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ORIGINAL ARTICLE

Air samplings in a *Campylobacter jejuni* positive laying hen flock

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Abstract

The air in laying hen houses contains high concentrations of airborne bacteria. The numbers of these bacteria can be influenced by the efficiency of the chosen sampling method. In the presented study, AGI-30 Impingers and the Coriolis®µ air Sampler were compared in terms of their efficiency in sampling aerobic mesophilic bacteria in a laying hen house. Measurements were conducted in a laying hen flock with high prevalences of *C. jejuni* in order to investigate if culturable cells of this organism can also be detected by the applied methods. Airborne dust was also analyzed for the presence of *C. jejuni* specific DNA to assess the possible occurrence of non-culturable *C. jejuni* in the hen house air. The numbers of mesophilic airborne bacteria ranged from $8 \times 10^4 - 2 \times 10^6$ CFU/m^{-3} when sampled using AGI-30 Impingers, and from $2 \times 10^5 - 4 \times 10^6$ CFU/m^{-3} when sampled using a Coriolis®µ air Sampler. The concentrations detected simultaneously by both devices correlated well ($r_{Pearson} = 0.755$), but the Coriolis®µ air Sampler showed a significantly higher sampling efficiency ($p < 0.001$). Although, the within flock prevalence of *C. jejuni* was high during the experiments (between 70-93%), neither of the air sampling methods could detect culturable *C. jejuni* from the air. However, *C. jejuni* specific DNA was detected in 15 out of 18 airborne dust samples by *mapA* PCR. Based on the results, it can be concluded that airborne culturable *C. jejuni* were not detectable, even with an efficient air sampler, because of their low concentration. Therefore, the risk of airborne infection to poultry workers on inhaling airborne *C. jejuni* seems negligible. Also, the transmission of culturable *C. jejuni* to neighboring farms by the airborne route is unlikely. Otherwise, the detection of airborne *C. jejuni* specific DNA suggests that non-culturable cells could appear in the hen house air, and in future it should be verified whether sampling stress of the air sampling methods could induce the non-culturable state.

Key words

AGI-30 impinger**,** Coriolis®µ air Sampler, airborne bacteria, *Campylobacter jejuni*, laying hens

INTRODUCTION

Campylobacter jejuni (*C. jejuni*) is an important zoonotic agent in Europe and many other countries worldwide [1, 2, 3]. The bacterium causes gastrointestinal infection after consumption of contaminated food. Poultry meat is the main source of infections and poultry houses can be a reservoir of *C. jejuni* [4, 5]. It is suggested that horizontal transmission plays a major role in the spread of *C. jejuni* within and between poultry flocks [6]. Probable sources of infection include colonized birds, contaminated faeces, feed, litter, water, equipment and transport vehicles, or even wild birds and insects [7, 8]. However, the role of airborne transmission is not well understood. Culturable airborne *C. jejuni* was isolated by Bull et al. [9] within and very close to – at a distance of in 30 m – a broiler barn. The concentration of *Campylobacter* spp. remained unknown because the authors used an enrichment method for the detection. Zhao et al. [10] tried to quantify culturable airborne *C. jejuni* with different air sampling techniques in an experimental room with infected broilers, but the detection failed. On the other hand, a poultry worker was probably infected by inhaling *C. jejuni* containing aerosols [11]. Therefore, it seems necessary to assess the risk of airborne infections at workplaces such as poultry houses. For this assessment, it would be useful to estimate the concentrations of airborne culturable *C. jejuni*. Unfortunately, so far there is no recommended air sampling technique for this purpose. A standardized method, which was used several times to measure concentrations of airborne culturable bacteria in poultry buildings, is the impingement with AGI-30 impingers [12, 13, 14]. This technique samples airborne microorganisms in a fluid (usually buffer or water) and allows detecting high microbial concentrations in animal house air [15]. If the sampling solution is filtered, the detection limited can be lowered to 12 cfu/m^3 [16]. Using AGI-30 impingers in poultry houses may also deliver comparable results to former studies [17, 18]. But, the detection of airborne *Campylobacter* with AGI-30 impingers has not been successful to date [10]. Therefore, a novel air sampler that recently showed a low detection limit for airborne bacteria cells Coriolis® µ [19] was used, taking the AGI-30 impinger as a reference method to compare the sampling efficiencies of the devices. Tests were conducted in a *C. jejuni* positive laying hen flock, expecting an airborne exposure to animals and humans. Air samples were tested for the total number of culturable mesophilic bacteria and culturable *C. jejuni*. Furthermore, airborne dust samples were taken simultaneously and analysed for the presence of *C. jejuni* specific DNA, which may indicate the occurrence of non-culturable *C. jejuni* cells in the poultry house air.

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MATERIALS AND METHODS

Sampling locations and sampling period. Samples were taken in a forced ventilation laying hen house equipped with an aviary system (NATURA 60, Big Dutchman, Germany). Altogether, 2,300 laying hens (breeding line 'Silver', Lohmann, Cuxhaven, Germany) were kept in this multilevel system with nest boxes at the sidewalls and a littered scratching area inside. The birds also had access to an outdoor scratching area (winter garden) via 6 openings in a sidewall. During the measurements, only a small number of hens (approx. 200) could be observed in this area. Samplings were carried out weekly between 10.00 – 13.00, beginning on the 14th and ending on the 19th week of one laying period. Air samples were taken lengthwise in the centre of each third of the laying hen house. The instruments were placed 1.5 m above the scratching area. Temperature and relative humidity (RH) were measured at the same height in the mid-position. During the air sampling, 30 cloacal swabs were taken from randomly selected hens during each farm visit to estimate the within flock prevalence of *C. jejuni*.

Sampling of airborne bacteria and dust. The impingement with all-glass impingers (AGI-30; Ace Glass Inc., Vineland, NJ, USA) and a wet cyclone technology (Coriolis®µ Air Sampler, Bertin Technologies, Montigny le Bretonneux, France) were used to sample airborne bacteria. On each sampling day, 3 impingers were operated simultaneously at the sampling locations for 30 min. Micro-organisms were collected in 30 ml phosphate buffered saline (PBS). The air flow (12.5 l min^{-1}) through the impingers was controlled before and after the end of the sampling time with a flow meter 044-14G from Analyt-MTC (Müllheim, Germany). In order to compare the impingement with the Coriolis®µ Air Sampler, one air sample was taken with the cyclone at each sampling position in parallel to the impingement. The cyclone was adjusted to sample 0.9 m^3 within 3 min. Airborne bacteria were collected in Coriolis®µ cones filled with 15 ml PBS.

Dust sampling was started at the same time of using the impingement. Therefore, one SKC pump with an IOM sampler (SKC Inc. Eighty-Four PA, USA) was located beside each AGI-30 impinger. The airflow of the pumps was adjusted to 2.5 l min^{-1}. Dust was sampled for 120 min. on polycarbonate membrane filters with 0.2 µm pores (Omnilab, Gehrden, Germany).

On each sampling day, one transport control for the impingement, one for the wet cyclone technology and one for the filtration method, were carried along with the air sampling. These controls were handled in the same way as the air samples with the exception that they were not exposed with air from the laying hen house. All samples were maintained at a temperature of 4°C-8°C when transporting to the laboratory in which analysis of the samples started the same day.

Laboratory analysis of air samples. Impingers and Coriolis®µ cones were shaken for 30 s at full speed with a Vortex-Genie2 (Scientific Industries Inc., USA) and 1 ml aliquots were taken from the sampling solutions to prepare serial dilutions (10^{-1} to 10^{-4}). Three times aliquots (0.1 ml) from the original sampling solution and from the dilutions were plated on blood agar base (Oxoid, Germany). The plates were incubated aerobically for 48 h at 36 °C. Subsequently, the average numbers of colony forming units (cfu) of one dilution step with countable colonies (30-300 colonies per plate) were used for calculating the total culturable airborne bacteria per cubic metre [c] by the following equation:

$$c = \frac{cfu}{V_{plated\ aliquote}\ [ml]} \times \frac{dilution\ factor \times V_{buffer\ after\ sampling}[ml]}{V_{airsample}\ [m^3]} \quad (1)$$

For the detection of airborne *C. jejuni*, 0.1 ml aliquot was taken from each of the previously shaken impingers and Coriolis®µ cones and plated directly on mCCAD and Brilliance CampyCount agar (Oxoid, Germany). Then, 1 ml aliquot of each impinger and Coriolis®µ cone was added to 9 ml Bolton broth (Oxoid, Germany) for enrichment of *C. jejuni*. Finally, the rest of the sampling buffers were filtered thought 0.2 µm pore size cellulose nitrate filters (Sartorius, Germany) placed on top of mCCDA agar. The plates and enrichment broth were incubated under anaerobic conditions, as described by Ahmed et al. [20].

Dust samples were analysed to detect the *C. jejuni* specific *mapA* gene by PCR. The polycarbonate filters were transferred from the IOM samplers to 1.5 ml Eppenndorf cups (Eppendorf, Germany) and stored at -20 °C over night. Next morning, the filters were washed with 0.5 ml cold TE buffer (10 mM Tris PH 8.0, 1 mM EDTA- disodium salt dihydrate) and vortexed for 5 min. The filters were then removed carefully without loosing buffer. The remaining dust within the Eppendorf cups was centrifuged at 25,000 × g for 5 min and the supernatant was discarded. The dust pellets were used for the isolation of DNA with a QIAamp DNA stool Mini Kit (Qiagen, Germany) according to manufacturer's instructions. The isolated DNA was used for the *C. jejuni* specific PCR, as described by Ahmed et al. [20].

Temperature and humidity measurements. Temperature and humidity were measured during the air samplings with a thermo-hygrometer (Rotronic Date logger Hydrolog-D HygroClipS Temperatur/RH (Rotronic GmbH, Ettlingen, Germany). Values were recorded 15 min after the impingement was started.

Prevalence of *C. jejuni* within the flock. In order to estimate the prevalence of *C. jejuni* within the laying hen flock, 30 randomly selected birds were captured and investigated by cloacal swabs (EUROTUBO®, DELTALAB, Spain) during each farm visit. One swab was obtained from each bird and streaked directly onto modified Charcoal Cefoperazone Desoxycholate Agar (mCCDA, Oxoid, Germany) and Brilliance CampyCount agar (Oxoid, Germany). Thereafter, eache swab was placed in a tube with 9 ml Bolton Broth (Oxoid, Germany). Samples were transported to the laboratory under cooled conditions together with air samples. Isolation and identification of *C. jejuni* from swab samples was carried out following the method recently described by Ahmed et al. [20]. The prevalence of *C. jejuni* within the laying hen flock was estimated by the number of *C. jejuni* positive hens (n_{pos}) in relation to the total number of tested hens (n = 30), as given by equation 2.

$$\text{Prevalence (\%)} = n_{pos} \times 100/n \quad (2)$$

Statical analysis. Statistical differences among the numbers of bacteria detected with the impingement and the

Coriolis®µ Air Sampler were assessed by using the Wilcoxon sum-rank test. The correlation (Pearson's correlation) among the bacteria concentrations measured with different sampling techniques was calculated with the SAS [21] software version 9.3 [SAS Institute Inc., Cary, NC, USA].

RESULTS

The concentrations of airborne mesophilic bacteria from impinger samples ranged from $8 \times 10^4 - 2 \times 10^6$ CFU/m^{-3} and the concentrations from the Coriolis®µ Air Sampler varied between $2 \times 10^5 - 4 \times 10^6$ CFU/m^{-3}. No bacteria growth was observed in any of the transport controls. In 17 out of 18 air samples the Coriolis®µ Air Sampler showed higher bacteria concentrations than the AGI-30 samplers (Fig. 1).

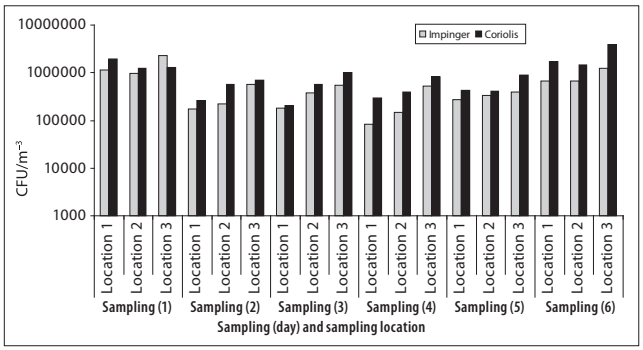

Figure 1. Airborne bacterial concentrations (cfu/m^{-3}) detected simultaneously with Impingers and Coriolis® µ Air Sampler on 6 different sampling days

The differences between the concentrations of both air samplers are highly significant ($p<0.001$). The tendencies of the concentrations of the different sampling methods are very similar and show a high correlation ($r_{Pearson} = 0.755$). There was no obvious coherence between climatic factors and the average bacteria concentrations of both sampling methods (Tab. 1). In this context, it is remarkable that the maximum variations of temperatures (± 3.6°C) and humidity (±17 %) were low among the different sampling days.

Table 1. Amounts of mesophilic bacteria × 10^4 CFU/m^3 (Mean ± SD) at different ages of laying hens and under different climatic conditions in the laying hen house

Sampling No. (week of laying period)	Air sampling methods		Temprature °C	RH %
	Impinger	Coriolis		
1 (14)	145±71	158±38	16.8	45.5
2 (15)	32±22	52±22	13.2	32.6
3 (16)	37±18	60±41	15.8	49.6
4 (17)	25±24	50±28	13.9	46.6
5 (18)	34±6	58±28	13.2	43.2
6 (19)	87±32	233±130	14.6	46.4

Culturable *C. jejuni* could not be detected in any of the air samples, neither by direct plating nor by the enrichment methods. On the other hand, *C. jejuni* specific DNA was detected in airborne dust by *mapA* PCR on each sampling day (Tab. 2). Overall, *C. jejuni* specific DNA was detected in 15 (83%) out of 18 airborne dust samples. The control samples showed no positive PCR results.

Table 2. Detection of *C. jejuni* DNA isolated from airborne dust

Sampling No.	Location		
	1	2	3
1	+	+	+
2	-	+	+
3	+	+	+
4	-	-	+
5	+	+	+
6	+	+	+

+ = positive dust sample;
- = negative dust sample

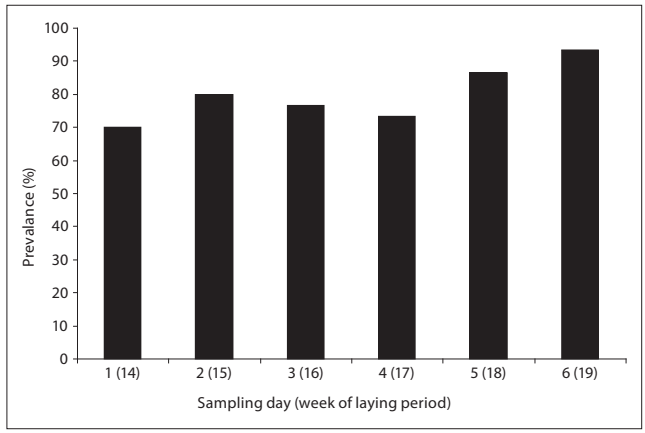

Figure 2. Prevalence (%) of *C. jejuni* isolated from 30 laying hens on 6 different sampling days

The estimated prevalence of *C. jejuni* within a laying hen flock during the measurements ranged between 70-93% (Fig. 2). It seems that the tendency of the prevalence increased from the 14th week (sampling day 1) to the 19th week (sampling day 6) of the laying period (Fig. 2).

DISCUSSION

Measurements with both applied air-sampling techniques showed a strong variation of bacteria concentrations (> one log step) between the 14th and 19th week of the laying period. Such different values of airborne bacteria were also detected with AGI-30 impingers in aviaries in a seasonal course by Springorum and Hartung [22]. The authors suggested that air exchange rates, animal activity and waste management are important factors having an impact on the concentrations of airborne microorganisms in a laying hen house. In the presented study it was also assumed that these factors affected the bacteria concentrations during the investigations. Interestingly, a high correlation was observed between the bacteria concentrations detected with different sampling methods. This indicates that probably the same factors (animal activity, ventilation rate, etc.) within the laying hen house influenced the results. However, the concentrations measured with the Coriolis®µ Air Sampler were significantly higher than the concentrations detected with AGI-30 impingers. One reason for this could be the difference in particle sizes sampled from the air – the Coriolis®µ Air Sampler samples larger particles compared to the AGI-30 impinger and these larger particles may carry more bacteria than the smaller

particles [23, 24] which could lead to a higher bacteria concentration in the sampling buffer of the Coriolis®µ cones. Another reason could be the sampling stress induced by the impingement and a minor loss through reaerosolization of particles within the cyclone [25, 26]. The reasons for these differences need to be clarified in future under laboratory conditions. However, due to the higher sampling efficiency of culturable bacteria and its lower detection limit compared to the AGI-30 impinger, the Coriolis®µ Air Sampler seems to be a suitable device for measuring bacteria concentrations in the air of poultry houses. Verification of this statement is recommended by conducting further experiments in different housing systems. Also, the potential impact of climatic conditions (temperature and relative humidity), which showed no obvious influence during the presented study, should be examined in more detail.

The sampling of airborne culturable *C. jejuni* was not successful, although a high prevalence of this bacterium was observed on all sampling days. Hens are known to shed *C. jejuni* at high rates [27] and that about 2-8% of airborne particles in poultry houses originate from faeces [28, 29, 30]. Therefore, airborne *C. jejuni* could be expected in the air of the laying hen house. Nevertheless, only *C. jejuni* specific DNA was detected in 83% of airborne dust samples. Washing and centrifugation of dust samples can lead to isolation of particular bound DNA (including bacteria cells), and the use of a stool kit for DNA extraction enables the detection of *C. jejuni* specific DNA in the presence of faecal particles [31, 32]. It was assumed that *C. jejuni* DNA detection in the air of the laying hen house refers to the presence of *C. jejuni* cells. Olsen et al. [33] arrived at the same conclusion when they detected *C. jejuni* DNA in the air of broiler houses. This means that culturable airborne *C. jejuni* does not occur or occurs in negligible amounts in flocks with a high prevalence. The theoretical detection limit of Coriolis®µ Air Sampler was 15 cfu/0.9 m^3 for the enrichment method, and 1 cfu/0.9 m^3 after filtration of the rest of the sampling solution. Assuming that only culturable *C. jejuni* are potentially infective, it seems that there is only a limited risk for farmers, veterinarians or workers in poultry houses to be infected by inhaling or swallowing *C. jejuni*. This is also supported by the findings of Berndtson et al. [34] and Chinivasagam et al. [35]. On the other hand, it is not known if sampling of airborne *C. jejuni* with the Coriolis®µ Air Sampler or with AGI-30 impingers may influence their culturability. Therefore, the survival of airborne *C. jejuni* during sampling with these samplers has to be investigated in suitable laboratories in future to exclude significant negative sampling effects. Furthermore, more samplings in high prevalent poultry flocks with higher sampling volumes or additives in sampling solutions that protect sensitive bacteria should be carried out to gain quantitative results for airborne culturable *C. jejuni*. This may confirm the assumptions about a low infection risk for persons by airborne *C. jejuni* in poultry houses, and could also help assess the risk of airborne transmission between poulty/animal houses.

CONCLUSIONS

The Coriolis®µ promises to be a useful technique for efficiently quantifying aerobic mesophilic bacteria in poultry houses. More investigations in different housing systems are necessary to confirm this hypothesis. The failure to detect culturable *C. jejuni* in a high prevalent laying hen flocks with an efficient air sampler indicates a low risk for persons to become infected by the airborne route. However, the role of non-culturable *C. jejuni,* which may occur in the animal house air, requires future investigation.

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