# Comparative performance of three sampling techniques to detect airborne *Salmonella* species in poultry farms

**Elisa Adell[1], Verónica Moset[2], Yang Zhao[3], Ana Jiménez-Belenguer[4], Alba Cerisuelo[2], María Cambra-López[1]**

[1] *Universitat Politècnica de València. Institute of Animal Science and Technology, Valencia, Spain*
[2] *Instituto Valenciano de Investigaciones Agrarias. Centro de Tecnología Animal, Segorbe, Castellón, Spain*
[3] *Iowa State University. Department of Agricultural and Biosystems Engineering, Iowa, USA*
[4] *Universitat Politècnica de València. Department of Biotechnology, Valencia, Spain*

## Abstract
Sampling techniques to detect airborne *Salmonella* species (spp.) in two pilot scale broiler houses were compared. Broilers were inoculated at seven days of age with a marked strain of *Salmonella enteritidis*. The rearing cycle lasted 42 days during the summer. Airborne *Salmonella* spp. were sampled weekly using impaction, gravitational settling, and impingement techniques. Additionally, *Salmonella* spp. were sampled on feeders, drinkers, walls, and in the litter. Environmental conditions (temperature, relative humidity, and airborne particulate matter (PM) concentration) were monitored during the rearing cycle. The presence of *Salmonella* spp. was determined by culture-dependent and molecular methods. No cultivable *Salmonella* spp. were recovered from the poultry houses' surfaces, the litter, or the air before inoculation. After inoculation, cultivable *Salmonella* spp. were recovered from the surfaces and in the litter. Airborne cultivable *Salmonella* spp. were detected using impaction and gravitational settling one or two weeks after the detection of *Salmonella* spp. in the litter. No cultivable *Salmonella* spp. were recovered using impingement based on culture-dependent techniques. At low airborne concentrations, the use of impingement for the quantification or detection of cultivable airborne *Salmonella* spp. is not recommended. In these cases, a combination of culture-dependent and culture-independent methods is recommended. These data are valuable to improve current measures to control the transmission of pathogens in livestock environments and for optimising the sampling and detection of airborne *Salmonella* spp. in practical conditions.

## Key words
Airborne transmission, air quality, bioaerosol, broiler housing, particulate matter

## INTRODUCTION

Airborne microorganisms are abundant in the air of livestock houses [1]. They can cause detrimental effects to the health of farmers and animals and can be responsible for infectious and non-infectious diseases [2, 3]. Although most airborne microorganisms in livestock houses are non-pathogenic, airborne pathogens can be found in small concentrations [4]. Seedorf et al. [5] reported concentrations of total airborne bacteria of 6 log colony forming units (CFU) per m³ in broiler houses, whereas the levels of *Enterobacteriaceae* (a family which includes pathogenic species) were 3 log CFU m$^{-3}$. When pathogens are zoonotic and airborne transmittable, long-distance transmission to nearby farms can occur, and the health of not only farmers but also people living near the livestock houses may be threatened [6].

In the environments in livestock houses, the biological survival of airborne microorganisms is affected by environmental conditions such as temperature, relative humidity, and ultraviolet radiation [7]. Moreover, the survival of microorganisms in air can be influenced to a large extent by airborne particulate matter (PM) [8, 9, 10] because their physical deposition is affected by particle characteristics, mainly the size of particle they attach to [11]. Although many bacteria and fungi have been recovered from airborne PM [12, 13, 14], the role of PM in the airborne transmission of specific pathogens is not fully understood.

Poultry production is a source of human pathogens such as *Salmonella* species (spp.), which are a major cause of foodborne illness throughout the world [15]. These bacteria are generally transmitted to humans through the consumption of contaminated food of animal origin, mainly meat, eggs, and milk. *Salmonella* spp. can cause adverse health effects such as fever, diarrhoea, abdominal cramps, and headache [16].

In poultry houses, *Salmonella* spp. can survive and remain viable in different reservoirs even after cleaning and disinfection [17, 18]. Several authors have isolated *Salmonella* spp. from surfaces or litter in poultry farms [19, 20, 21, 22]. Marin et al. [18] reported that farm surfaces, faeces, and settled dust were the most relevant sources of *Salmonella* spp. contamination in poultry flocks. Furthermore, *Salmonella* spp. can become airborne and remain viable in the air. Their presence in the air has been confirmed inside poultry farms [19, 23]. Additionally, it has been recognised that the airborne transmission of *Salmonella* spp. among animals over short distances can occur [23, 24]. David and Morishita [23] also recovered airborne *Salmonella* spp. 12 meters from a layer farm, thus indicating that the spread of *Salmonella* spp. to

16

Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 1

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…

the outside environment may also occur through ventilation exhausts.

Nevertheless, to determine whether a certain airborne pathogen can furthermore cause infection, not only its presence, but also its concentration in the air is necessary [25]. Research has dealt with the detection and quantification of *Salmonella* spp. in different reservoirs in poultry environments, including the air, and using several sampling techniques and culture-dependent as well as culture-independent methods such as polymerase chain reaction (PCR) [19, 20, 26, 27, 28]. Literature shows that these results can vary considerably depending on the sampling techniques and method for analysis. Moreover, although practical measures to control airborne transmission of *Salmonella* spp. in poultry environments are necessary, the behaviour of *Salmonella* spp. in the air still remains unpredictable. Furthermore, there is currently a lack of standardised techniques to detect and quantify airborne pathogens, specifically airborne *Salmonella* spp.

The problem concerns the control of airborne pathogens, which is complicated because sampling and analytical techniques have been developed and validated in other matrices, such as water [29], which differ from air, where airborne pathogens are found in low concentrations. At present, only limited efforts have been made to compare the different techniques and to apply them to livestock-derived pathogens in the air. Therefore, to improve current measures to control the transmission of pathogens in livestock environments, the performance of sampling techniques and analytical methods under different housing and environmental conditions needs to be assessed.

The objective of the presented study was to compare the performance of techniques to sample and detect airborne *Salmonella* spp. in broiler (poultry for meat production) farms. The study was conducted in two pilot scale broiler houses during a summer rearing cycle in experimentally inoculated birds. Air sampling techniques based on impaction, gravitational settling, and impingement followed by culture-dependent and molecular methods were tested. The comparison between techniques will provide insight into the advantages and disadvantages of the sampling techniques and analytical methods used to detect pathogens found at low concentrations in the air. Additionally, the relationship between airborne *Salmonella* spp. and airborne PM characteristics and the processes leading to *Salmonella* spp. becoming airborne were examined. These data will be useful to improve current control measures for pathogenic and non-pathogenic airborne bacteria inside and outside livestock houses.

## MATERIALS AND METHODS

**Experimental poultry houses and broilers.** The study was conducted in two identical poultry houses in the pilot scale broiler farm at the Animal Technology Centre (CITA-IVIA) located at Segorbe (Castellón, Spain). Each poultry house measured 13 x 6 meters. The houses were heated by a central heating system and mechanically ventilated with two ventilators suspended from the ceiling.

At the start of the rearing cycle, 288 one-day-old male broiler chicks (Hubbard) were introduced in each house. The birds were placed randomly into 24 floor group pens with an area of 1.3 $m^2$ for a pen in each house (12 pens per house and 12 animals per pen). Each pen contained wood shavings as litter to a depth of 10 centimetres. The rearing cycle lasted 42 days during the summer. Broilers had free access to feed and drinking water.

**Animal inoculation with *Salmonella* spp.** On day 7 of the rearing cycle, broilers were orally inoculated with 1 mL of a bacterial solution containing $10^8$ CFU *Salmonella enteritidis* with kanamycin resistance, a clinical isolate from faeces, wild-type mutant strain 3934 yhjL-km (Instituto Universitario de Agrobiología y Recursos Naturales y Departamento de Producción Agraria, Universidad Pública de Navarra-Consejo Superior de Investigaciones Científicas, Spain; deposited in the Spanish Type Culture Collection [CECT], Accession No. CECT 7236). It was previously confirmed that this mutant behaved like a standard *S. enteritidis* strain and that the resistance was stable. This protocol was revised and accepted by the Animal Welfare Committee of the Instituto Valenciano de Investigaciones Agrarias.

**Animal productive parameters.** Bird weight was recorded at the beginning and at the end of the rearing cycle by weighing the animals in each house. Feed consumption was recorded at the end of the rearing cycle in each house. With these data, total average daily gain, average daily feed intake, and feed conversion in each house were calculated. Mortality was supervised daily and was used in the calculation of the productive parameters.

**Environmental parameters and airborne particulate matter.** Temperature and relative humidity were recorded in each house using data loggers (HOBO U12-O13, Onset Computer Corp, Pocasset, MA, USA). Two data loggers were placed inside each house, and two were placed outside. Data were recorded at 5-minute intervals. The ventilation rate in each house was calculated using a carbon dioxide ($CO_2$) balance [30]. The $CO_2$ concentration was measured every 5-minutes inside each house, at a representative sample point near one of the two exhaust ventilators in each house, using a $CO_2$ sensor with a measurement range from 0 – 10,000 ppm (Vaisala GMT-222, Vaisala Oyj., Helsinki, Finland) coupled with a data logger (HOBO U12-O13, Onset Computer Corp, Pocasset, MA, USA). The $CO_2$ concentration of the inlet air was considered constant and equal to 350 ppm (clean air) as a result of previous measurements conducted at our installations.

Additionally, concentrations of PM10 (particles smaller than 10 μm) and PM2.5 (particles smaller than 2.5 μm) were simultaneously determined using a 'tapered element oscillating microbalance' (TEOM model 1405-D, Thermo Fisher Scientific, Franklin, MA, USA). This device operated on changes in the resonant frequency of an oscillating element as a function of increases in the particle mass collected on a filter. Changes in the recorded resonant frequency of the element provide continuous and time-averaged measurements of mass accumulation. The TEOM device was located indoors, close to the ventilation exhaust in each poultry house. Measurements were conducted at a height of 2 meters. Particulate matter concentrations were measured weekly in each house. The sampling duration was 24 hours, and recordings were stored every minute.

Average one-minute records were summarised to calculate the 24-hour PM concentrations.

**Sampling and microbiological analysis of *Salmonella* spp. on surfaces.** Prior to the arrival of the chicks, the absence of *Salmonella* spp. on the farm surfaces (floor and wall), feed, and litter was confirmed following the ISO 6579:2002 method [31].

During the rearing cycle, settled dust on surfaces was collected by means of sterile wet gauze pads (AES Chemunex, Bruz Cedex, France). Samples were collected on two days of the rearing cycle (days 23 and 37) at eight different points distributed randomly across the feeders, drinkers, and walls in each poultry house. The presence of *Salmonella* spp. in these samples was tested following the ISO 6579:2002 method [31]. Isolated colonies were further confirmed for *Salmonella* spp. using biochemical confirmation (API-20E, bioMérieux, Madrid, Spain). The same biochemical confirmation of *Salmonella* spp. was performed for all the samples that were analysed with a culture-based method in the presented study.

**Sampling and microbiological analysis of *Salmonella* spp. in the litter.** Litter was sampled weekly in each poultry house, starting on day 3 pre-infection. Litter samples were collected in each house by randomly sampling 24 spots per house to a depth of 1 – 4 cm. Samples were pooled per house, homogenised to achieve a uniform sample, stored in sterile bags and refrigerated between 4°C – 8°C until transport to the laboratory.

A 25-g aliquot of each litter sample was prepared in 225 mL of buffered peptone water. Each sample was manually shaken, and 1 mL of appropriate serial dilutions was inoculated into 9 mL of buffered peptone water. *Salmonella* spp. colonies were determined by culturing 1 mL of the continuous dilutions in duplicate brilliant green agar (BGA) (Liofichen, Roseto degli Abruzzi, Italy) plates with 50 μg mL$^{-1}$ of kanamycin (kanamycin sulphate, Sigma-Aldrich, Steinheim, Germany). Plates were incubated at 37°C for 24 h, and then CFUs were counted on plates containing between 30 – 300 colonies [33]. The colonies were further confirmed to be *Salmonella* spp. using biochemical confirmation.

Additionally, the dry matter content of the litter was determined. A sample of 80 – 100 g of litter was dried in an oven at 104°C for 24 hour according to AOAC International [32]. Dry matter analyses were conducted in triplicate per house.

**Sampling and microbiological analysis of airborne *Salmonella* spp.** The air in each poultry house was sampled weekly, on the same day, using 3 techniques: impaction, gravitational settling, and impingement. Impaction and impingement samplings were conducted within a 20-minute interval between houses. Gravitational settling was conducted simultaneously in both houses.

Air sampling by impaction was conducted with a 6-stage viable Andersen Impactor (Thermo Scientific, Franklin, MA, USA). The Andersen sampler had 6 stages, each of which consisted of a plate with agar placed under a screen with 400 holes. The diameter of the holes decreased in each successive stage. Airborne microorganisms were retained on the agar plates in different stages according to their size. From the first stage to the sixth stage, bacterial particles larger than 7 μm, from 4.7 – 7.1 μm, from 3.3 – 4.7 μm, from

2.1 – 3.3 μm, from 1.1 – 2.1 μm, and from 0.65 – 1.1 μm in size, were collected. Plates containing BGA (Liofichen, Roseto degli Abruzzi, Italy) with 50 μg mL$^{-1}$ of kanamycin (kanamycin sulphate, Sigma-Aldrich, Steinheim, Germany) were used in the Andersen sampler. The sampling airflow rate was 28.3 L min$^{-1}$. Three repetitions were conducted in the centre of each house at different heights: 10–30 cm (animal breathing height), 150 cm (human breathing height) and 200 cm (exhaust fan height). Sampling duration was 90 seconds per repetition and height. Plates were directly incubated at 37°C for 24 hours and then CFUs were counted and divided by the volume of the sampled air. Colonies were further confirmed to be *Salmonella* spp. using biochemical confirmation. Plates positively confirmed for *Salmonella* spp. were considered positive plates.

The gravitational settling technique was used to sample airborne *Salmonella* spp. across the whole house space. Gravitational settling sampled microorganisms adhered to coarse particles or particle aggregates, which settled by gravitational forces, without using forced air. Therefore, it allowed sampling for airborne *Salmonella* spp. without size discrimination (as for impaction or impingement) and for longer sampling durations, overcoming the short sampling times required for impaction and impingement. Thirty-six Petri plates with BGA (Liofichen, Roseto degli Abruzzi, Italy) and 50 μg mL$^{-1}$ of kanamycin (kanamycin sulphate, Sigma-Aldrich, Steinheim, Germany) were placed open, at 3 different heights, 12 plates per height: 10–30 cm (animal breathing height), 150 cm (human breathing height) and 200 cm (exhaust fan height). The sampling time was 24-hours. The plates were directly incubated at 37°C for 24 hours, after which the CFUs were counted. Colonies were further confirmed to be *Salmonella* spp. using biochemical confirmation. Plates positively confirmed for *Salmonella* spp. were considered positive plates.

Air sampling using liquid impingement was conducted with AGI-30 samplers (Ace Glass Co., Vineland, NJ, USA). The AGI-30 sampler worked by accelerating airborne particles through a narrow orifice placed at a fixed distance from the bottom of a flask containing a liquid. A pressure drop is created in the flask and forces the air to enter through the inlet of the impinger. The AGI-30 sampler worked with a cut-off diameter of 0.31 μm. Each sampler contained 20 mL of buffered peptone water, 0.01% of Tween, and 0.005% of anti-foam and was operated at a flow rate of 12.5 L min$^{-1}$ for 15 minutes. Sampling was performed in triplicate at a height of 1.5 meters in the centre of each house, near the exhaust air. The 3 samples were then pooled and refrigerated between 4°C – 8°C until transport to the laboratory (within 2 hours). The final volume was measured and corrected for evaporation before using culture-dependent and molecular methods.

A schematic diagram of one poultry house showing the sampling locations is provided in Figure 1. The sampling locations were the same in each house.

**Culture-dependent analysis of airborne *Salmonella* spp.** For the selective detection of *Salmonella* spp. in air samples collected using liquid impingement, 3 methods were used: serial dilutions and plating, most probable number (MPN), and the ISO 6579:2002 method. First, the sampled liquid was serially diluted 10-fold in buffered peptone water, and then 0.1-mL samples were plated onto duplicate BGA plates (Liofichen, Roseto degli Abruzzi, Italy) with 50 μg mL$^{-1}$
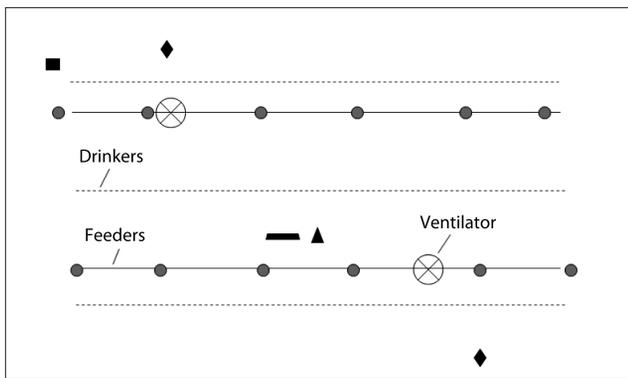
18     Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 1

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…



**Figure 1.** Schematic diagram of a poultry house showing sampling locations (■ Particulate Matter sampler (TEOM); ▬ Andersen cascade impactor; ▲ Impingers; ◯ Gravitational plates; ◆ Temperature and relative humidity sensors)

of kanamycin. Plates were incubated at 37°C for 24 hours, after which the CFUs were counted. Colony forming units were counted on plates containing between 30 – 300 colonies [33]. The concentrations of *Salmonella* spp. in the liquid samples were determined by multiplying the CFU by the dilution volume and dividing by the volume plated (0.1 mL). The concentrations of *Salmonella* spp. in the air were then calculated by introducing the volume of sampled air. Colonies were further confirmed to be *Salmonella* spp. using biochemical confirmation. Plates positively confirmed for *Salmonella* spp. were considered positive plates.

Second, a 5-tube MPN analysis was performed for *Salmonella* spp. One mL of liquid impingement was used to make decimal dilutions ($10^1 – 10^{-4}$) in buffered peptone water and incubated at 37°C for 24 hours. Aliquots of 0.1 mL from each incubated broth were inoculated onto 10 mL of Rappaport Vassiliadis (RV), followed by incubation at 42°C for 24 hours. Positive tubes were cultured onto duplicate BGA with 50 µg mL$^{-1}$ of kanamycin and xylose lysine deoxycholate agar (XLD, Difco, Le Pont de Claix, France) plates and incubated at 37°C for 24 hours. Colonies were further confirmed using biochemical confirmation (API-20E, bioMérieux, Madrid, Spain).

Third, the samples from the liquid impingement were also analysed following the ISO 6579:2002 method to confirm the presence of *Salmonella* spp.

Finally, the remaining liquid was stored under refrigeration at 4°C and then processed for DNA extraction.

**Molecular methods.** Approximately 25 mL of the liquid impingement was centrifuged at 4,200 rpm in a microcentrifuge tube for 20-minutes at 4°C. The supernatant was discarded and the pellet was suspended in 1 mL of PBS 1X (phosphate-buffered saline, 10 mM Tris HCl, 1 mM EDTA, pH 8) and stored at -20°C prior to DNA extraction. The DNA was extracted with Real Pure Genomic DNA Extraction (Durviz, Valencia, Spain). The total extracted DNA was suspended in a final volume of 100 µL and stored at -20°C.

*Salmonella* species-specific PCR primers, ST11 (5'-AGCCAACCATTGCTAAATTGGCGCA-3') and ST15 (5'- GGTAGAAATTCCAGCGGGTACTG-3'), purchased at Roche Diagnostics (Roche Diagnostics, Mannheim, Germany) and published previously by Aabo et al. [34], were used to amplify a 429-bp fragment. The PCRs were performed in a PTC°-100 thermocycler (BioRad, Hercules,

CA, USA). A 25-µL PCR mixture contained the following concentrations of the reagents: 0.4 µM of each primer, 200 µM of each dNTP (Bioline, London, UK), 1X PCR buffer (20 mM Tris-HCL[pH 8,4], 50 mM KCl), 1.5 mM MgCl$_2$ 0.75 U BIOTAQ™ polymerase (Bioline, London, UK), and 5 µL of sample DNA. The incubation conditions were 95°C for 1 minute, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. A final extension of 72°C for 4 minutes was used. The PCR products were visualised by agarose gel electrophoresis. Four repetitions of the PCR analyses of the extracted DNA were conducted. The sample was considered positive as long as one repetition was positive.

A summary of the sampling techniques used and the analytical method to detect *Salmonella* spp. during the rearing cycle is presented in Table 1.

**Table 1.** Summary of evaluated parameters, sampling techniques, description of sampling, and analytical methods used in the study to detect *Salmonella* spp. per sampling event. Sampling frequency was weekly for all parameters

| Parameter | Sampling technique (sampling points) | No. of samples per house and sampling event | Sampling duration | Airflow (L min$^{-1}$) | Analytical method |
|---|---|---|---|---|---|
| *Salmonella* spp. on surfaces | Sterile wet gauze pads (8 points) | 1 | - | - | ISO 6579:2002 |
| *Salmonella* spp. in litter | Litter sampling (24 points) | 3 | - | - | Direct count (dilutions) |
| Airborne *Salmonella* spp. | Impaction (3 points) | 18 | 90 s | 28.3 | Direct count |
| | Gravitational settling (36 points) | 36 | 24 h | - | Direct count |
| | Impingement (1 point) | 3 | 15 min | 12.5 | Direct count (dilutions) |
| | | | | | Most Probable Number |
| | | | | | ISO 6579:2002 |
| | | | | | Polymerase Chain Reaction |

**Data analyses.** All data were summarised and analysed per house and week. The airborne distribution of *Salmonella* spp. obtained with gravitational plates was compared among the different heights using analysis of variance with SAS software [35], comparing the average *Salmonella* spp. counts per height, house, and week using the Tukey test with a significance level of 5%.

The detection limits for each culture-dependent sampling technique were calculated assuming a single CFU in the agar plate considering the sampler's airflow and the sampling duration [36]. For impingement, the detection limit was calculated from the total volume of the liquid plated.

The relationship between *Salmonella* spp. concentration in the litter and in the air (using gravitational settling) with the productive and environmental parameters was investigated using Pearson's correlation coefficient for the whole sampling period with SAS software [35].

Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 1

19

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…

## RESULTS

**Productive and environmental parameters.** The animals performed similarly in each poultry house. The average productive parameters are shown in Table 2.

**Table 2.** Average daily gain (ADG), average daily feed intake (ADFI), and feed conversion in each poultry house during broiler rearing cycle

| House | ADG (g day$^{-1}$) | ADFI (g day$^{-1}$) | Feed conversion (g feed g weight$^{-1}$) |
|---|---|---|---|
| 1 | 63.2±3.4 | 103.7±4.5 | 1.64±0.03 |
| 2 | 67.6±2.2 | 110.5±3.0 | 1.63±0.04 |

Ventilation increased throughout the rearing cycle and varied from 0.04 – 1.56 m$^3$ h$^{-1}$ animal$^{-1}$ (house 1), and from 0.04 – 1.09 m$^3$ h$^{-1}$ animal$^{-1}$ (house 2). Outdoor temperature varied from 16.2°C – 27.1°C and outdoor relative humidity varied from 38.3% – 72.5%. Average indoor temperature varied from 24.5°C – 31.2°C in house 1 and from 24.5°C – 30.7°C in house 2. Average indoor relative humidity varied from 24.3% – 72.6% in house 1 and from 27.3% – 71.1% in house 2.

Average concentration of PM during the whole cycle for both houses was 0.019±0.008 mg m$^{-3}$ for PM2.5 and 0.189±0.104 mg m$^{-3}$ for PM10. Both PM2.5 and PM10 concentrations generally increased during the rearing cycle in both houses. The maximum PM2.5 concentrations registered during the whole cycle were 0.082 mg m$^{-3}$ for house 1 and 0.079 mg m$^{-3}$ for house 2. The maximum PM10 concentrations were 1.14 mg m$^{-3}$ for house 1 and 1.79 mg m$^{-3}$ for house 2 (data not shown). Weekly averages for indoor and outdoor temperature and relative humidity and the indoor PM2.5 and PM10 concentrations during the rearing cycle in each house are shown in Table 3.

**Table 3.** Average outdoor temperature (out T) and relative humidity (out RH), indoor T (in T) and RH (in RH), PM2.5 and PM10 concentration, and standard deviation in each poultry house during broiler rearing cycle

| Day | House | out T (°C) | out RH (°C) | in T (°C) | in RH (%) | PM2.5 (mg m$^{-3}$) | PM10 (mg m$^{-3}$) |
|---|---|---|---|---|---|---|---|
| 3 | 1 | 18.7 ±4.2 | 56.5 ±9.0 | 33.0±0.9 | 33.6±3.2 | 0.007±0.002 | 0.045±0.014 |
| | 2 | | | 30.8±3.2 | 45.8±8.7 | 0.009±0.009 | 0.051±0.030 |
| 10 | 1 | 21.0 ±4.9 | 65.9 ±11.9 | 32.5±0.6 | 41.6±3.1 | 0.013±0.010 | 0.091±0.123 |
| | 2 | | | 29.6±0.1 | 46.0±3.2 | 0.011±0.005 | 0.107±0.064 |
| 17 | 1 | 26.4 ±5.4 | 45.8 ±10.7 | 29.1±1.4 | 45.6±3.4 | 0.019±0.011 | 0.270±0.208 |
| | 2 | | | 28.4±1.0 | 47.6±4.3 | 0.019±0.007 | 0.179±0.123 |
| 24 | 1 | 26.2 ±5.2 | 54.1 ±12.2 | 27.7±1.9 | 60.2±4.4 | 0.022±0.010 | 0.263±0.169 |
| | 2 | | | 28.4±2.4 | 54.6±4.7 | 0.024±0.014 | 0.337±0.250 |
| 31 | 1 | 25.5 ±4.0 | 63.5 ±8.8 | 25.3±1.4 | 77.0±5.8 | 0.021±0.008 | 0.178±0.152 |
| | 2 | | | 26.2±1.6 | 77.0±3.9 | 0.030±0.011 | 0.300±0.287 |
| 38 | 1 | 25.5 ±3.7 | 64.3 ±11.5 | 24.6±0.9 | 64.0±6.1 | 0.023±0.021 | 0.304±0.287 |
| | 2 | | | 25.6±0.7 | 80.0±2.0 | 0.028±0.012 | 0.153±0.090 |

**Salmonella spp. on surfaces.** Before the arrival of the birds, analyses of the farm facilities (floor and wall), feed, and litter resulted in the absence of *Salmonella* spp. in the facilities.

The settled dust that was collected on surfaces (feeders, drinkers, and walls) using sterile wet gauze pads on days 23 and 37 of the rearing cycle was positive for *Salmonella* spp. in both days and houses.

**Salmonella spp. in the litter.** *Salmonella* spp. in the litter was not detected on day 3 of the rearing cycle, previous to the experimental infection. After the experimental infection, *Salmonella* spp. was detected and quantified in both houses, showing no clear pattern along the rearing cycle. Ten days post-infection (day 17 of the rearing cycle), the levels of *Salmonella* spp. in the litter were equal to 4.4 log CFU g$^{-1}$ (house 1) and 3.2 log CFU g$^{-1}$ (house 2). Overall, the concentrations of *Salmonella* spp. in the litter ranged from 3 log CFU g$^{-1}$ – 4.6 log CFU g$^{-1}$. Figure 2 shows the evolution of log CFU of *Salmonella* spp. per g of litter throughout the rearing cycle in each poultry house.
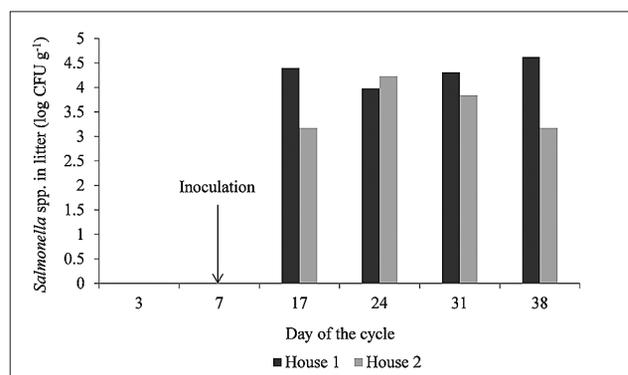


**Figure 2.** *Salmonella* spp. counts in the litter (log CFU g$^{-1}$ l) during the cycle in each poultry house

The dry matter content of the litter decreased during the rearing cycle in both houses. The dry matter percentage varied from 86% (day 3 of the rearing cycle) to 69% (day 31 of the rearing cycle) in house 1, and from 85% to 61% in house 2. Dry matter values were similar between houses (data not shown).

**Airborne Salmonella spp.** Differences in the detection of airborne *Salmonella* spp. were recorded using the impaction, gravitational settling, and impingement sampling techniques.

By means of impaction using the Andersen cascade impactor, positive samples for *Salmonella* spp. were only randomly detected at the end of the cycle (days 24 and 38) in house 2, in size ranges between 0.65 – 1 μm (1.97 log CFU m$^{-3}$ at 200 cm), 3.3 – 4.7 μm (1.38 log CFU m$^{-3}$ at 150 cm), and 7 μm or higher (1.38 log CFU m$^{-3}$ at 150 cm). No positive samples for *Salmonella* spp. were observed in house 1 using impaction. The calculated detection limit of the Andersen cascade impactor (90-seconds sampling duration) was 1.38 log CFU m$^{-3}$.

Using gravitational plates at different heights, *Salmonella* spp. were detected towards the end of the rearing cycle only on day 38 (house 1) and on days 24 and 38 (house 2). Nevertheless, the analysis and counting of the gravitational plates was complicated due to the accumulation of dirt during the 24-hours of exposure, and the overgrowth of other Gram-negative bacteria (e.g., coliforms) that differed from *Salmonella* spp. in the culture plates. For these reasons, only those results corresponding to days 3, 24, and 38 of the rearing cycle are shown (Table 4). The results are shown as a percentage of *Salmonella* spp.-positive plates out of 12 plates used in each of the sampled heights, per sampling day and house. In the first 3 weeks of the rearing cycle, *Salmonella* spp. were not detected in either of the two houses. *Salmonella*'s

20

Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 1

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…

prevalence increased at the end of the cycle, and house 2 presented a higher percentage (64%) of positive plates than house 1 (11%). No statistically significant differences among heights were observed per house.

**Table 4.** Percentage of positive plates of *Salmonella* spp. using gravitational settling, and p-values at different heights per day in the cycle, by poultry house and height

| House | Days of the cycle | | Height | No. of samples | No. of *Salmonella*-positive plates (%) | p-value |
|---|---|---|---|---|---|---|
| 1 | Before inoculation | 3 | 30 cm | 12 | 0 | - |
| | | | 150 cm | 12 | 0 | |
| | | | 200 cm | 12 | 0 | |
| | Post-inoculation | 24 | 30 cm | 12 | 0 | - |
| | | | 150 cm | 12 | 0 | |
| | | | 200 cm | 12 | 0 | |
| | | 38 | 30 cm | 12 | 1 (8.3%) | 0.140 |
| | | | 150 cm | 12 | 0 | |
| | | | 200 cm | 12 | 3 (25%) | |
| 2 | Before inoculation | 3 | 30 cm | 12 | 0 | - |
| | | | 150 cm | 12 | 0 | |
| | | | 200 cm | 12 | 0 | |
| | Post-inoculation | 24 | 30 cm | 12 | 0 | 0.140 |
| | | | 150 cm | 12 | 3 (25%) | |
| | | | 200 cm | 12 | 1 (8.3%) | |
| | | 38 | 30 cm | 12 | 7 (58.3%) | 0.887 |
| | | | 150 cm | 12 | 8 (66.7 %) | |
| | | | 200 cm | 12 | 8 (66.7%) | |

No *Salmonella* spp. were detected by liquid impingement during the whole cycle in any house by culture-dependent methods using dilutions and plating, MPN, or the ISO 6579:2002 method. Using impingement, the calculated detection limit (15-minute sampling duration) was 3.48 log CFU m$^{-3}$.

Positive results for *Salmonella* spp., however, were obtained using PCR. Table 5 presents the results of samples from the AGI-30 analysed by PCR in both poultry houses during the cycle, showing a positive detection of *Salmonella* spp. in all samples, except for day 24 of the rearing cycle in house 1. Samples were positive on day 3 (pre-infection). Figure 3 shows the expected PCR products visualised by agarose gel electrophoresis.

**Table 5.** Positive (+) and negative (-) results for *Salmonella* spp. detection using PCR for 4 repetitions from impingement samples

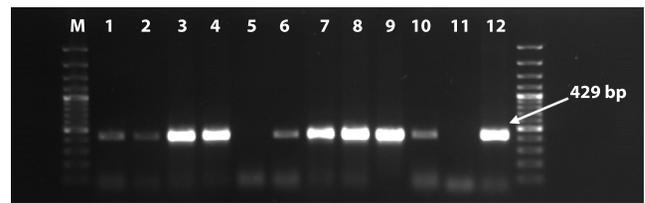| Days of the cycle | | House | *Salmonella* spp. detection |
|---|---|---|---|
| Before inoculation | 3 | 1 | + |
| | | 2 | + |
| Post-inoculation | 17 | 1 | + |
| | | 2 | + |
| | 24 | 1 | - |
| | | 2 | + |
| | 31 | 1 | + |
| | | 2 | + |
| | 38 | 1 | + |
| | | 2 | + |



**Figure 3.** PCR amplification profiles. Lane 1-10: houses 1 and 2 on different days of rearing cycle; Lane 11: negative control; Lane 12; positive control 3934 yhjL-km, kanamycin-resistant strain; M lane contains the 100-bp molecular size ladder marker

The correlation coefficients between *Salmonella* spp. concentration in the litter and in the air (using gravitational settling) and productive and environmental parameters were low (data not shown), except for ambient relative humidity and airborne *Salmonella* spp. which showed the strongest correlation (correlation coefficient equal to 0.80, P=0.06). Airborne *Salmonella* spp. also correlated fairly well with the dry matter content of the litter (correlation coefficient equal to 0.72, P=0.11), with PM2.5 (correlation coefficient equal to 0.64, P=0.17), and with animal weight (correlation coefficient equal to 0.69, P=0.12). A negative correlation was found between ambient temperature and concentration of airborne *Salmonella* spp. (correlation coefficient equal to -0.55, P=0.25). The concentration of *Salmonella* spp. in the litter showed no strong correlation with any of the measured environmental or productive parameters (correlation coefficients below 0.42). The correlation between *Salmonella* spp. concentration in the litter and *Salmonella* spp. concentration in the air was -0.82 (P=0.18).

## DISCUSSION

Our results revealed differences using three sampling techniques and two analytical methods to detect airborne *Salmonella* spp. in a broiler farm. Experimentally inoculated birds released *Salmonella* spp. in variable amounts during the rearing cycle, which could be detected along the experimental period mainly in litter and dust reservoirs. *Salmonella* spp. have been reported to survive desiccation better than other *Enterobacteriaceae* [37]. It can survive in old fan dust up to 30 weeks after depopulation in poultry houses [38], and in litter, dry faeces, and feed, it can survive up to 26 months after depopulation [20]. Its behaviour in the air, however, remains unpredictable. Its survival in the air is probably different from other more appropriate substrates and may lead to nutrient stress and shock.

During the broiler rearing cycle, with regards to the animal productive parameters, these were generally found within the upper ranges of other studies [39, 40]. This could be due to the controlled environmental conditions in this study conducted in a pilot scale broiler house. Environmental parameters, such as ventilation rates and outdoor temperature and relative humidity, were typical of summer conditions in the study area.

During the experimental period, the detection of airborne cultivable *Salmonella* spp. occurred towards the end of the rearing cycle, coinciding with the highest ventilation rates, the highest airborne PM2.5 and PM10 concentrations, and the highest indoor relative humidity, along with the lowest indoor temperatures. We found a strong correlation between airborne *Salmonella* spp. and ambient relative humidity. Research has reported that primarily temperature and relative

Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 1

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…

21

humidity affect the survival of airborne microorganisms and that temperatures above 24°C can decrease airborne bacterial survival [41]. Additionally, Zhao et al. [42] reported that airborne microorganisms were associated with the PM. A higher PM concentrations could have enhanced bacteria's growth, although we only found a small correlation between airborne *Salmonella* spp. and PM2.5. Adell et al. [8] reported that airborne mesophilic bacteria were generally attached to particles from 3.3 to >7 μm. These authors reported increasing PM and airborne mesophilic bacteria concentrations in the air in a broiler farm as a function of time, showing a high correlation coefficient (0.78 – 0.89) between both variables. In our study, however, airborne *Salmonella* spp. were randomly found attached to particles from 0.65 to > 7 μm in diameter.

Before the infection of the chicks, no cultivable *Salmonella* spp. were recovered from the poultry houses' surfaces or in the litter, indicating that the experimental inoculation was probably the only source of cultivable *Salmonella* spp. Recovery of cultivable *Salmonella* spp. on settled dust collected using sterile wet gauze pads from the houses' surfaces, was positive in all samples taken post-infection. These results are also in accordance with Marin et al. [18] who identified settled dust collected on surfaces as a relevant risk of *Salmonella* spp. contamination among poultry flocks.

Positive samples for *Salmonella* spp. were first obtained in the litter ten days post-infection (day 17 of the rearing cycle). Chinivasagam et al. [19] obtained similar concentrations of *Salmonella* spp. in broiler litter in a commercial broiler house. *Salmonella* spp. in the litter was probably a result of bacterial shedding in faeces by inoculated birds. Animal faeces found in the litter have been reported to be one of the main sources of pathogens in the air in livestock houses [19, 21]. Furthermore, particles from broiler excreta have been identified as one of the major sources of airborne fine and coarse PM in broiler houses [43].

There was a delay in the detection of *Salmonella* spp. in the air compared with the litter of one or two weeks, depending on the poultry house. This could possibly be explained by the time needed for the faeces in the litter to dry, disintegrate, and become airborne. Although we found no correlation between dry matter content of the litter and *Salmonella* spp. concentration in the litter as described by Hayes et al. [44]; when litter is dry it is more prone to becoming airborne as a consequence of increased ventilation rate or birds movement [9]. In fact, we found strong correlations between airborne *Salmonella* spp. and dry matter litter content as well as with *Salmonella* spp. in the litter. Therefore, our results suggest that the airborne process may take some time and that the excretion of *Salmonella* spp. from inoculated chicks occurs earlier than its presence in the air. Adell et al. [8] reported a higher bacterial concentration of airborne mesophilic bacteria near the litter than at higher levels at the beginning of a broiler rearing cycle. Our results, however, showed no differences in *Salmonella* spp. distribution in the poultry house space. In practical conditions, the detection of infected animals, as well as the detection of *Salmonella* spp. in the litter above certain thresholds, as shown in our results, can be considered a surrogate indicator of possible air contamination and a useful preventive measure of airborne transmission. Nevertheless, further research is necessary to better understand the processes leading to airborne *Salmonella* spp. under practical conditions in non-inoculated, *Salmonella* spp.-free birds.

The different air sampling techniques used in this study to detect airborne *Salmonella* spp. were impaction, gravitational settling, and impingement. The differences in the results among techniques could be explained by differences related to the sampling technique used: (i) the use of forced air and direct impaction on agar or not; (ii) the cut-off diameter of each sampling device (i.e., the size of sampled particles); and (iii) their detection limits.

The Andersen impactor and impingers used forced air, whereas gravitational settling plates worked without forced air. Moreover, using impaction and gravitational settling, air impacted directly onto the agar, reducing problems associated with sample processing in the laboratory compared with impingement, where air was sampled into liquid media and then transferred onto agar. The Andersen impactor could discriminate between particle sizes of 0.65 μm in diameter up to a maximum of 7 μm in diameter. Gravitational settling plates, however, sampled all airborne microorganisms adhered to coarse particles that can settle by gravity and probably large particle aggregates as well. Impingement had a cut-off diameter of 0.31 μm.

These differences related to the sampling techniques used resulted in cultivable *Salmonella* spp. being positive using impaction and gravitational settling in some cases and negative using impingement. Nevertheless, using impaction, *Salmonella* spp. were only recovered in a few samples at the end of the rearing cycle. Using impingement, no cultivable *Salmonella* spp. were recovered by the different culture-dependent methods during the experimental period. The results show that the performance of the sampling techniques can be improved when the sampling devices sample directly onto agar (i.e., impaction or gravitational settling). The sampling performance and collection efficiency using the impingement technique could be affected by the sampling stress caused when cells are accelerated in the nozzle at high velocities (equal to 313 m s$^{-1}$) [45] and particles bounce and re-aerosolise from the liquid [46]. This could result in a loss of culturability and reduced collection efficiency. Additionally, the survival of *Salmonella* spp. in the impingement liquid and the competition between other bacteria (in peptone water), together with the nutrient stress and shock caused by *Salmonella* spp. inhabiting the air, could also explain these unexpected results. The manipulation and processing of the liquid impingement in the laboratory may also influence the detection of cultivable *Salmonella* spp. Using impingement, Brooks et al. [47] reported the difficulties in isolating *Salmonella* spp. from air samples. These authors could only isolate *Salmonella* spp. once from 38 impinger samples in a commercial broiler house, although *Salmonella* spp. were quantified in the litter.

Although most common airborne microorganism sampling techniques involve filtration, impaction and/or impingement [7], our results show that no sampling approach can be considered universally suitable for *Salmonella* spp. Therefore, although air sampling by impingement has been recognised as an appropriate sampler for assessing other airborne microorganisms, it has not been fully validated for airborne *Salmonella* spp. According to our results, and in agreement with Brooks et al. [47], the use of impingement for quantification or detection of cultivable airborne *Salmonella* spp. is not recommended.

With regards to the sampler detection limits, these could also partly explain the controversial results among sampling

22     Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 1

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…

techniques. In our study, the detection limit calculated for impaction showed better sensitivity (1.38 log CFU m$^{-3}$) than using impingement (3.48 log CFU m$^{-3}$). Therefore, when reporting negative results, the lowest sampler detection limit should be considered.

In addition to intrinsic sampling characteristics, differences in results among techniques could also be explained by intrinsic microbiological factors, such as shifts between modes of survival and competition with other microorganisms. The concentration of airborne microorganisms could be underestimated with culture-dependent methods because airborne bacteria may utilise survival strategies, such as the formation of biofilms, resistance to low water activity, rugose formation, and entry into a viable but non-culturable (VBNC) state [48], in which viable bacteria have lost their ability to form colonies in a reversible process. Additionally, interferences and competition with other microorganisms can occur, especially when airborne pathogen concentrations are low [49]. In inoculated animals, Lever and Williams [50] reported airborne cultivable *Salmonella* spp. concentrations to be relatively low at 1 log CFU m$^{-3}$. Therefore, such low concentrations could favour the growth of competitors of *Salmonella* spp. Consequently, culture methods can greatly underestimate the real populations of pathogenic bacteria and their health threat to workers and animals [51].

For these reasons, most authors have used more sensitive laboratory methodologies to detect pathogens in the air, such as the ISO 6579:2002 presence and absence technique or the semi-quantitative technique of MPN [23, 52]. These techniques, however, are qualitative and are not valid for quantifying microorganisms. In our study, using the ISO 6579:2002 presence and absence technique and the semiquantitative technique of MPN, *Salmonella* spp. were not detected in impingement samples. Eriksson and Aspan [26] affirmed that the qualitative ISO 6579:2002 presence and absence technique was the most sensitive and specific method among presence/absence, PCR or ELISA to detect *Salmonella* spp. in faeces. The fact that the prevalence of *Salmonella* spp. in the litter is higher than in the air [19, 53] could explain such differences. In practice, other authors also reported difficulties in detecting airborne *Salmonella* spp. using culture-dependent methods in poultry farms when other airborne pathogens, such as *Escherichia coli*, were detected in concentrations ranging from 2 to 5 log CFU m$^{-3}$ [19]. Nevertheless, the use of standardized ISO technique in this study, provides the possibility to further compare culture quantification between institutions and researchers.

When attempting to detect pathogens in the air, such as *Salmonella* spp., the use of PCR can provide rapid and sensitive results [54]. The detection limit of PCR is lower than that of culture-dependent techniques because it can detect a single cell in the sample aliquot [54]. Although our results were obtained using conventional PCR and real-time PCR is considered the gold standard nowadays, conventional PCR proved to be sufficiently sensitive in this study at the tested concentrations. The results using PCR analyses of the liquid impingement in this study demonstrated the presence of *Salmonella* spp. in the air, contrary to the results obtained with culture-dependent methods. Zhao et al. [55] obtained similar results with airborne *Campylobacter*. These authors did not detect airborne *Campylobacter* by culture-dependent methods, but they obtained positive results using PCR. Furthermore, Hospodsky et al. [56] reported that the

accuracy, precision, and method detection limits of real-time PCR for airborne microorganisms are influenced by several factors during the sampling, DNA extraction, and analytical phases.

In addition to the advantages of molecular methods, analytical methods to detect airborne pathogens based on PCR can have drawbacks related to their limited ability to provide information on pathogen viability and ability to cause infection. When monitoring airborne pathogens, an assessment of viability to investigate whether they pose a threat to human or animal health is necessary [57]. Bacterial pathogens are able to infect animals and humans, but molecular methods cannot easily differentiate between viable and dead pathogens [57], and in our study, some samples were positive for *Salmonella* spp. prior to bird inoculation. Stojek et al. [58] in a study to detect *Legionella* spp. in water concluded that PCR cannot be a substitute for the culture methods, nonetheless it could be regarded as an useful complementary method. Although the analyses of *Salmonella* spp. in farm facilities, feed, bedding, and animals before the arrival of the animals were all negative for cultivable *Salmonella* spp., *Salmonella* spp. from a previous flock could have remained in VBNC form, or bacterial DNA from dead cells could also be detected.

Moreover, the presence of a certain pathogen does not necessarily mean infection will occur. For infection to occur, a human must be exposed to a pathogen's infective dose (the amount that will cause 50% of exposed individuals to suffer illness) [25]. The infective dose for *Salmonella* spp. has been reported to range between $10^3$ and $10^5$ organisms, being dependent upon the strains used, and the age and physical condition of the individuals [16, 59].

Overall, Table 6 summarises the detection results using the different sampling techniques and analytical methods in this study, and it presents recommendations for optimising the sampling and detection of airborne *Salmonella* spp. in practical conditions. Although the use of gravitational settling was complicated in this study, it can still be recommended for viability assessment in combination with other culture-dependent method (i.e. impaction) because they are simple and easy to use and can sample during long periods (hours). From our results, overall recommendations include a combination of culture-dependent and culture-independent methods to overcome the limitations of a single method.

## CONCLUSIONS

We evaluated the performance of air sampling techniques based on impaction, gravitational settling, and impingement, followed by culture-dependent and molecular methods to detect airborne *Salmonella* spp. in experimentally inoculated birds in two pilot scale broiler houses. Our results revealed differences using three sampling techniques and two analytical methods and that no sampling approach is universally suitable for airborne *Salmonella* spp. These data are valuable to improve current measures to control the transmission of pathogens in livestock environments. From our results, we can conclude the following:

- During the experimental period, the detection of airborne *Salmonella* spp. occurred towards the end of the rearing cycle (from day 24 onwards). The environmental conditions

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…

**Table 6.** Detection results (positive, + or negative, -) and recommendations for sampling and detecting airborne *Salmonella* spp. according to techniques and analytical sampling methods used in the study

| Parameter | Technique | Sampling duration | Airflow (L min⁻¹) | Collection medium | Analytical method | *Salmonella* spp. detection | Recommendation |
|---|---|---|---|---|---|---|---|
| **Before arrival of chicks** | | | | | | | |
| *Salmonella* spp. on water, feed, and litter | 1 mL sample for water 25 g sample for feed and litter | - | - | - | Culture- dependent ISO 6579:2002 | - | Viability assessment. |
| **After arrival of chicks, weekly** | | | | | | | |
| *Salmonella* spp. on surfaces | Sterile gauze pads | - | - | - | Culture- dependent ISO 6579:2002 | + | Viability assessment. |
| *Salmonella* spp. in litter | 25 g sample | - | - | - | Culture- dependent Direct count (dilutions) | + | Viability assessment. Could serve as indicator of presence in the air. |
| Airborne *Salmonella* spp. | Impaction | 90s | 28.3 | Agar | Culture- dependent Direct count | + | Viability assessment |
| | Gravitational settling | 24 h | - | Agar | Culture- dependent Direct count | + | Viability assessment |
| | Impingement | 15 m | 12.5 | Liquid | Culture- dependent Direct count (dilutions) | - | Not recommended |
| | | | | | Most Probable Number | - | Not recommended |
| | | | | | Culture- dependent ISO 6579:2002 | - | Not recommended |
| | | | | | Culture-independent Polymerase Chain Reaction | + | Detection combined with culture-dependent method for viability assessment |

at the end of the rearing cycle could have positively influenced bacteria survival and growth, especially ambient relative humidity, litter dry matter content, and PM2.5 concentration. Airborne *Salmonella* spp. were randomly found attached to particles ranging from 0.65 to > 7 μm in diameter.

- There was a delay of one or two weeks in the detection of *Salmonella* spp. in the air compared with in faeces (litter). Further research, however, is necessary to better understand the processes leading to *Salmonella* spp. becoming airborne under practical conditions in non-inoculated, *Salmonella* spp.-free birds.
- Positive samples for airborne cultivable *Salmonella* spp. were obtained by sampling directly onto agar (i.e., impaction or gravitational settling), while samples were negative using impingement. At low airborne concentrations, the use of impingement for the quantification or detection of cultivable airborne *Salmonella* spp. is not recommended.
- A combination of culture-dependent and culture-independent methods is recommended to prevent undetected pathogen concentrations; however, when monitoring airborne pathogens, an assessment of viability to investigate whether they pose a threat to human or animal health is necessary.

## Acknowledgements

## REFERENCES

1. Dungan RS. Board-invited review: Fate and transport of bioaerosols associated with livestock operations and manures. J Anim Sci. 2010; 88: 3693–3706.
2. Bonlokke JH, Meriaux A, Duchaine C, Godbout S, Cormier Y. Seasonal variations in work-related health effects in swine farm workers. Ann Agric Environ Med. 2009; 16: 43–52.
3. Donham KJ, Cumro D, Reynolds SJ, Merchant JA. Dose-response relationships between occupational aerosol exposures and cross-shift declines of lung function in poultry workers: Recommendations for exposure limits. J Occup Environ Med. 2000; 42: 260–269.
4. Zucker BA, Trojan S, Muller W. Airborne gram-negative bacterial flora in animal houses. J Vet Med B Infect Dis Vet Public Health. 2000; 47: 37–46.
5. Seedorf J, Hartung J, Schroder M, Linkert KH, Phillips VR, Holden MR, et al. Concentrations and emissions of airborne endotoxins and microorganisms in livestock buildings in Northern Europe. J Agr Eng Res. 1998; 70: 97–109.
6. Radon K, Schulze A, Ehrenstein V, van Strien RT, Praml G, Nowak D. Environmental exposure to confined animal feeding operations and respiratory health of neighboring residents. Epidemiol. 2007; 18: 300–308.
7. Cox CS, Wathes C. Bioaerosols handbook. C CRC Press. Boca Raton, Florida, U.S.A, 1995.
8. Adell E, Moset V, Zhao Y, Cerisuelo A, Cambra-López M. Concentración, distribución espacial y por tamaño de bacterias aerobias mesófilas en el aire de granjas de broilers. ITEA-Información técnica económica agraria. 2011; 107: 77–93.
9. Cambra-López M, Aarnink AJA, Zhao Y, Calvet S, Torres AG. Airborne particulate matter from livestock production systems: A review of an air pollution problem. Environ Pollut. 2010; 58: 1–17.
10. Nimmermark S, Lund V, Gustafsson G, Eduard W. Ammonia, dust and bacteria in welfare-oriented systems for laying hens. Ann Agric Environ Med. 2009; 16: 103–113.
11. Just N, Duchaine C, Baljit S. An aerobiological perspective of dust in cage-housed and floor-housed poultry operations. J Occup Med Toxicol. 2009; 4: 13.
12. Andersson AM, Weis N, Rainey F, Salkinoja-Salonen MS. Dust-borne bacteria in animal sheds, schools and children's day care centres. J Appl Microbiol. 1999; 86: 622–634.
13. Curtis SE, Drummond JG, Kelley KW, Grunloh DJ, Meares VJ, Norton HW, Jensen AH. Diurnal and annual fluctuations of aerial bacterial and dust levels in enclosed swine houses. J Anim Sci. 1975; 41: 1502–1511.

24 Annals of Agricultural and Environmental Medicine 2014, Vol 21, No 1

Elisa Adell, Verónica Moset, Yang Zhao, Ana Jiménez-Belenguer, Alba Cerisuelo, María Cambra-López. Comparative performance of three sampling techniques…

14. Martin WT, Zhang Y, Willson P, Archer TP, Kinahan C, Barber EM. Bacterial and fungal flora of dust deposits in a pig building. Occup Environ Med. 1996; 53: 484–487.

15. WHO. (2011). World Health Organization. Health topics. *Salmonella*. http://www.who.int/topics/salmonella/en/ (acces: 2012.06.12)

16. Blaser MJ, Newman LS. A review of human salmonellosis.1. Infective dose. Rev Infect Dis. 1982; 4: 1096–1106.

17. Davies RH, Breslin M. Observations on *Salmonella* contamination of commercial laying farms before and after cleaning and disinfection. Vet Rec. 2003; 152: 283–287.

18. Marin C, Hernandiz A, Lainez M. Biofilm development capacity of *Salmonella* strains isolated in poultry risk factors and their resistance against disinfectants. Poult Sci. 2009; 88: 424–431.

19. Chinivasagam HN, Tran L, Maddock L, Gale A, Blackall PJ. Mechanically ventilated broiler sheds: a possible source of aerosolized *Salmonella*, *Campylobacter*, and *Escherichia coli*. Appl Environ Microbiol. 2009; 75: 7417–7425.

20. Davies, R. H., and M. Breslin. Persistence of *Salmonella* Enteritidis Phage Type 4 in the environment and arthropod vectors on an empty free-range chicken farm. Environ Microbiol. 2003; 5: 79–84.

21. Mallinson ET, De Rezende CE, Tablante NL, Carr LE, Joseph SW. A management technique to identify prime locations of *Salmonella* contamination on broiler and layer farms. J Appl Poultry Res. 2000; 9: 364–370.

22. Wales A, Breslin M, Davies R. Semiquantitative assessment of the distribution of *Salmonella* in the environment of caged layer flocks. J Appl Microbiol. 2006; 101: 309–318.

23. Davis M, Morishita TY. Relative ammonia concentrations, dust concentrations, and presence of *Salmonella* species and *Escherichia coli* inside and outside commercial layer facilities. Avian Dis. 2005; 49(1): 30–35.

24. Oliveira CJB, Carvalho LFOS, Garcia TB. Experimental airborne transmission of *Salmonella* Agona and *Salmonella* Typhimurium in weaned pigs. Epidemiol Infect. 2006; 134(1): 199–209.

25. Blackall PJ, Chinivasagam HN, Ristovski Z. Evaluating risks posed by pathogen and dust emissions from meat chicken sheds. Rural Industries Research and Development Corporation, Australia, 2010.

26. Eriksson E, Aspan A. Comparison of culture, ELISA and PCR techniques for *Salmonella* detection in faecal samples for cattle, pig and poultry. BMC Vet Res. 2007; 3: 21.

27. Gradel KO, Jorgensen JC, Andersen JS, Corry JEL. Laboratory heating studies with *Salmonella* spp. and Escherichia coli in organic matter, with a view to decontamination of poultry houses. J Appl Microbiol. 2003; 94: 919–928.

28. Adell E, Moset V, Zhao Y, Jiménez-Belenguer A, Cerisuelo A, Cambra-López M, Detection of airborne *Salmonella* spp. in poultry farms using impingement: culture-dependent vs. culture-independent methods. In: Proceedings of the 9th International Livestock Environment Symposium (ILES IX). Paper No. 1300. M. Cambra-Lopez, E.F. Wheeler, D. J. Moura, K. Janni, and R.S. Gates (eds.). St. Joseph, Mich.: ASABE.

29. Peccia J, Hernandez M. Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review. Atmos Environ. 2006; 40: 3941–3961.

30. Calvet, S., F. Estellés, M. Cambra-López, A. G. Torres, and H. Van den Weghe. The influence of broiler activity, growth rate and litter on carbon dioxide balances for the determination of ventilation flow rates in broiler production. Poult Sci. 2011; 90: 2449–2458.

31. ISO 6579:2002. Microbiology of food and animal feeding stuffs. Horizontal methods for the detection of *Salmonella* spp. International Organization for Standardization, Geneve,Switzerland.

32. AOAC International. Official methods of analysis of AOAC international. Official Method 945.18. Cereals Adjuncts, 17th ed. 2nd revision. 2003. Association of Analytical Communities, Gaithersburg, MD, USA.

33. Thorne PS, Kiekhaefer MS, Whitten P, Donham KJ. Comparison of bioaerosol sampling methods in barns housing swine. Appl Environ Microbiol. 1992; 58: 2543–2551.

34. Aabo S, Rasmussen OF, Rossen L, Sorensen PD, Olsen JE. *Salmonella* Identification by the Polymerase Chain-Reaction. Mol. Cell. Probes. 1993; 7: 171–178.

35. SAS. SAS User's Guide: Statistics. Cary NC. USA, SAS Institute Inc. 2001.

36. Buttner M, Stetzenbach L. Evaluation of four aerobiological sampling methods for the retrieval of aerosolized pseudomonas syringae. Appl Environ Microbiol. 1991; 57: 1268–1270.

37. Carrique-Mas JJ, Davies RH. Sampling and bacteriological detection of *Salmonella* in poultry and poultry premises: a review. Rev Sci Tech OIE. 2008; 27(3): 665–677.

38. Davies RH, Wray C. Persistence of *Salmonella* enteritidis in poultry units and poultry food. Br Poult Sci. 1996; 37: 589–596.

39. Al Homidan A, Robertson JF, Petchey AM. Effect of environmental factors on ammonia and dust production and broiler performance. Br Poult Sci. 1998; 39, 9–10.

40. Feddes JJ, Emmanuel EJ, Zuidhoft MJ. Broiler performance, body weight variance, feed and water intake, and carcass quality at different stocking densities. Poult Sci. 2002; 81: 774–779.

41. Tang JW. The effect of environmental parametres on the survival of airborne infectious agents. J Roy Soc Interface. 2009; 6, 737–746.

42. Zhao Y, Aarnink AJA, De Jong MCM, Ogink NWM, Koerkamp PWGG. Effectiveness of multi-stage scrubbers in reducing emissions of air pollutants from pig houses. Trans ASABE. 2011; 54: 285–293.

43. Cambra-Lopez M, Hermosilla T, Lai HTL, Aarnink AJA, Ogink NWM. Particulate matter emitted from poultry and pig houses: source identification and quantification. Trans ASABE. 2011; 54: 629–642.

44. Hayes JR, Car LE,Mallinson ET, Douglas LW, Joseph SW. Characterization of water activity and moisture content to the population distribution of *Salmonella* spp. in comercial poutry houses. Poult Sci. 2000; 79: 1557–1561.

45. Lin XJ, Reponen T, Willeke K, Wang Z, Grinshpun SA, Trunov M. Survival of airborne microorganisms during swirling aerosol collection. Aerosol Sci Technol. 2000; 32: 184.

46. Grinshpun SA, Willeke K, Ulevicius V, Juozaitis A, Terzieva S, Donnelly J, Stelma GN, Brenner KP. Effect of impaction, bounce and reaerosolization on the collection efficiency of impingers. Aerosol Sci Technol. 1997; 26: 326–342.

47. Brooks JP, McLaughlin MR, Scheffler B, Miles DM. Microbial and antibiotic resistant constituents associated with biological aerosols and poultry litter within a commercial poultry house. Sci Total Environ. 2010; 408(20): 4770–4777.

48. Gupte AR, de Rezende CLE, Joseph SW. Induction and resuscitation of viable but nonculturable *Salmonella* enterica serovar typhimurium DT104. Appl Environ Microbiol. 2003; 69: 6669–6675.

49. Qasem JA, Al Mouqati S, Rajkumar G. Comparison of DNA probe, PCR amplification, ELISA and culture methods for the rapid detection of *Salmonella* in poultry. In: Makkar H, Viljoen G, editors. Applications of Gene-Based Technologies for Improving Animal Production and Health in Developing Countries. Springer Netherlands; 2005, 529–541.

50. Lever MS, Williams A. Cross-infection of chicks by airborne transmission of *Salmonella* enteritidis PT4. Lett Appl Microbiol. 1996; 23(5): 347–349.

51. Chi MC, Li C-H. Analysis of bioaerosols from chicken houses by culture and non-culture method. Aerosol Sci Technol. 2006; 40, 1071–1079.

52. Gast RK, Mitchell BW, Holt PS. Evaluation of culture media for detecting airborne *Salmonella* enteritidis collected with an electrostatic sampling device from the environment of experimentally infected laying hens. Poult Sci. 2004; 83: 1106–1111.

53. Kwon YM, Woodward CL, Pillai SD, Pena J, Corrier DE, Byrd JA, Ricke SC. Litter and aerosol sampling of chicken houses for rapid detection of *Salmonella* typhimurium contamination using gene amplification. J Ind Microbiol Biotechnol. 2000; 24: 379–382.

54. Alvarez A, Buttner M, Stetzenbach L. PCR for bioaerosol monitoring: sensitivity and environmental interference. Appl Environ Microbiol. 1995; 61: 3639–3644.

55. Zhao Y, Aarnink AJA, Groot Koerkamp PWG, Hagenaars TJ, Katsma WEA, De Jong MCM. Detection of airborne *Campylobacter* with three bioaerosol samplers for alarming bacteria transmission in broilers. Biol eng. 2011; 3: 177–186.

56. Hospodsky D, Yamamoto N, Peccia J. Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. Appl Environ Microbiol. 2010; 76: 7004–7012.

57. Keer JT, Birch L. Molecular methods for the assessment of bacterial viabiliy. J Microbiol Meth. 2003; 53: 175–183.

58. Stojek NM, Wojcik-Fatla A, Dutkiewicz J. Efficacy of the detection of *Legionella* in hot and cold water samples by culture and PCR. II. Examination of native samples from various sources. Ann Agric Environ Med. 2012; 19: 295–298.

59. Kothary MH, Babu US. Infective dose of foodborne pathogens in volunteers: A review. J Food Safety. 2001; 21: 49–68.