (15) and by qPCR (18).

MATERIALS AND METHODS

Sampling. Sampling in Denmark was performed in chicken houses in rearing weeks 0 to 2 and 2 to 5. Each sampling consisted of a 10-min particle count (28 liters of air) with the AeroTrak Optical Particle Counter 8220 (TSI, Shoreview, MN), collection of a fecal sample on two boot swabs as previously described (8, 26), and air sampling on three gamma-irradiated, Campylobacter-free gelatin membrane filters (diameter of 80 mm, Sartorius Stedim Nordic, Taastrup, Denmark) sampled consecutively with the AirPortMD8 (Sartorius) (at an air volume of 750 liters, flow rate of 50 liters/min, and height of approximately 50 cm above floor level for each filter).

Sampling under Polish farming conditions was carried out from July to October 2012 in two chicken houses in mideastern Poland. Three chicken flocks of approximately 17,500 animals were sampled, two in parallel and one in sequence. Nine to 10 samplings per flock were performed, one in week 0, i.e., before the flock was introduced to the chicken house, and one per week throughout the rearing period (approximately 7 to 8 weeks), plus an extra sampling performed in week 3. The litter was changed after each rearing period only, and entry to the chicken house was through an anteroom where clothes were changed and disinfection mats were used.

Analysis of air samples. The gelatin filters were analyzed by culture followed by colony PCR and by direct DNA extraction followed by qPCR (see below). Culture enrichment (at 41.5 ± 1°C under microaerobic conditions [6% O2, 7% CO2, 7% H2, and 80% N2; these conditions applied throughout the study]) was performed on one-fourth of a filter by adding it to 10 ml of complete Bolton broth (Oxoid, Roskilde, Denmark). After a brief vortexing, the tubes were incubated for 24 ± 3 h. A 10-μl inoculum was plated on modified charcoal cefoperazone deoxycholate agar (Oxoid), which was incubated for 24 to 48 h, and five colonies were subcultured on blood agar plates (24 to 48 h). Five colonies were analyzed with qPCR as previously described (18) to verify the presence of Campylobacter.

Analysis of boot swab samples. Boot swabs were placed in a stomacher bag, and buffered peptone water (Oxoid) added to achieve a dilution of 1:9 (wt/vol). The sample was homogenized in a Stomacher 400 (Seward Laboratory Systems, Port Saint Lucie, FL), and 10 ml was transferred to 90 ml of complete Bolton broth and incubated for 24 h. After enrichment, samples were analyzed as described above for air samples. The homogenate was allowed to sediment for 10 min, and 1-ml aliquots were drawn for DNA extraction.

DNA extraction. One-fourth of a gelatin filter was dissolved in 1,750 μl of Milli-Q H2O, and 50 μl of alkaline protease (Protex 6L, Genencor International, AE Leiden, The Netherlands) was added. This sample was vortexed and incubated twice at 37°C for 3 min. A 1-ml volume of boot swab homogenate or 1,800 μl of dissolved filter solution was centrifuged at 3,000 × g for 5 min at 4°C, and DNA extraction performed on the pellet in a KingFisher (Thermo Labsystems, Helsinki, Finland) as previously described (18), using a DNA isolation kit for blood, stool, cells, and tissue (MagneSil KF, Genomic system, Promega, Nacka, Sweden) as specified by the manufacturer. An amount of 10 μl was used as the template in the qPCR.

qPCR analysis. Quantification of thermotolerant Campylobacter was performed on an Mx3005P (Stratagene, Agilent Technologies, Hørsholm, Denmark) as previously described (18) and analyzed using the MxPro software (version 4.10). The threshold for each run was assigned as previously described (18), while the threshold for collected runs (projects) was set to 1,500 baseline-corrected fluorescence units based on visual inspection of the amplification curves.

In every run, a Campylobacter jejuni standard for absolute quantification was included in duplicate, together with controls comprising a nontemplate control, a DNA extraction process control, a negative control (5 ng of Escherichia coli DNA), and a positive control (5 ng of C. jejuni DNA).

Preparation of standard curves. C. jejuni CCUG 11284 was recovered on blood agar (Statens Serum Institut, Copenhagen, Denmark) and isolated on modified charcoal cefoperazone deoxycholate agar (Oxoid). Buffered peptone water (Oxoid) was used to produce 24-h cultures for preparing the Campylobacter quantification standards.

Suspensions from gelatin filters were used to prepare 10-fold serial dilutions in 0.9% NaCl, and Campylobacter was enumerated by plate suspending onto blood agar plates (Statens Serum Institut) in duplicate. One-quarter pieces of gelatin filters were inoculated with 100 μl containing 4.8 × 102 to 4.8 × 103 CFU of C. jejuni CCUG 11284 from the appropriate dilutions. DNA was extracted from the filters as described above. One biological and six qPCR replicates were used to produce the filter standard curve.

Campylobacter cells from boot swabs were harvested and enumerated as described above for the filter standards. Two boot swabs (approximately 15 g) were mixed with 30 g of Campylobacter-negative chicken feces, 405 ml of buffered peptone water (Oxoid) was added, and homogenization was performed in a Stomacher 400 (Seward). After sedimentation for 10 min, 1-ml aliquots were drawn and inoculated with 1 × 103 to 1 × 105 CFU Campylobacter. DNA was extracted as described above. Three biological and six qPCR replicates were used to produce the boot swab standard curve.

Data analysis. Standard curves were produced by plotting the cycle threshold (Cq) values obtained in the qPCR against the log-transformed number of CFU in the standards. The amplification efficiency was calculated from this linear relationship (18). The quantifiable range was established by visual evaluation of data points in the standard curves (Fig. 1). For Cq values within this range, the standard curves were used to estimate the number of Campylobacter cell equivalents (CCE). For values outside the quantifiable range, the samples were assessed qualitatively.

The particle counts were analyzed by plotting the average particle counts per cubic meter against the age of the chickens and making a visual comparison to the number of Campylobacter.

RESULTS

Optimization of air sampling and qPCR methods. In order to optimize the combined air sampling and qPCR protocols, a trial was conducted in two Danish chicken houses (data not shown). The results showed a good agreement between air sampling coupled with qPCR and boot swab samples with regard to the contamination status.
of the flocks. The detection limit for the air sampling coupled with qPCR detection was found to be approximately 100 CCE/m³ of air when sampling a volume of 0.75 m³ air and analyzing a one-eighth section of the filter. However, this sensitivity is dependent on the air volume sampled, amount of filter analyzed, DNA extraction protocol, and qPCR protocol and is therefore an approximation. For the analysis of the Polish chicken flocks, the same volume of air was sampled, but a one-quarter section of the filter was analyzed to increase sensitivity.

Detection of *Campylobacter* in Polish chicken houses. To assess the ability to use air sampling to isolate and enumerate *Campylobacter* under different farming conditions, three Polish chicken flocks were sampled using air sampling and boot swabs and analysis of the samples by qPCR and culture in parallel. It was not possible to recover viable *Campylobacter* from any of the samples (data not shown). The qPCR performed on the DNA extractions from gelatin filters and boot swabs showed that *Campylobacter* was present in all sample types for all three flocks from week 2 until the end of the sampling period (Fig. 2). The boot swab samples were negative before the introduction of a new flock and during the first week of rearing, while the air samples for the same weeks showed that *Campylobacter* was present at levels near the limit of detection, i.e., having high (37.5 to 39.9) or no C₇ values for replicate qPCR analysis. To determine whether samples from weeks 0 and 1 were truly positive, they were reanalyzed by qPCR in triplicates. The boot swab samples remained negative, while at least one filter from each air sampling was found to be positive (Fig. 2).

FIGURE 1. The standard curves used to estimate the number of CCE from artificially contaminated filters (●) or boot swabs (△), with cultures enumerated by counting CFUs. Data points show average C₇ values with 95% confidence intervals.

FIGURE 2. The log-transformed *Campylobacter* cell equivalents (log CCE) per sample (e.g., per filter [●] or boot swab [△]). (A to C) The data represented are the CCE per sample for each of the three flocks for each week (0, week 0, before introduction of chickens to the house; 1 to 8, weeks 1 through 8, the remaining sampling period). The error bars show the standard deviations related to the qPCR analysis. The data points marked with an asterisk are from samples that gave C₇ values outside the quantifiable range and for the value 0 on the y axis, meaning no detection of *Campylobacter*. 

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Quantification of Campylobacter in Polish chicken houses. qPCR standard curves, prepared separately for the two sample types, were used to assess the number of Campylobacter cells in the samples (Fig. 1). The limit of quantification was found to be 10^2 CCE per sample. Although the three flocks sampled showed too much variance to be considered as one population, there was a tendency for the CCE levels per sample to be 1 to 2 log higher in the air samples than in boot swabs (Fig. 2). In weeks 0 and 1, all flocks were found to be positive for Campylobacter with air sampling, although the levels were close to the limit of detection, with CT values close to the maximum possible value of 40 (37.5 to 39.3).

Analysis of particle counts. The initial particle counts performed in Danish chicken houses showed that the smaller particles (0.3 to 5 μm) were most abundant in the beginning of the rearing period, but after 1 to 2 weeks, the levels of larger particles (5 to >10 μm) were almost as high. The levels of airborne particles were 10^9 to 10^11 particles per m^3 (data not shown).

The particle counts from all three Polish chicken houses showed the same overall distribution of particle sizes, with the smallest particles recorded (0.3 to 0.5 μm) being 1 to 2 log more abundant throughout the entire rearing period (Fig. 3). The larger particle sizes (0.5 to >10 μm) showed 1- to 2-log increases from week 0 (before the chickens were introduced to the chicken house) to week 2, and the levels of airborne particles were 10^6 to 10^7 particles per m^3. The CCE was found not to correlate with one specific particle size but, rather, followed the overall tendency of all the particle size intervals recorded (Fig. 3).

DISCUSSION

This study evaluates the use of air sampling to monitor the Campylobacter prevalence on a flock level under different European farming conditions to verify the generality of the approach. Air sampling coupled with real-time PCR for detection of Campylobacter has previously been tested under Danish farming conditions using an integrated sampling and qPCR device (23). However, as the device used is no longer commercially available, an air sampler using gelatin filters for collection of airborne bacteria (1) was combined with a validated, noncommercial qPCR assay (18).

It has previously been found that contamination with Campylobacter could be detected in air samples as much as 11 days before it was detected in the boot swab samples, which is consistent with the results from the Polish chicken flocks tested in this study, where Campylobacter was detected in the air samples up to 2 weeks before it was found in the boot swab samples (23). The ability to reveal contamination of a flock earlier with air sampling is relevant for investigations of the epidemiology of Campylobacter, as well as to establish an on-line monitoring system. We found the contamination, in both the Polish and Danish chicken houses studied, to be well established by week 2, whereas other studies have shown contamination and/or colonization to occur around week 3 (5, 23).

FIGURE 3. Results from the optical particle counts for three Polish chicken flocks plotted against the age of the chickens in weeks. Particle size intervals: 0.3 to 0.5 μm (diamond), 0.5 to 1.0 μm (square), 1.0 to 3.0 μm (triangle), 3.0 to 5.0 μm (×), 5.0 to 10.0 μm (×), and >10.0 μm (+). The dotted (●) curve shows the average Campylobacter cell equivalents per cubic meter (CCE/m^3) as determined by the qPCR analysis of the air samples.

However, because it was not possible to isolate any living Campylobacter from either the filter samples or the boot swab samples, the proportion of dead airborne Campylobacter is not known. The lack of culturability in these samples is most likely due to the long storage time and transportation, as Campylobacter cells are known to die or enter into a VBNC state when encountering stress (16). For the boot swab samples taken at the Danish poultry houses, the levels of culturable campylobacters in samples were found to be approximately 10^5 CFU/ml when contamination was established in flocks of around 1 to 2 weeks of age. Culturing, however, only gives an estimate of the number of culturable Campylobacter cells, while providing no information about VBNC Campylobacter cells in the samples. In future studies, alternative strategies could be applied to quantify both the viable and the VBNC fractions (4, 18). On the other hand, to establish the contamination status of the flock, it is not necessary to know the viability status of the
Campylobacter, as this is only used as a marker for colonization.

An advantage of using air sampling combined with qPCR, as described in this study, is that as well as being noninvasive, cost effective, and highly sensitive, it is not limited to Campylobacter detection in chicken houses. It has previously been used for various types of housed poultry (6, 20), as well as other farmed animals (1). Moreover, the flock contamination status for multiple bacterial pathogens could potentially be determined simultaneously by this form of air sampling (24), as evaluated by others, e.g., to test for bioterror-related microorganisms (11) and to monitor the quality of indoor air (13).

In this study, no correlation between a specific size interval of sampled particles and the CCE measured via the air sampling was found, which is the opposite of a peak in the amount of particles of 2 to 5 μm at the end of the rearing period that was previously reported, with the conclusion that Campylobacter was likely to be found in this fraction (23). What contradicts this theory is the fact that Campylobacter was detected in dust samples almost at the same time as in the air samples, suggesting that particles carrying Campylobacter are large enough to sediment, i.e., >5 μm (21). Considering the size of Campylobacter cells (between 0.2 to 0.9 and 0.5 to 5 μm (22)) and the fact that airborne bacteria are often found in aggregates (27), the particle counts from this study suggest that airborne Campylobacter can be associated with variable particle sizes, though probably not below 2 μm in diameter.

Considerable differences in the particle size distributions, probably related to differences in ventilation systems and sizes of houses, were found when comparing the results from Danish and Polish chicken houses. This could have influenced the detection using air sampling, but these results showing that air sampling worked well in both countries, as well as previous results for the detection of methicillin-resistant Staphylococcus aureus (MRSA) in pig herds. Epidemiol. Infect. doi:10.1017/S095026881300280X.


