Detection of Airborne *Campylobacter* with Three Bioaerosol Samplers for Alarming Bacteria Transmission in Broilers

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ABSTRACT. *In an airborne transmission experiment,* Campylobacter *in the air was sampled by three types of bioaerosol samplers (all-glass impinger AGI-30, Andersen six-stage impactor, and OMNI-3000) in four broiler rooms. In each room, five 14-day-old broilers inoculated with* Campylobacter jejuni *were kept in a central cage located in the middle of the room. Another ten broilers, as susceptible animals, were kept individually in ten cages surrounding the central cage at a distance of approximately 75 cm. Air samples were taken on eight days: the day before inoculation (BI) as a negative control, and 1, 3, 6, 9, 14, 21, and 29 days post-inoculation (PI). Presence of* C. jejuni *was investigated with the culture method for culturable bacteria and with the PCR test for bacterial DNA. Results showed that* Campylobacter *infection of susceptible broilers occurred in all four rooms; however, no culturable* C. jejuni *could be detected in any of the air samples. This might have been the result of the low number of broilers in the room and the unfavorable conditions for* Campylobacter *survival, leading to* Campylobacter *concentrations below the detection limits of the bioaerosol samplers. The PCR test showed that DNA of* C. jejuni *was detected in the air samples on the first day PI, but no bacterial DNA was detected on the following days. It is concluded that the three samplers used in this study are not able to alarm* Campylobacter *outbreaks through an airborne route when low bacterial concentrations are present. Developments of new sampling techniques with low detection limits are required for biosecurity assessment.*

Keywords. *Aerial, Bacteria,* Campylobacter*, Poultry, Transmission.*

Campylobacter species are recognized as an important cause of human illness, such as diarrhea and Guillain-Barré syndrome, and sporadic morbidity (Keener et al., 2004; Humphrey et al., 2007). It was reported that 50% to 70% of *Campylobacter* infections are caused by consumption of poultry products in the U.S., Europe, and Australia (Allos, 2001). The prevalence of *Campylobacter* is high in the

Submitted for review in October 2010 as manuscript number BE 8855; approved for publication by the Biological Engineering Editorial Board of ASABE in May 2011.

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  ISSN 1934-2799

poultry industry. Approximate 20% of broiler flocks in 1999-2002 (Van de Giessen et al., 2006) and 35% of organic broiler flocks in 2003 (Rodenburg et al., 2004) were *Campylobacter*-positive in The Netherlands. For biosecurity, an obligatory monitoring program has been implemented in the European Union to identify the presence of *Campylobacter* in poultry and to develop control strategies (EU, 2003).

Campylobacter transmission in poultry was thought to be caused mainly by contacting contaminated feed, water, infected animals, and insects (Jacobs-Reitsma et al., 1995). Recently, airborne transmission of *Campylobacter* has been suspected as another mechanism for the spread of disease based on the recovery of culturable bacteria from the air in poultry processing plants and an infection case of a poultry worker via the air (Berrang et al., 2004; Wilson, 2004; Johnsen et al., 2007). If this mechanism is valid, then detection of airborne bacteria with bioaerosol samplers is required to be included in the monitoring program for *Campylobacter*. However, it is not clear whether or not effective monitoring, with respect to alarming the airborne transmission of *Campylobacter*, can be achieved by sampling using bioaerosol samplers.

The techniques for sampling airborne microorganisms include impingement, impaction, filtration, and cyclone (Eduard and Heederik, 1998). In practical measurements, none of these techniques can fully recover all the microbial species from the air due to non-isokinetic sampling and sampling stresses on the target organisms (Stewart et al., 1995). These limitations make precise quantification of airborne microorganisms a challenge, especially for stress-sensitive species. So far, the all-glass impinger (AGI-30) and Andersen six-stage impactor are the most commonly used bioaerosol samplers and are recommended as standard samplers for airborne microorganisms. However, their utility is limited in environments with low concentrations of microorganisms due to their relatively low airflow rates (12.5 L min^{-1} for AGI-30 and 28.3 L min^{-1} for Andersen impactor). In order to perform air sampling in low-concentration environments, samplers with higher air volumes have been used, e.g. OMNI-3000 (300 L min^{-1}) and SAS Super 90 impaction air sampler (90 L min^{-1}) (Gast et al., 2003), to sample a large amount of air in a short time.

Previous studies on airborne transmission of pathogenic microorganisms have been performed with infected and healthy animals in lab-scaled experiments (Mars et al., 1999; Berthelot-Herault et al., 2001; Brockmeier and Lager, 2002). Airborne infection can be shown when healthy susceptible animals are infected by infected animals that are physically, but not aerially, separated from them. A similar experimental setup could be applied to study the airborne transmission of *Campylobacter*.

To analyze *Campylobacter*, a culture method or polymerase chain reaction (PCR) technology can be used. With the culture method, culturable *Campylobacter*, which is capable of multiplying, can be quantified, but this method is time consuming and labor intensive. A PCR test is fast compared to the culture method; however, because the test detects only a part of a certain gene, *Campylobacter* DNA is determined without differentiation between culturable and dead bacteria.

The objective of this study was to test three bioaerosol samplers (AGI-30, Andersen impactor, and OMNI-3000) for detecting *Campylobacter jejuni* (culturable and DNA) in the air for alarming its airborne transmission by broilers in an experimental setup.

Materials and Methods

Experimental Rooms

The study was conducted in four climate-controlled (temperature and relative humidity) rooms, each 7.40 m in length 4.75 m in width, and 2.80 m in height. Room temperature gradually decreased from 25°C on the day before inoculation (BI) (13-day bird age) to 18°C on 17 days post-inoculation (PI) and then remained constant until the end of the experiment (35 days PI). Relative humidity was set at a constant level of 55%. The rooms were ventilated by an overpressure system in the roof of the building. The inlet air was filtered by an absolute filter to prevent interference from outside. The air outlet was installed on one side wall. The average ventilation rate of each room was 1000 m^3 h^{-1}; thus, the air changes per hour (ACH) in the rooms was about 10 ACH.

Before the experiment, all rooms were disinfected with formaldehyde gas and then high-pressure water. Samples from 12 different surfaces were taken to ensure the absence of both culturable *C. jejuni* and bacterial cells.

Animals and Cages

Fifteen 12-day-old *Campylobacter*-free broilers were introduced into each room. Five broilers were reared in a cage (2.25 m in length and 1.00 m in width) located in the middle of the room. After two days, these five broilers were orally inoculated with 10^6 colony forming units (CFU) of *C. jejuni* strain 356. As susceptible animals, the other ten broilers were reared separately in ten individual cages surrounding the central cage. The individual cages (1.00 m in length and 0.75 m in width) for the susceptible broilers were placed at a distance of 0.75 m from the central cage. Figure 1 shows a schematic of the room. All broilers were reared on wood shavings on the ground. Water was supplied by a nipple drinking system, using separate systems for the central cage and the other cages.

From the first day PI until 35 days PI, cloacae swabs were taken on a daily basis. When the inoculated broilers tested positive for *C. jejuni* on three consecutive post-

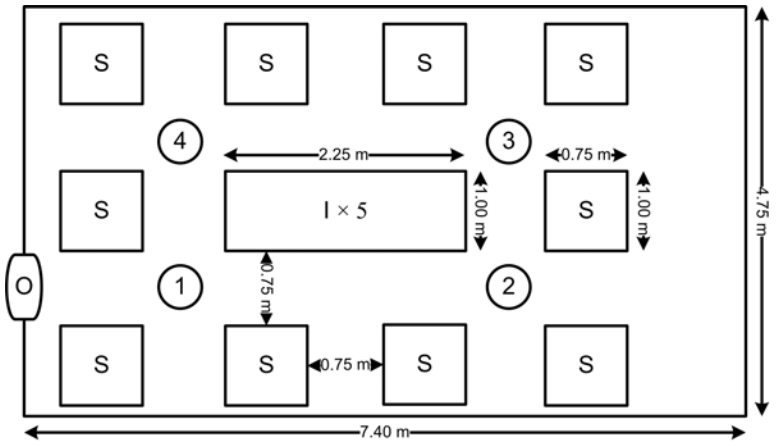

Figure 1. Schematic of the room: I = inoculated broilers in central cage, S = susceptible broiler cage, O = air outlet, and 1 through 4 = air sampling locations.

inoculation days, swabs were taken weekly instead of daily. The susceptible broilers were tested every day. Whenever a susceptible broiler turned positive for *C. jejuni*, it was ruled out from the room.

Bioaerosol Samplers

Three bioaerosol samplers were used: AGI-30, Andersen impactor, and OMNI-3000. The AGI-30 and Andersen impactor are able to preserve viability of *C. jejuni* due to their low sampling stresses and therefore have been suggested as samplers for airborne culturable *C. jejuni* (Zhao et al., 2011a, 2011b). However, when bacterial DNA, which could be from both culturable and dead bacteria, is of concern, the AGI-30 and Andersen impactor may be not suitable because of their low airflow rates. A new type of high airflow rate sampler, the OMNI-3000 (300 L min^{-1}), was also used in this study to detect bacterial DNA at low concentrations in the air.

The AGI-30 (7540, Ace Glass, Inc., Vineland, N.J.) collects bacteria into a liquid medium by impingement. It is operated at an airflow rate of 12.5 L min^{-1}. A 20 mL volume of physiological salt water (PSW, bioTRADING Benelux B.V., Mijdrecht, The Netherlands) was used as the collection medium in this study.

The Andersen impactor (TE-10-800, Pacwill Environmental, Ltd., Beamsville, Ontario, Canada) consists of six stages, each of which contains a glass Petri dish under a screen with 400 holes. The number of holes is the same for each stage, but the diameter of the holes becomes smaller at each stage. At an airflow rate of 28.3 L min^{-1} provided by an external vacuum pump, the air speed through the holes increases from the first stage to the sixth stage. The bacterial particles in the air stream are differentiated according to their size and impact on the Petri dishes. From the first stage to the sixth stage, bacterial particles >7.1 μm, 4.7 to 7.1 μm, 3.3 to 4.7 μm, 2.1 to 3.3 μm, 1.1 to 2.1 μm, and 0.65 to 1.1 μm in size, respectively, are collected. Normally, a Petri dish with agar is positioned at each stage. In this study, 2.5 mL buffered peptone water (BPW, bioTRADING Benelux B.V., Mijdrecht, The Netherlands) with a gelatin filter (17528-80-ACD, Sartorius, Göttingen, Germany) was used as the collection medium in each Petri dish. This is because airborne *Campylobacter* was successfully recovered from the air in slaughterhouses by using this medium (Jacobs-Reitsma, 2002).

The OMNI-3000 (Evogen, Inc., Kansas City, Mo.) is operated at a high airflow rate of 300 L min^{-1}. It collects bacteria in 10 mL phosphate-buffered saline (PBS, Evogen, Inc., Kansas City, Mo.). Before sampling, the PBS is sucked from a cartridge into the contactor. Airflow provided by an inner pump forces the PBS to rotate and contact the incoming air in the contactor. In this way, the bacteria are trapped in the PBS. To compensate for the evaporation of PBS during sampling, sterilized water is continuously supplemented into the contactor to maintain the sample volume at 10 mL. After sampling, the PBS is drained from the contactor back to the cartridge. The cost of the OMNI-3000 instrument is $9875 U.S., plus $784 for the battery pack. The cost of each sampling run is estimated as $10 (including $7 for one PBS cartridge, and $3 for the rinsing liquid).

Sampling Schedule

The bioaerosol samplers were used for sampling for 10 to 30 min on each sampling day at a level of approximately 0.25 m above the floor in the broiler rooms. The sampling locations and dates are given in figure 1 and table 1.

Table 1. Air sampling duration, airflow rate, sample volume, location, and date in broiler rooms.

Sampler	Sampling Duration (min)	Airflow Rate (L min^{-1})	Sample Volume (mL)	Sampling Location[a]	Sampling Date (days PI)[b]
AGI-30	30	12.5	20	1, 2, 3, 4	Control[c], 1, 3, 6, 9, 14, 21, 29
Andersen	10	28.3	12.5 × 6	1	3, 9, 21[d], 29
OMNI-3000	10	300.0	10	1, 3	3, 9, 14, 21, 29

[a] See figure 1 for sampling locations. Samples from locations 1 and 3 were analyzed by PCR.
[b] Days PI = days post-inoculation.
[c] The control air sampling was taken on the day before inoculation (13-day bird age).
[d] On this day, the air samples were not analyzed by PCR.

Microbiological Analysis

After air sampling with the Andersen impactor, the gelatin filter in each Petri dish was moved to a 15 mL sterilized vial. The Petri dish was carefully washed with 10 mL of BPW, which was pipetted into the vial with the gelatin filter. These vial samples were centrifuged at 2500×*g* for 10 min. The samples from the AGI-30 and the OMNI-3000 were sent directly for analysis.

Culturable bacteria and bacterial DNA of *C. jejuni* were analyzed with the culture method and PCR test, respectively. For culturable *C. jejuni*, 1 mL of each liquid sample was used to make decimal dilutions (10^{-1} to 10^{-6}) in PSW, and 0.1 mL of each dilution was plated on a charcoal cefoperazone deoxycholate agar (CCDA) plate and incubated at 41.5°C for 48 h under micro-aerial condition (6% O_2, 10% CO_2, and 84% N_2). If colonies formed after incubation, the CFU on the agar plate (normally 30 to 300 colonies) was counted and the CFU in the liquid sample could be calculated (ISO, 2006). Another 1 mL liquid sample was added into 10 mL of Bolton broth (bioTRADING Benelux B.V., Mijdrecht, The Netherlands) for enrichment at 37°C for 4 to 6 h and then at 41.5°C for 48 h under micro-aerial conditions. The culturable *C. jejuni* count in the enriched sample was also analyzed with the culture method.

Half of the air samples from the AGI-30 (those from location 1 and 3, see fig. 1) and all samples from the Andersen impactor and the OMNI-3000 were analyzed for DNA of *C. jejuni* with the PCR test. The liquid air sample was filtrated by a polytetrafluoroethylene (PTFE) filter (pore size 0.5 μm, Sartorius, Nieuwegein, The Netherlands). The filter and the filtrated bacteria were then totally immersed in 0.4 mL lysis buffer in a sample tube and stored at -80°C before the PCR test.

Airborne Dust Sampling

The PM_{10} (particulate matter smaller than 10 μm aerodynamic diameter) and $PM_{2.5}$ were determined for 24 h with a DustTrak sampler (TSI, Inc., Shoreview, Minn.), which measures the mass concentration of aerosols using a photometric principle, in two of the four rooms on 28 days PI. Two DustTrak samplers were placed at the same level as the bioaerosol samplers at sampling location 1.

Data Analysis

The non-100% specificity of the PCR test also reported positive results for the control air samples (1 day BI), which were collected with the AGI-30. Thus, the amounts of DNA of *C. jejuni* sampled by each type of bioaerosol sampler on different sampling days were separately compared to the control samples with a non-parametric test (Mann-Whitney U-test, SPSS ver. 15, SPSS, Inc., Chicago, Ill.). The reason for using a non-parametric test was the skew distribution of the data. Positive samples were rec-

ognized as those whose mean rank values were higher than the control samples at $p < 0.05$.

Results and Discussion

Infection in Broilers

The cloacae swabs showed that all broilers in the central cages of the four experimental rooms suffered from infection within two days after orally inoculation with *C. jejuni*. This result was comparable with other studies (Beery et al., 1988; Shanker et al., 1988) in which chickens were positive at one day PI. Bacterial infection of susceptible broilers was noticed in all four rooms: six susceptible broilers in room 1 were infected between 15 and 29 days PI, one broiler in room 2 was infected at 30 days PI, one broiler in room 3 was infected at 35 days PI, and two broilers in room 4 were infected at 16 and 34 days PI (Van Bunnik et al., 2009). The infections of susceptible broilers revealed that airborne transmission of *Campylobacter* had occurred.

Detection of Airborne *C. jejuni*

The PCR test showed that DNA of *C. jejuni* was present in the air samples collected with the AGI-30 at 1 day PI (table 2). No bacterial DNA was detected in the air samples collected by the three samplers from 3 to 29 days PI, and all air samples were negative for culturable *C. jejuni*, no matter whether it was directly cultured or cultured after enrichment in broth. The different results between the PCR and culture tests show that only dead *C. jejuni* was present in the air. Moreover, the PCR test seems more sensitive to indicate airborne transmission of *Campylobacter*.

The outcome that susceptible broilers were infected, but no culturable airborne *C. jejuni* was recovered, is probably to the low concentrations of the bacteria in the air, which were below the detection limits of the three bioaerosol samplers. Five infected broilers were reared in the experimental rooms, and therefore a low amount of *C. jejuni* was shed. Only the air samples collected at 1 day PI were positive in the PCR test, and the chance of recovering culturable *C. jejuni* was even less. Some researchers have assumed that microorganisms can be transmitted by dust particles and are therefore somehow positively correlated with the airborne dust concentration (Cambra-Lopez et al., 2010). Our dust measurements showed that the average PM_{10} and $PM_{2.5}$ concentrations in the rooms were 118 and 43 $\mu g\ m^{-3}$, respectively, at 28 days PI. These concentrations were much lower than those reported for commercial poultry farms (Takai et al., 1998). Furthermore, *Campylobacter* had difficulty surviving in the air in the experimental broiler rooms (Luechtefeld et al., 1981; Doyle and Roman, 1982). The optimal temperature for *Campylobacter* multiplication is approximately 42°C (Keener et al., 2004), and the optimal temperature for preservation of viability is 4°C

Table 2. Culturable *Campylobacter jejuni* (tested by culture method) and DNA of *C. jejuni* (tested by PCR) in air samples of four replicated broiler rooms.

	C. jejuni Count (culturable/DNA)							
Sampler	Control	1 day PI	3 days PI	6 days PI	9 days PI	14 days PI	21 days PI	29 days PI
AGI-30	Neg/Neg	Neg/Pos	Neg/Neg	Neg/Neg	Neg/Neg	Neg/Neg	Neg/Neg	Neg/Neg
Andersen	-/-[a]	-/-	Neg/Neg	-/-	Neg/Neg	-/-	Neg/-	Neg/Neg
OMNI-3000	-/-	-/-	Neg/Neg	-/-	Neg/Neg	Neg/Neg	Neg/Neg	Neg/Neg

[a] Not done.

to 10°C (Buswell et al., 1998). In this experiment, the room temperature was controlled at 25°C to 30°C, which is neither within the optimal growing temperature nor within the optimal survival temperature. The relative humidity in the broiler room (55%) was lower than the preferred humidity of 98.7% for bacteria (Koop et al., 2000; NZFSA, 2001). The susceptible broilers were exposed to the bacterial atmosphere throughout the entire period. They served as "integrated samplers" for airborne *Campylobacter*. Compared to the broilers, the samplers took air samples only one time per measurement day for short period; thus, the chance of capturing bacteria was lower.

The bioaerosol samplers may fail to collect bacteria, and *Campylobacter* may lose culturability due to sampling stresses. When standard Petri dishes with agar are used, the Andersen impactor has a cut-off diameter (D_{50}, defined as the particle diameter corresponding to 50% physical collection efficiency) of 0.65 μm for the sixth stage (Andersen, 1958). In this experiment, we replaced the agar with a gelatin filter with 2.5 mL BPW because a previous study reported that airborne *Campylobacter* was successfully recovered with this medium (Jacobs-Reitsma, 2002). However, this adaptation might have increased the cutoff diameter of the Andersen impactor and thereby reduced its sampling efficiency (Andersen, 1958).

From a biological perspective, the AGI-30 seems a suitable sampler for airborne *C. jejuni* in that it collects bacteria in a liquid medium, which provides a humid environment. Zhao et al. (2011b) proved that the sampling stress of the AGI-30 on *C. jejuni* was lower compared to the OMNI-3000. In addition, the AGI-30 has a low cut-off diameter of 0.31 μm (Nevalainen et al., 1992) and therefore has a relatively high physical efficiency (Zhao et al., 2011b; Zhao et al., 2011c). The problem with the AGI-30 is that it has the lowest sampling airflow rate (12.5 L min^{-1}) among the three bioaerosol samplers. This restricts its application in environments with low bacteria concentrations.

The OMNI-3000 was originally expected to have the greatest chance of capturing *Campylobacter* because it samples the air at a high airflow rate (300 L min^{-1}), which provides 8 and 10.6 times the air volume of the AGI-30 and Andersen impactor, respectively. However, the OMNI-3000 failed to collect both culturable *C. jejuni* and its DNA from the air. This might be because of the low physical efficiency of the OMNI-3000 and its severe sampling stress on the microorganisms. In another study, only 1% of *C. jejuni* could be recovered under the high sampling stress of the OMNI-3000 (Zhao et al., 2011b).

The detection limits of the three samplers used in this experiment for culturable *C. jejuni* were calculated with the physical and biological efficiency of the samplers (Zhao et al., 2011b) and by assuming 1 CFU on the cultured Petri dish with an undiluted sample (table 3). It remains unclear whether the detection limit could be lowered by increasing the sampling duration. The possibility for capturing bacterial particles increases when the sampling duration is extended; however, the collected bacteria

Table 3. Detection limits of AGI-30, Andersen impactor, and OMNI-3000 for culturable airborne *C. jejuni*.

Sampler	Detection Limit (CFU m^{-3})	Sampling Duration (min)
AGI-30	5.3×10^2	30
Andersen	4.4×10^2	10
OMNI-3000	6.8×10^3	10

suffer sampling stress for a longer time, which may adversely affect their survival. To reach a lower detection limit, further research needs to be carried out for a suitable sampling duration.

Could airborne transmission occur by susceptible broilers inhaling particles with viable but non-culturable (VNC) *Campylobacter* (Cappelier et al., 1999)? Two additional questions need to be taken into consideration for this to be the case. First, can VNC bacteria exist in airborne particles? The existence of a VNC state for other bacteria in aerosols has been proven (Heidelberg et al., 1997). Federighi et al. (1998) also verified the presence of VNC *Campylobacter* in microcosm water with plate culturing, staining, and microscope scanning methods. However, there is currently a lack of evidence of VNC *Campylobacter* in airborne particles. Secondly, can VNC *Campylobacter* infect animals? This remains a controversial issue. Some studies have successfully infected eggs, chickens, and mice with VNC *Campylobacter* (Cappelier et al., 1999; Talibart et al., 2000). The disputable point of their findings is that the authors were not completely sure that the inoculation medium was free of viable *Campylobacter*. Other studies have failed to infect birds with VNC *Campylobacter*, which was suspended in water at room temperature for 7, 10, or 14 days (Ziprin et al., 2003). However, the conclusion that VNC inoculation did not produce infection also seems doubtful. An investigation by Thomas et al. (2002) indicated that there were no VNC, only dead bacteria, in the aqueous medium after a 7-day suspension.

Conclusion

In this experiment, none of the bioaerosol samplers could recover culturable *C. jejuni* from the air, despite the fact that the susceptible broilers were infected by the infected broilers, probably by airborne transmission. This finding is probably due to airborne bacterial concentrations that were lower than the detection limits of the bioaerosol samplers. It is therefore suggested that negative results from these bioaerosol samplers cannot ensure a safe air environment. Sampling techniques with low detection limits must be developed in order to detect low concentrations of microorganisms for biosecurity assessment.

Acknowledgements

We thank Mr. Frans Putirulan and Mr. Nico Bolder for their assistance in the analysis of bacteria, Mr. Bastiaan Vroegindeweij for helping in the sampling, and Mr. Eef Lovink for calibrating the equipments.

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