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

KERSTIN FALLSCHISSEL[1], PETER KÄMPFER[1] and UDO JÄCKEL[2]*

[1]*Institute for Applied Microbiology, Justus-Liebig University of Giessen, Heinrich-Buff-Ring 26-32, 34637 Giessen, Germany;* [2]*Federal Institute for Occupational Safety and Health, Nöldnerstraße 40-42; 10317 Berlin, Germany*

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 2.2×10^1 to 3×10^6 *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

*Author to whom correspondence should be addressed.
Tel: +49 3051548-4788; fax: +49 3051548-4171;
e-mail: jaeckel.udo@baua.bund.de

methods are unsuitable for the detection of dead or slow-growing microorganisms and for viable but non-cultivable (VBNC) microbes. In addition, selective media may not restrict growth of undesired organisms (Albrecht and Kämpfer 2006). It is also widely accepted that the vast majority of naturally occurring microorganisms cannot be cultivated using standard cultivation techniques (Parkes and Taylor 1985; Amann *et al.* 1995).

An air sampling by personal carried devices during a whole working day, which is a basic requirement for an ideal exposure measurement, is also not feasible in cultivation-based approaches because many bacteria are not resistant to desiccation and sampling stress (Marthi *et al.* 1990; Potts 1994; Durand *et al.* 2002).

Molecular biological methods like polymerase chain reaction (PCR) may offer the advantage of a more sensitive and specific detection method. These methods, which target the DNA, have already been applied successfully to investigate microorganisms in different environmental samples (Stach *et al.* 2001; Stubner 2002; Kolb *et al.* 2005). Real-time quantitative PCR (qPCR) is a potential method for species- or genus-specific quantification. While this method has rarely been applied to bioaerosols (Makino *et al.* 2001; Makino and Cheun 2003; Zeng *et al.* 2006; Cayer *et al.* 2007; Dutil *et al.* 2007; Oppliger *et al.* 2008), it has not been standardized for occupational exposure measurements. Therefore, the purposes of this study were (i) to develop a fast and reliable method to determine concentrations of airborne bacteria in animal houses by application of a real-time qPCR, (ii) to determine possible losses during DNA extraction and air sampling, and (iii) to evaluate the methods for suitability of rapid exposure measurements in the air of livestock stables.

The genus *Salmonella* was chosen as a model organism because the genus has been frequently reported in livestock stable aerosols especially in poultry confinements (Hoover *et al.* 1997; Venter *et al.* 2004; Lues *et al.* 2007).

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 29522[T] 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.

Because for all *Salmonella* strains specific PCR products were detected (data not shown), all following experiments and preparation of quantification standards for real-time PCR were carried out using the strain *Salmonella enterica* subps. *enterica* serovar Typhimurium CIP 60.62[T].

Determination of total cell count by fluorescence staining and microscopy

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 air-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 (Axiophot, 1000-fold magnification; 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

Table 1. Bacterial strains used in this study

Species	Strain no.	Specific PCR product[a]
Salmonella enterica subsp. *enterica* serovar Typhimurium	CIP 60.62[T], CCUG 42060[T]	+
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 25922[T]	−

[a]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 aluminum stacks; Sartorius, Germany). An air volume of 0.50–0.55 m^3 (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 downside 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, ∅ 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 1.5×10^8 and 1.1×10^{11} 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 4.9×10^3 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 (6.02×10^{23} 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. The following thermal program was used for amplification: 15 min initial denaturation at 95°C, followed by 40 cycles of denaturation (95°C, 1 min), annealing (54°C, 15 s), elongation (72°C, 20 s, first acquisition), and a second acquisition step at 80°C for 20 s to identify primer dimers. After cycling, a melt curve, ranging from 60 to 95°C at 0.5°C steps, was added, with fluorescence acquisition at each temperature rising.

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).

$$\text{Log concentration} = m \times \log C_T + b \quad (1)$$

$$\text{Efficiency} = 10^{-1/m} - 1, \quad (2)$$

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 10^8 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 $1.1 \times 10^8 \pm 3.1 \times 10^7$ and $4.2 \times 10^7 \pm 1.6 \times 10^7$ *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 rate ranged 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
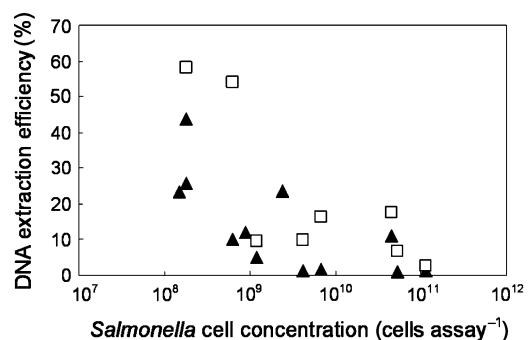

Fig. 1. DNA extraction efficiency (%) as a function of total applied *Salmonella* cell number. Extraction efficiency was calculated based on photometric (□) and fluorometric (▲) DNA quantification.

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 μl^{-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%.
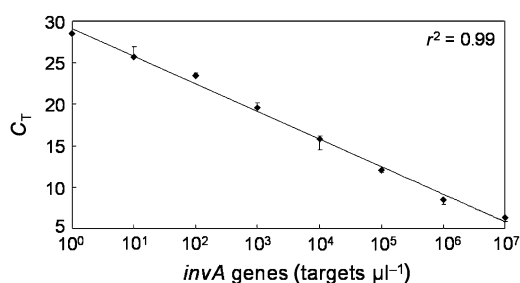

Fig. 2. Linear correlation of *Salmonella invA* gene concentrations from 10^0 to 10^7 targets μl^{-1} amplified by real-time qPCR and C_T values. Values are means of $n = 4 \pm$ standard deviation.
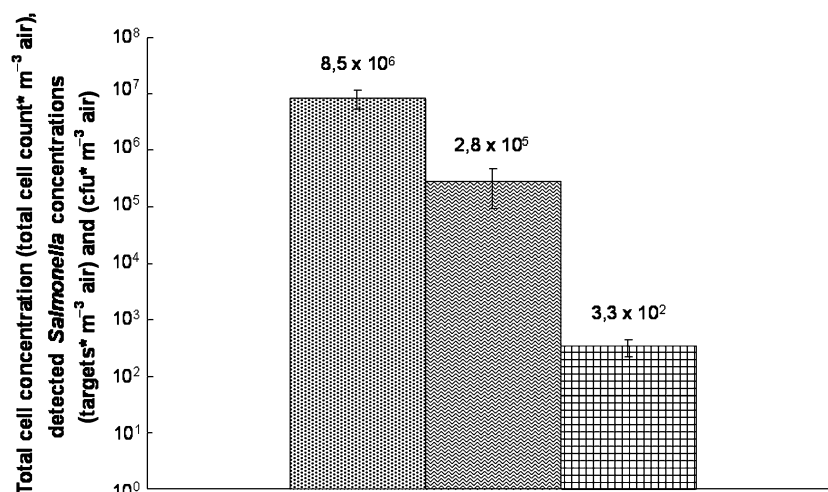
Application of the developed real-time PCR detection system to common bioaerosol samples

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 $2.8 \times 10^5 \pm 1.9 \ 10^5$ *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 2.5×10^1 and 3×10^6 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 ($\sim$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 (▦) 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.

sequences of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (EU348365; Fig. 5).

Biological sampling efficiency

For investigating the pathogenic potential of bio-aerosol 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 by-products 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;
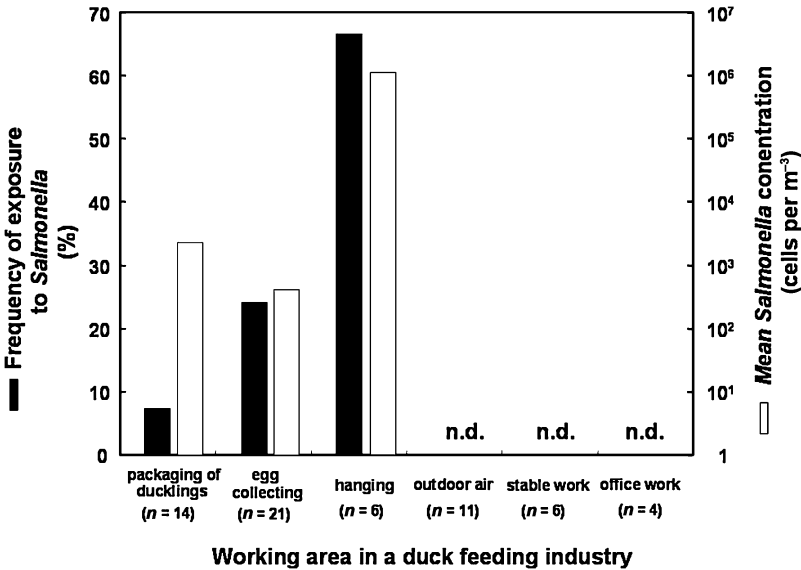
Fig. 4. Frequency of exposure to *Salmonella* (%) and mean airborne *Salmonella* concentration (cells m^{-3}) subject to the working areas in a duck feeding industry. Concentrations were determined by real-time qPCR using the primer system described by Rahn *et al.* (1992). Bioaerosol samples were obtained by personal air samplers during a whole working day. Values are means ± standard deviation. n.d., not detectable.
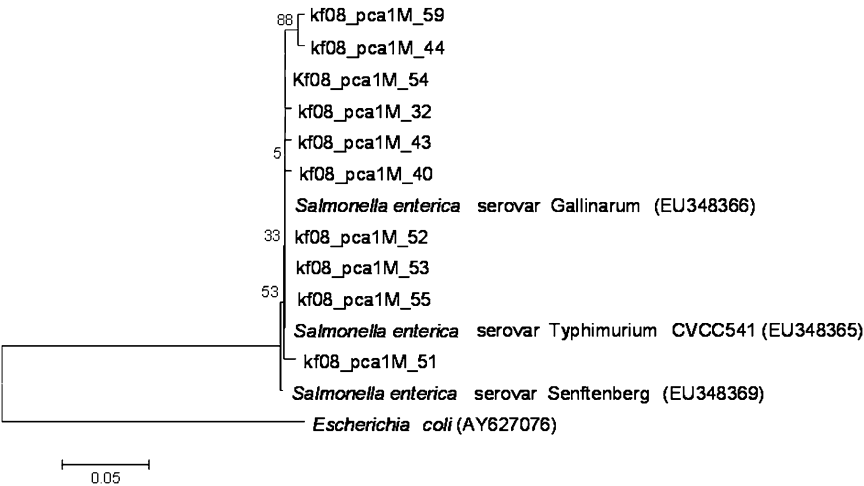
Fig. 5. Neighbor-joining tree of sequences (295 bp) of the operational taxonomic units obtained from bioaerosol samples of the broiler houses after cloning analysis. Distances were calculated and the tree was constructed in MEGA4 after BLAST search to ensure correct placement of the obtained partial *invA* gene sequences. Stability of the tree branches was assessed with the bootstrap method using 1000 replicates. Bar, 0.05 nucleotide substitutions per nucleotide position.

Zucker *et al.* 2000; Radon *et al.* 2002; Venter *et al.* 2004; Lee *et al.* 2006). The organic dusts at these working places are known as causative agents for various pulmonary health effects, e.g. allergic reactions such as occupational asthma or allergic alveolitis. Therefore, bioaerosol monitoring should be carried out for risk assessment at these working places.

Many different sampling systems can be used for bioaerosol collection. The most common systems are based on impaction, filtration, and impingement. Because of the differences of these systems in biological and physical sampling efficiency, the results cannot be compared directly.

One major problem for exposure measurements is the lack of a standardized methodology measuring total bacterial burden. Up to now, bioaerosol monitoring assays at workplaces are carried out by analyzing sum parameters like endotoxins, inhalable dust, and total cultivable bacteria or fungi without consideration of the composition of the microbial communities. However, from a microbiological point of view, a species-specific exposure–response relationship can be assumed (Thurston *et al.* 1979; Baseler *et al.* 1983; Fogelmark *et al.* 1991; Dutkiewicz 1997; Milanowski *et al.* 1998). For this reason, a species-specific detection is highly desirable. At present, standardized guidelines, e.g. in Germany for analysis of airborne microorganisms, are based on a cultivation-based approach (Kommission Reinhaltung der Luft im VDI und DIN 2004a,b,c,d). Because of the known methodological limitation of cultivation-based approaches, molecular methods 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 working places.

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 $<10^9$ cells per extraction assay. Lower extraction efficiencies were obtained for cell numbers $>10^9$ 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 $1–10^7$ targets μl^{-1} of DNA extracted from a pure culture ($r^2 = 0.99$; Fig. 2). In contrast, environmental samples may contain inhibitory substances. For bioaerosol samples from animal stables, this may be associated with animal dander, feces, 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 $3.3 \times 10^2 \pm 1.2\ 10^2$ cfu m^{-3} of air and $2.8 \times 10^5 \pm 1.9 \times 10^5$ 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 $\sim 10^6$ 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 Górny 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).

FUNDING

REFERENCES

Albrecht A, Kämpfer P. (2006) Potential overestimation of the actinomycetes concentration by growth of thermophilic species of Bacillus and Geobacillus on selective media for thermophilic actinomycetes. Air Quality Control; 9: 365–7.

Amann R, Binder BJ, Olson RJ *et al.* (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol; 56: 1919–25.

Amann RI, Ludwig W, Schleifer KH. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev; 59: 143–69.

Baseler MW, Fogelmark B, Burell R. (1983) Differential toxicity of inhaled Gram-negative bacteria. Infect Immun; 40: 133–8.

Carlile FS. (1984) Ammonia in poultry houses: a literature review. World's Poultry Science Journal; 40: 99–113.

Cayer M-P, Veillette M, Pageau P *et al.* (2007) Identification of mycobacteria in peat moss processing plants: application of molecular biology approaches. Can J Microbiol; 53: 92–9.

Clark SC, Rylander R, Larson L. (1983) Airborne bacteria, endotoxin and fungi in dust poultry and swine confinement buildings. Am Ind Hyg Ass J; 44: 537–541.

Cormier Y, Tremblay G, Meriaux A, Brochu G, Lavoie J. (1990) Airborne microbial contents in two types of swine confinement buildings in Quebec. Am Ind Hyg Assoc J; 51: 304–9.

Crozier-Dodson BA, Fung DYC. (2002) Comparison of recovery of airborne microorganisms in a dairy cattle facility using selective agar and thin agar layer resuscitation media. J Food Prot; 65: 1488–92.

Darlow HM, Bale WR, Carter GB. (1961) Infection of mice by the respiratory route with Salmonella typhimurium. J Hyg; 59: 303–8.

Deutsches Institut für Normung. (1993) DIN EN481: 1993 Workplaces atmospheres; size fraction definitions for measurement of airborne particles. Berlin, Germany: Beuth Verlag.

Deutsches Institut für Normung. (1999) Verfahren zum Nachweis von Salmonellen mit der Polymerase-Kettenreaktion (PCR). Vol. DIN10135. Berlin, Germany: Deutsches Institut für Normung.

Durand KTH, Muilenberg ML, Burge HA *et al.* (2002) Effect of sampling time on the culturability of airborne fungi and bacteria sampled by filtration. Ann Occup Hyg; 46: 113–8.

Dutil S, Veillette M, Mériaux A *et al*. (2007) Aerosolization of mycobacteria and legionellae during dental treatment: low exposure despite dental unit contamination. Environ Microbiol; 9: 2836–43.

Dutkiewicz J. (1997) Bacteria and fungi in organic dust as potential health hazard. Ann Agric Environ Med; 4: 11–6.

Dutkiewicz J, Krysińska-Traczyk E, Skórska C *et al*. (2000) Exposure of agricultural workers to airborne microorganisms and endotoxin during handling of various vegetable products. Aerobiologia; 16: 193–8.

Eduard W, Douwes J, Omenaas E *et al*. (2004) Do farming exposures cause or prevent asthma? Results from a study of adult Norwegian farmers. Thorax; 59: 381–6.

Einen J, Thorseth IH, Ovreas L. (2008) Enumeration of Archaea and Bacteria in seafloor basalt using real-time quantitative PCR and fluorescence microscopy. FEMS Microbiol Lett; 282: 182–7.

Ellerbroek L. (1997) Airborne microflora in poultry slaughtering establishments. Food Microbiol; 14: 527–31.

Fey A, Eichler S. (2004) Establishment of real-time PCR-based approach for accurate quantification of bacterial RNA-targets in water, using Salmonella as a model organism. Appl Environ Microbiol; 70: 3618–23.

Fogelmark B, Lacey J, Rylander R. (1991) Experimental allergic alveolitis after exposure to different microorganisms. Int J Exp Pathol; 72: 387–95.

Galan JE, Ginocchio C. (1992) Molecular and functional characterization of the *Salmonella* invasion gene invA: homology of InvA to members of a new protein family. J Bacteriol; 174: 4338–49.

Górny R, Dutkiewicz J. (2002) Bacterial and fungal aerosols in indoor environment in Central and Eastern European countries. Ann Agric Environ Med; 9: 17–23.

Haas D, Posch J, Schmidt S *et al*. (2005) A case study of airborne culturable microorganisms in a poultry slaughterhouse in Styria, Austria. Aerobiologia; 21: 193–201.

Heederik D, Brouwer R, Biersteker K *et al*. (1991) Relationship of airborne endotoxin and bacteria levels in pig farms with the lung function and respiratory symptoms of farmers. Int Arch Occup Environ Health; 62: 595–601.

Higgins D, Thompson J, Gibson T *et al*. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res; 22: 4673–80.

Hoover NJ, Kenney PB, Amick JD *et al*. (1997) Preharvest sources of Salmonella colonization in turkey production. Poult Sci; 76: 1232–8.

Kolb S, Carbrera A, Kammann C *et al*. (2005) Quantitative impact of CO2 enriched atmosphere on abundances of methanotrophic *bacteria* in a meadow soil. Biol Fert Soils; 41: 337–42.

Kommission Reinhaltung der Luft im VDI und DIN. (2004a) Richtlinie VDI 4252 Blatt 2: Erfassen luftgetragener Mikroorganismen und Viren in der Außenluft; Aktive Probenahme von Bioaerosolen; Abscheidung von luftgetragenen Schimmelpilzen auf Gelatine/Polycarbonat-Filtern. Berlin, Germany: Beuth Verlag.

Kommission Reinhaltung der Luft im VDI und DIN. (2004b) Richtlinie VDI 4252 Blatt 3: Erfassen luftgetragener Mikroorganismen und Viren in der Außenluft; Aktive Probenahme von Bioaerosolen—Abscheidung von luftgetragenen Bakterien mit Impingern nach dem Prinzip der kritischen Düse. Berlin, Germany: Beuth Verlag.

Kommission Reinhaltung der Luft im VDI und DIN. (2004c) VDI Richtlinie 4253 Blatt 2: Erfassen luftgetragener Mikroorganismen und Viren in der Außenluft—Verfahren zum kulturellen Nachweis der Schimmelpilz-Konzentrationen in der Luft—Indirektes Verfahren nach Probenahme auf Gelatine/Polycarbonat-Filtern. Berlin, Germany: Beuth Verlag.

Kommission Reinhaltung der Luft im VDI und DIN. (2004d) VDI Richtlinie 4253 Blatt 3: Erfassen luftgetragener Mikroorganismen und Viren in der Außenluft—Verfahren zum quantitativen kulturellen Nachweis von Bakterien in der Luft—Verfahren nach Abscheidung in Flüssigkeiten. Berlin, Germany: Beuth Verlag.

Krsek M, Wellington EMH. (1999) Comparison of different methods for the isolation and purification of total community DNA from soil. J Microbiol Methods; 39: 1–16.

Lee S-A, Adhikari A, Grinshpun SA *et al*. (2006) Personal exposure to airborne dust and microorganisms in agricultural environments. J Occup Environ Hyg; 3: 118–30.

Lever MS, Williams A. (1996) Cross-infection of chicks by airborne transmission of *Salmonella enteritidis* PT4. Lett Appl Microbiol; 23: 347–49.

Liebers V, Brüning T, Raulf-Heimsoth M. (2006) Occupational endotoxin-exposure and possible health effects on humans (review). Am J Ind Med; 49: 474–91.

Lues JFR, Theron MM, Venter P *et al*. (2007) Microbial composition in bioaerosols of a high-throughput chicken-slaughtering facility. Poult Sci; 86: 142–49.

Makino S, Cheun HI. (2003) Application of the real-time PCR for the detection of airborne microbial pathogens in reference to the anthrax spores. J Microb Meth; 53: 141–147.

Makino SI, Cheun HI, Watarai M *et al*. (2001) Detection of *anthrax* spores from the air by real-time PCR. Lett Appl Microbiol; 33: 237–40.

Marthi B, Fieland VP, Walter M *et al*. (1990) Survival of bacteria during aerosolization. Appl Environ Microbiol; 56: 3463–67.

McClelland M, Sanderson KE, Spieth J. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature; 413: 852–6.

Milanowski J, Dutkiewicz J, Potoczna H *et al*. (1998) Allergic alveolitis among agricultural workers in Eastern Poland: a study of twenty cases. Ann Agric Environ Med; 5: 31–43.

Mumy KL, Findlay RH. (2004) Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. J Microbiol Methods; 57: 259–68.

Nielsen B, Breum N. (1995) Exposure to air contaminants in chicken catching. Am Ind Hyg Assoc J; 56: 804–8.

Oppliger A, Charriere N, Droz P-O *et al*. (2008) Exposure to bioaerosols in poultry houses at different stages of fattening; use of real-time PCR for airborne bacterial quantification. Ann Occup Hyg; 52: 405–12.

Parkes RJ, Taylor J. (1985) Characterization of microbial populations in polluted marine sediments. J Appl Bacteriol; 59: 155S–73S.

Pitcher DG, Saunders NA, Owen RJ. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol; 8: 151–6.

Porter KG, Feig YS. (1980) The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr; 25: 943–8.

Potts M. (1994) Desiccation tolerance of prokaryotes. Microbiol Rev; 58: 755–805.

Radon K, Danuser B, Iversen M *et al*. (2002) Air contaminants in different Europe farming environments. Ann Agric Environ Med; 9: 41–8.

Rahn K, De Grandis SA, Clarke RC *et al*. (1992) Amplification of an InvA gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol Cell Probes; 6: 271–9.

Roy SR, Schiltz AM, Marotta A. (2003) Bacterial DNA in house and farm barn dust. J Allergy Clin Imm; 112: 571–8.

Rylander R. (1986) Lung diseases caused by organic dusts in the farm environment. Am J Ind Med; 10: 221–7.

Sambrook J, Russel DW. (2001) Molecular cloning—a laboratory manual. Vol. 1. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Seedorf J, Hartung J. (2002) Stäube und Mikroorganismen in der Tierhaltung. Kuratorium für Technik und Bauwesen in der Landwirtschaft e. V. (KTBL-Schrift 393). Münster, Germany: Landwirtschaftsverlag; pp. 166

Stach JEM, Bathe S, Clapp JP *et al*. (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. FEMS Microbiol Ecol; 36: 139–51.

Stubner S. (2002) Enumeration of 16S rDNA of *Desulfotomaculum* lineage 1 in rice field soil by real-time PCR with Sybr-Green detection. J Microbial Methods; 50: 155–64.

Tamura K, Dudley J, Nei M *et al*. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol; 24: 1596–9.

Thurston JR, Cysewski SJ, Richard JL. (1979) Exposure of rabbits to spores of *Aspergillus fumigatus* or *Penicillium* sp.: survival of fungi and microscopic changes in the respiratory and gastrointestinal tracts. Am J Vet Res; 40: 1443–9.

TRBA 230:2007-11. (2008) Technische Regel für biologische Arbeitsstoffe: Schutzmaßnahmen bei Tätigkeiten mit biolo-gischen Arbeitsstoffen in der Land- und Forstwirtschaft und vergleichbaren Tätigkeiten. Gemeinsames Ministerial-blatt. Vol. 4, 71–83 Available from: http://www.baua.de/nn_15074/de/Themen-von-A-Z/Biologische-Arbeitsstoffe/TRBA/pdf/TRBA-230.pdf?

Venter P, Lues JF, Theron H. (2004) Quantification of bioaerosols in automated chicken egg production plants. Poult Sci; 83: 1226–31.

Wathes CM. (1995) Bioaerosols in animal houses. In Cox CS, Wathes CM, editors. Bioaerosols handbook. Boca Raton, FL: CRC Press; pp. 547–77.

Wathes CM, Zaidan WAR, Pearson GR *et al*. (1988) Aerosol infections of calves and mice with *Salmonella typhimurium*. Vet Rec; 123: 590–4.

Zeng Q, Westermark S, Rasmuson-Lestander A *et al*. (2006) Detection and quantification of *Cladosporium* in aerosols by real-time PCR. J Environ Monit; 8: 153–60.

Zucker B-A, Trojan S, Müller W. (2000) Airborne Gram-negative bacterial flora in animal houses. J Vet Med B Infect Dis Vet Public Health; 47: 37–46.