Direct Detection of Salmonella Cells in the Air of Livestock Stables by Real-Time PCR

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A SYBR® Green real-time quantitative polymerase chain reaction (qPCR) assay for specific detection and quantification of airborne Salmonella cells in livestock housings is presented. A set of specific primers was tested and validated for specific detection and quantification of Salmonella-specific invA genes of DNA extracted from bioaerosol samples. Application of the method to poultry house bioaerosol samples showed concentrations ranging from $2.2 \times 10^1$ to $3 \times 10^6$ Salmonella targets m$^{-3}$ of air. Salmonella were also detected by a cultivation-based approach in some samples, but concentrations were two to three magnitudes lower than the concentrations detected by molecular biological results. Specificity of results was demonstrated by cloning analyses of PCR products, which were exclusively assigned to the genus Salmonella. However, by molecular methods, microorganisms are detected independently of their viability status, leading to an overestimation of concentration. Hence, the survival rate of Salmonella cells was measured on filter surfaces during filtration samplings where 82% of the cells died within 20 min of filtration. The results clearly show the specificity and practicability of the established qPCR assay for analysis and quantification of salmonellae in bioaerosols. The results demonstrate airborne Salmonella workplace concentrations in poultry production of up to 3.3% of 4′,6-Diamidino-2-phenylindole-counted total cell numbers.

Keywords: bioaerosol; DNA extraction efficiency; livestock stables; poultry; real-time PCR; Salmonella; sampling efficiency; SYBR Green

INTRODUCTION

Employees in modern agriculture environments are exposed to many different agents such as organic and inorganic dusts containing, e.g. endotoxins, bacteria, fungi, gases, and chemicals (Clark et al. 1983; Carlile 1984; Radon et al. 2002; Roy et al. 2003). These substances mainly affect the respiratory system and can lead to, e.g. asthma, asthma-like syndrome, mucous membrane irritation or chronic bronchitis, and hypersensitivity pneumonitis (Rylander 1986; Heederik et al. 1991; Eduard et al. 2004; Liebers et al. 2006).

In densely stocked and enclosed animal production buildings, bioaerosols mainly consist of microorganisms as well as their metabolic products and microbial cell constituents. Exposure assessment shows that the concentration of airborne microorganisms in livestock stable air can reach values up to $10^{10}$ cells m$^{-3}$ (Radon et al. 2002). Staphylococcus and Streptococcus have been described as the predominant bacterial genera in these agricultural environments (Nielsen and Breum 1995; Seedorf and Hartung 2002; Haas et al. 2005), whereas sensitizing, toxin-producing, or infection-causing bacteria like Listeria monocytogenes, Mycobacterium avium, Mycobacterium tuberculosis, Mycoplasma hyopneumoniae, Salmonella enterica subsp. enterica serovar Typhimurium var., and Pantoea agglomerans (formerly Enterobacter agglomerans) have been found as well (Cormier et al. 1990; Wathes 1995). Nevertheless, airborne microbial communities found at agricultural working places are rarely characterized in detail.

Usually, microbial communities are analyzed by cultivation-based methods. Here, exposure levels are examined on low selective agar media. By these approaches, only viable microorganisms that are able to grow at the selected nutrient and cultivation conditions (medium, pH, temperature, humidity etc.) are detectable. For this reason, cultivation-based

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MATERIAL AND METHODS

Bacterial strains and growth conditions

Bacterial strains used for inclusivity testing of the invA gene-specific real-time PCR assay are listed in Table 1. Additionally, Escherichia coli ATCC 29522T was used as a negative control. All Salmonella strains and E. coli were grown in Tryptic Soy Broth (Difco) at 37°C with shaking (Innova4000; New Brunswick Scientific, 110 rpm). Bacterial growth was monitored by determination of optical density at 600 nm.

For quantification of total cell count (TCC), 4′,6-Diamidino-2-phenylindole (DAPI) fluorescence staining (Porter and Feig 1980) was applied to pure cultures and bioaerosol samples. Cells were washed two times with ×1 phosphate-buffered saline, followed by paraformaldehyde fixation (Amann et al. 1990). Aliquots of the cell extract were mixed with 50 μl of DAPI solution (10 μM) and incubated in the dark for 20 min. Subsequently, cells were filtered onto a black polycarbonate filter (Nucleopore, 0.2-μm pore size; Whatman, Kent, UK) using a vacuum filtration unit (Schleicher and Schüll, Dassel, Germany). The dried filters were immersed in Citifluor (AF1 Citifluor Ltd, London, UK) on a glass slide. Analysis of the filters was carried out microscopically with an epifluorescence microscope (Axioskop; Zeiss, Germany). Blue fluorescent cells of 20 randomly chosen microscopic fields on the filter were counted manually using a counting ocular (Zeiss). By taking into account the effective filter surface, the dilution, and the sampled air volume, the cell concentration was calculated m⁻³ of air.

Sampling resistance of Salmonella cells during filtration by live/dead staining

To study the effect of the filtration sampling, cells were added onto a sterile polycarbonate filter (Isopore™ ATTP 0.8-μm pore size, Ø 80 mm; Millipore), followed by sampling of increasing volumes of cell-free air (0, 362, and 724 l). The cells were then detached and homogenized in 10 ml NaCl

| Species | Strain no. | Specific PCR product |
|---------|-----------|---------------------|
| Salmonella enterica subsp. enterica serovar Typhimurium | CIP 60.62T, CCUG 42060T | + |
| Salmonella enterica subsp. arizonae | CCUG 1743 | + |
| Salmonella enterica subsp. enterica serovar Enteritidis | CCUG 32352, ATCC 25928 | + |
| Salmonella enterica subsp. enterica serovar Choleraesuis | CIP 58.57, CCUG 49677, ATCC 13312 | + |
| Escherichia coli | ATCC 25922T | – |

*DNA of the species showed specific PCR products after amplification with the 139/141 primer system.
0.9% (w/v) using a stomacher (Stomacher 80 lab systems; Seward, London, UK) for 60 s. The proportions of ‘living/dead’ (intact/damaged cell membranes) cells were determined using the BacLight™ Live/Dead Kit for quantitative assays (Invitrogen Corp., USA) according to the manufacturer’s instructions. A 1:10 dilution with 0.9% NaCl (w/v) of both dyes was used for staining. Stained samples were analyzed immediately but at least within 24 h as described for TCC by fluorescence microscopy.

**Sampling site**

Samples were taken at different working places in a conventional farmed duck fattening facility and two broiler chicken houses (each accommodated with a flock of 26 000 animals) in Germany between January and September 2007. The birds were allowed to move freely on the litter, which consisted of sawdust.

The broiler chicken confinement plant consisted of two equally constructed compartments. In one of the compartments, an aerocleaner system (LH Agrartechnik, Germany) was installed, spraying an emulsion of water and essential oils into the air every 15 min to reduce dust and microorganisms. Air inlets were located at the gable wall and air outlets at the ceiling and were both automatically controlled.

The bioaerosols in a duck feeding facility (anonymous) were collected by personal carried sample devices at different working locations and during different activities of the employees during the entire work shift on different days—(i) in the duck stables: (a) during egg collecting and (b) during general stable work (straw dispersing, cleaning); (ii) in the slaughterhouse: during shackling/hanging; (iii) in the duck hatchery: during packaging of ducklings; (iv) outdoor (upwind direction); and (v) in the office: at desk work. Locations (i–v) were spatially separated, so that a direct exchange of bioaerosols could be excluded.

**Bioaerosol sampling**

In the broiler chicken house, airborne microorganisms were sampled using filtration devices (MD8 aluminium stacks; Sartorius, Germany). An air volume of 0.50–0.55 m³ (MP2/39; Umweltanalytik Holbach, Germany) was collected by filtration through polycarbonate membrane filters (Isopore™ ATTP 0.8-μm pore size; Millipore) within 20 min (27.2 l min⁻¹). The exact sampled volume was monitored by calibrated gas meters (Remus4; GSA, Germany, and Gallus 2000; Actaris). For each measurement, the downsize facing filtration devices were mounted on a tripod (1.5-m height) in the middle of the stables. One blank field sample was taken in the same way for each sampling method (samplers were not operated in these cases).

In the duck fattening facility, personal air samplers (PGP/GSP-3.5; BIA, Germany) in combination with specific pumps SG-10 (GSA) were used. The sampling system collected the inhalable dust fraction as defined by EN481 (Deutsches Institut für Normung 1993) at a sampling rate of 3.5 l min⁻¹ during the entire work shift with working activities as given previously. Here, bioaerosols were collected on polycarbonate filters (0.8-μm pore size, Ø 3.7 cm; Whatman), which were fixed onto the filter cassette of the PGP system.

**Preparation of bioaerosol samples for cultivation and molecular methods**

Collected cells were detached and homogenized as described previously. For selective detection of *Salmonella* cells, resuspended cells were serially diluted. Bismuth sulfite agar plates (Difco; colony-forming units (cfu) *Salmonella*) were inoculated with 0.1-ml aliquots of each dilution in triplicates. Characteristic colonies were counted after an incubation of 24 h at 37°C. Concentrations were calculated as cfu per volume of sampled air (cfu m⁻³).

For DNA extraction and TCC determination, 4.5-ml cell suspension was concentrated by centrifugation at 17 400 g for 10 min.

**Extraction of total community DNA from bioaerosol samples**

Extraction of total genomic DNA was performed according to Pitcher et al. (1989). Total extracted DNA was resuspended in a final volume of 75 μl TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8). Only DNAse-, RNAse-, and DNA-free solutions and consumables were used.

**Determination of DNA extraction efficiency**

To analyze the DNA extraction efficiency, varying cell numbers between 1.5 × 10⁹ and 1.1 × 10¹¹ cells of *Salmonella* (of the stationary growth phase) were used for a DNA extraction assay. Cell numbers at this point of time were determined by the DAPI staining method as described previously. DNA extraction was done as previously described and total DNA concentration was determined by ultraviolet photometric as well as by fluorometric measurements. *Salmonella* cell equivalents were calculated based on the genome size of 4.9 × 10⁶ kb and a GC content of 53% (McClelland *et al.* 2001) according to the following equation: genome weight W = (% GC × genome length) × 615.4/100 + (100 – % GC × genome length) × 616.4/100 + 61. Genome copies per nanogram of DNA were calculated as follows: ng DNA = (NL/W × 10⁻⁹), where NL is the Avogadro constant (6.02 × 10²³ molecules mol⁻¹). Extraction efficiencies (%) were calculated by the quotient of determined and assumed DNA concentrations multiplied by 100.

**Real-time qPCR**

Real-time PCR was performed using the Rotor-Gene 3000 (Corbett Research, UK). Primers 139/141 were those described by Rahn *et al.* (1992) and...
purchased from MWG Biotech (Germany). Assays were performed in a total volume of 10 μl mixing 1 μl of DNA with 5 μl SYBR® Green Mix (ABGene Absolute QPCR SYBR® Green Mix), 3.6 μl molecular grade water, and 0.2 μl of each primer. All samples and standards were analyzed in quadruplicates.

Development of a real-time PCR standard curve for Salmonella quantification

For preparation of quantification standards, genomic DNA from a Salmonella culture was extracted and later quantified by fluorometric measurement. The DNA concentration in the real-time PCR assay was adjusted to gene target numbers between 1 and 10^7 targets μl^-1 under the assumption of one invA gene per genome (Fey and Eichler 2004). For each concentration, the cycle threshold (C_T) value was plotted against the log value of corresponding Salmonella invA genes. The calibration curve was generated by the RotorGene software version 6.0.16. Consequently, initial target copy numbers in the samples were computed by the slope of the resulting linear equation (1). Then, the slope of each calibration curve was used (equation 2) to determine the reaction efficiency (perfect exponential amplification has an efficiency of 1).

Log concentration = m × logC_T + b
Efficiency = 10^-(1/m - 1),

where m is the slope and b the intercept.

Recovery rate of Salmonella by real-time PCR

To examine possible inhibitory effects, the following experiments were conducted to analyze the recovery of real-time PCR Salmonella detection.

To study inhibition by non-target DNA, 10^8 E. coli cells were supplemented with declining Salmonella cell numbers between 10^4 and 10^4 cells. Assuming a total cell count of 10^8–10^9 cells m^-3 of air, this would correspond to a theoretical proportion of 0.01–100% Salmonella cells covering a wide proportion range. From these mixtures, DNA was extracted and invA gene copy number determined by real-time PCR.

To analyze the recovery of Salmonella cells from bioaerosol samples, bioaerosol samples from a turkey stable were spiked with 1.1 × 10^9 ± 3.1 × 10^7 and 4.2 × 10^7 ± 1.6 × 10^7 Salmonella cells.

Construction of an invA gene clone library and sequencing of the plasmid inserts

Specificity of PCR products was tested by cloning analyses of amplicons obtained from bioaerosol samples collected in the broiler houses. The PCR products retrieved with the 139/141 primer system were cloned into E. coli using the pGEM®-T Vector System Kit (Promega Corp., USA) according to the manufacturer’s instructions. Ten clones were randomly chosen from the library. Cultures were grown overnight in liquid Lysogeny broth medium at 37°C and cells harvested by centrifugation. Pellets were used for plasmid extraction by alkaline lysis (Sambrook and Russel 2001). Plasmid inserts were sequenced (remittance work by the Institute of Microbiology, Justus-Liebig University of Giessen) using the M13 primers (Invitrogen Corp.).

Sequence data were analyzed using MEGA version 4 (Tamura et al. 2007). First, a BLAST search (www.ncbi.nlm.nih.gov) was carried out to ensure the correct placement of the obtained partial invA gene sequences. Subsequently, the closest BLAST matches were aligned with ClustalW (Higgins et al. 1994) provided by MEGA4. Distances between sequence pairs were calculated and trees were constructed with the neighbor-joining method included in MEGA4. Stability of branches was assessed with the bootstrap method using 1000 replicates.

RESULTS

DNA extraction efficiency

A decreasing DNA extraction efficiency was found with increasing applied cell concentrations (Fig. 1). The efficiency range between 58 and 1%.

Standard curve for quantification by real-time PCR

For molecular detection of salmonellae, the primer system described in DIN 10135 (Deutsches Institut für Normung 1999) was optimized for real-time
qPCR. These primers induce the amplification of a fragment of the invA gene, specific for the genus Salmonella. A linear correlation of $C_T$ value and target concentrations ($r^2 = 0.99$) was found for concentrations between $10^0$ and $10^7$ targets $\mu l^{-1}$ (Fig. 2). The initial number of equivalent Salmonella in environmental samples was calculated using this calibration curve.

Recovery rate of Salmonella by real-time PCR

In a first set of experiments, defined cell numbers of Salmonella were mixed with constant E. coli numbers. Here, a clear linear correlation of applied cell number and quantified target number was found ($r^2 = 0.99$). The recovery rate for this approach ranged between 15 and 28%.

In the second assay, defined numbers of S. enterica cells were added to bioaerosol samples collected by impingement or filtration in poultry houses. Depending on the sampling method, the recovery rate varied between 26 and 66%.

![Fig. 2. Linear correlation of Salmonella invA gene concentrations from $10^0$ to $10^7$ targets $\mu l^{-1}$ amplified by real-time qPCR and $C_T$ values. Values are means of $n = 4 \pm$ standard deviation.](image)

Application of the developed real-time PCR detection system to common bioaerosol samples

For testing the established analytical PCR protocol, bioaerosol samples of a broiler chicken house with prior salmonellosis finding of the animals were analyzed. A cultivation-based approach was carried out in parallel.

In the samples collected by filtration, Salmonella were detected both by cultivation and by real-time qPCR. Concentrations found with the cultivation-based approach were $3.3 \times 10^2 \pm 1.2 \times 10^2$ cfu Salmonella $m^{-3}$ of air and for the molecular approach $2.8 \times 10^5 \pm 1.9 \times 10^5$ invA gene targets $m^{-3}$ of air (Fig. 3). Salmonella were not found in bioaerosol samples of the reference house (without salmonellosis finding), neither by the cultivation-based approach nor by the real-time qPCR.

With the established protocol, specific invA gene PCR products were obtained by real-time PCR in 16% of 83 bioaerosol samples from different working places in the duck fattening industry. Depending on the examined working area, concentrations ranged between $2.5 \times 10^1$ and $3 \times 10^6$ targets $m^{-3}$ of air (Fig. 4).

Testing of primer specificity by cloning analyses

The 139/141 primer pair originally described by Rahn et al. (1992) was used in real-time PCR reactions containing SYBR Green as fluorescent marker. Results from gel electrophoresis confirmed the correct molecular size of the amplicon (~250 bp), indicating primer specificity (data not shown). PCR products from the Salmonella-containing bioaerosols were used for construction of an invA clone library. Sequence analyses of the library showed sequence similarities between 98.9 and 99.6% to invA gene.

![Fig. 3. Total cell count $m^{-3}$ of air (square) determined after DAPI staining for bioaerosol samples obtained by filtration of a broiler chicken house. Furthermore, Salmonella concentrations $m^{-3}$ of air determined by real-time qPCR (○) and cultivation (●) are shown. Values are means of three bioaerosol samples ± standard error.](image)
sequences of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (EU348365; Fig. 5).

**Biological sampling efficiency**

For investigating the pathogenic potential of bioaerosol microorganisms, the biological sampling efficiency of different sampling systems is of high importance. This was studied for filtration by the use of live/dead staining. Results showed differences in the effect of *Salmonella* cells correlated to sampling method and sampled air volume. More than 40% and >80% of the cells were detected ‘dead’ after 10 and 20 min, respectively.

**DISCUSSION**

Compared with background exposure levels, cultivable and dead microorganisms as well as their byproducts like endotoxins and mycotoxins can be detected in very high concentrations at working places in agriculture (Clark *et al.* 1983; Cormier *et al.* 1990; Nielsen and Breum 1995; Ellerbroek 1997;...
dependent. Krsk and Wellington (1999) found, e.g., that the extraction efficiency was found to be species dependent. The variation of biochemical components in different organisms may present an attractive alternative for both quantification and differentiation of microorganisms. In this study, the genus Salmonella was chosen as a model organism group to prove the applicability of real-time qPCR for a species-specific bioaerosol monitoring at workplaces.

As a first step, DNA has to be extracted quantitatively from investigated samples. For qualitative analysis, a preferably high DNA extraction efficiency is desirable. For standardization, a reproducible protocol with known extraction efficiency is needed. The variation of biochemical components in different samples, the different extraction protocols used, and also the presence of different organisms usually lead to variable efficiencies of DNA extraction methods. To our knowledge, DNA extraction efficiency has not been evaluated consistently for microorganisms. Einen et al. (2008) showed a DNA extraction efficiency of ~1.6% for E. coli by application of the Bio101 Kit (Qbiogen) to pure cultures. Another study showed DNA extraction efficiencies of overall undetectable to 43.3%, depending on the protocol used (Mumy and Findlay 2004). It must be stressed that not only the overall efficiency varied but also that the extraction efficiency was found to be species dependent. Krsk and Wellington (1999) found, e.g., differences in denaturing gradient gel electrophoresis fingerprint patterns depending on the DNA extraction method used. In our study, basic examinations were conducted concerning DNA extraction efficiency for Salmonella. An inverse proportionality of adjusted cell number and extraction efficiency was detected (Fig. 1). The DNA extraction efficiencies varied between 10.1 and 58.5%, if cell numbers were <10⁹ cells per extraction assay. Lower extraction efficiencies were obtained for cell numbers >10⁹ cells per assay. This can be due to the limitations imposed by chemicals and enzymes. For estimation of the initial exposure, the loss of DNA by extraction has to be considered as aforementioned. The actual exposure may be higher by a multiple.

Basically, selectivity and specificity of PCR depend on the primer system and amplification conditions used. The primers used in this study target the Salmonella invA gene, which is assumed to be genus specific (Galan and Ginocchio 1992) with one copy per genome (Fey and Eichler 2004). The calibration curve needed for quantification has been carried out for 1–10⁷ targets μl⁻¹ of DNA extracted from a pure culture (r² = 0.99; Fig. 2). In contrast, environmental samples may contain inhibitory substances. For bioaerosol samples from animal stables, this may be associated with animal dander, feathers, pollen, or dust particles from feeding stuff or straw. The qualitative and quantitative chemical composition can vary highly and may be affected by animal species, season, stable climate, or architecture. The different chemicals are co-extracted and may cause inhibitory effects.

In our experiments for inhibition analysis, defined amounts of Salmonella cells were mixed both with constant numbers of E. coli cells and with bioaerosol samples of a turkey stable. The recovery was determined by real-time qPCR. The recovery of Salmonella in the mixed assays of E. coli and Salmonella ranged between 15 and 28%, and 26 and 66% in the supplemented bioaerosol samples. The loss of DNA is most probably due to an inefficient DNA extraction because the obtained recovery rates correspond to those in the pure culture experiments.

Bioaerosol samples from a poultry confinement with prior Salmonella findings of the animals were investigated to validate the implemented system. Salmonella were found by both the cultivation-based approach and the real-time PCR. In contrast to the cultivation-based quantification, the counts of airborne microorganisms by real-time PCR were between 4- and 100-fold higher. Concentrations were 3.3 × 10⁵ ± 1.2 10⁵ cfu m⁻³ of air and 2.8 × 10⁶ ± 1.9 × 10⁵ cells m⁻³ of air, respectively (Fig. 3). For estimation of the initial exposure, the loss of DNA by extraction has to be considered as aforementioned. Therefore, the actual exposure is expected to be 5-fold higher.
The clear differences of detected *Salmonella* concentrations by cultivation-based and molecular methods can basically be explained by the limitation of the determination method. On the one hand, cell agglomerates, particle-bound microorganisms, dead cells, and VBNC microbes lead to an underestimation of the total cell concentration by the non-equivalent number of cfu. On the other hand, molecular methods ideally determine all cells, without differentiation between cultivable and non-culturable organisms (dead cells and VBNC cells). In this context, our results show that 40% of *Salmonella* cells placed on a filter were stained by the ‘dead’ dye from the live/dead staining kit after a 10-min sampling period. These results indicate membrane damages to *Salmonella* cells due to the sampling procedure, which may result in a loss of cultivability. To alleviate those stress effects, a resuscitation step could be very helpful (Crozier-Dodson and Fung 2002).

Bioaerosol samples may contain a lot of unknown organisms with genes never analyzed before. Therefore, for evaluation of a new PCR system, it has to be ensured that the gene fragments amplified from environmental origin (e.g. soil or organic matter) belong to the targeted species. In this study, a clone library of PCR products gained from the *Salmonella*-positive broiler chicken houses was generated. All sequences analyzed showed high similarities (>98.9) to sequences from *Salmonella enterica* subsp. *enterica* serovar Typhimurium (EU348365), indicating a high specificity of the PCR protocol used.

With the implemented and validated PCR protocol, a total of 62 bioaerosol samples collected at six different working environments in a duck fattening industry during the entire working day by personal aerosol samplers were analyzed. Exposure to *Salmonella* was determined in samples collected during three working activities: egg collection, general work in the duck stables, and during shackling of birds before slaughter. The incidence of airborne bacteria as well as the exposure levels showed the highest values in the samples collected during shackling of ducks. Here, in 66% of air samples, *Salmonella* were detected at a mean concentration of ~10^6 cells m^-3 of air (Fig. 4).

Despite frequently observed workers’ respiratory disorders, till now there are no general accepted occupational exposure limits at agricultural working places. On the one hand, this fact can be drawn back to the mentioned lack of generally accepted standardized quantification methods. On the other hand, by insufficient knowledge about dose–response relationships that highly depend on the individual disposition of occupants.

Dutkiewicz (2000) and Górný and Dutkiewicz (2002) proposed a maximum load of 10^5 cfu bacteria (total cultivable bacteria) m^-3 of air. As shown previously, these exposure levels are exceeded in the duck fattening industry even by the potential pathogenic *Salmonella* serotypes at duck shackling activity.

In particular for mice, calves, and chicken, an airborne transmission of *Salmonella* has already been shown; the infection dose via respiration was assumed to be even lower than for the oral route of infection (Darlow *et al.* 1961; Wathes *et al.* 1988; Lever and Williams 1996). Workers at duck shackling are presumably exposed during the entire work shift to airborne *Salmonella*. The risk for infection at these working places therefore seems quite probable.

In future, next to technical and organizational measures, an adequate breathing protection is recommended for protection of workers at these working environments (Ausschuss für Biologische Arbeitsstoffe 2007).

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