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REVIEW

Qualitative and quantitative methodologies for determination of airborne microorganisms at concentrated animal-feeding operations

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Abstract The generation of airborne microorganisms from concentrated animal-feeding operations (CAFOs) is a concern from a human and animal health perspective. To better understand the airborne microorganisms found in these environments, a number of collection and analytical techniques have been utilized and will be discussed in this review. The most commonly used bioaerosol collection method is the liquid impingement format, which is suitable with a number of culture-based and non-culture molecular-based approaches, such as polymerase chain reaction. However, the vast majority of airborne microorganism studies conducted at CAFOs utilize culture-based analyses. Because of the limitations often associated with culture-based analyses, we focused our discussion on the application of molecular-based techniques to identify and/or quantify microorganisms, as they have promising application in bioaerosol research. The ability to rapidly characterize airborne microorganisms will help to ensure protection of public and environmental health.

Keywords Airborne microorganisms · Bioaerosol · Concentrated animal-feeding operations · Impaction · Impingement · Nucleic acid · Polymerase chain reaction · Real-time PCR

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Introduction

Modern animal husbandry has changed from one that was low density pasture-based to one that predominately employs confinement of animals at high stocking density. Confined or concentrated animal-feeding operations (CAFOs) concentrate a large population of single species in one area to increase production and reduce costs. During recent decades, CAFOs have become common in many countries including The Netherlands, Denmark, France, USA, Canada, China, Germany, and Poland (Schulze et al. 2006). A consequence of high stocking densities combined with enclosed rearing facilities, in some cases, is that the air may contain bioaerosol levels that are sufficiently high to cause adverse health effects in both animals and workers (Thorne et al. 1992). Crook and Sherwood-Higham (1997) indicated that inhalation of airborne microorganisms and their constituents can be detrimental to health through infection, allergy, or toxicosis. As the environment within CAFOs can be potentially hazardous to both human and animal health at the facility as well as in surrounding areas, research is being pursued in order to quantify, characterize, and control the release of bioaerosols from CAFOs.

Bioaerosols is a term commonly used to describe viable and non-viable airborne biological particles, such as fungal spores, bacteria, pollen, and viruses and their fragments and byproducts (Grinshpun et al. 2007). Fungal spores, bacteria, and pollen are typically 1–30, 0.25–8, and 17–58 μm in diameter, respectively, while viruses generally have diameters <0.3 μm (Jones and Harrison 2004). Matthais-Maser et al. (2000) suggested that up to 28% (by volume) of the particulate matter suspended over remote land surfaces is comprised of biological particles. Womiloju et al. (2003) concluded that fungal cells and pollen accounted for 4–11% of the total mass of airborne

particulate matter <2.5 μm ($PM_{2.5}$). Although microorganisms are ubiquitous in the ambient environment, previous studies have shown higher airborne microorganism concentrations in animal houses than in industrial, residential, or ambient settings (Clark et al. 1983; Thorne et al. 1992; Griffiths et al. 1997).

Bioaerosols are typically associated with particulate matter or surrounded by a thin layer of water, having an aerodynamic diameter range of 0.5–100 μm (Lighthart 1994; Cox 1995). Bioaerosol particles 1–5 μm in diameter present the most concern since they are readily transported into the lung, with the greatest retention of the 1–2 μm particles in the alveoli (Salem and Gardner 1994). The microbial component of respirable bioaerosols contributes significantly to the pulmonary diseases associated with inhalation of agricultural dusts (Merchant 1987; Lacy and Crook 1988). The allergenic, toxic, and inflammatory responses are caused by exposure to not only viable but also non-viable microorganisms present in bioaerosols (Robbins et al. 2000; Gorny et al. 2002). An estimation of occupational and residential risks from bioaerosol exposure have been addressed by Brooks et al. (2005a, b) and Tanner et al. (2008). As the generation of bioaerosols from CAFOs is a concern from a human and animal health perspective, the sampling and analysis of airborne microorganisms is of great interest. Protection of public and environmental health is dependent upon the ability to efficiently collect bioaerosol samples, then accurately identify and quantify the airborne microorganisms.

In this concise review, we focus our discussion on bioaerosol sampling and sample processing methods that are most suitable to quantitatively and qualitatively determine airborne microorganisms at CAFOs, although their application to other situations is not limited. The major findings of bioaerosol studies conducted at CAFOs are also discussed. While this is not meant to be an exhaustive review of the literature, the reader will find an excellent array of peer-reviewed articles on aerosol science and molecular biology and their application to studies of air quality. This review will be very useful to those interested in conducting bioaerosol research using both traditional microbiological and molecular techniques.

Airborne microorganism sampling

The collection of airborne microorganisms is performed through active air sampling, which results in the efficient removal and collection of biological particles from the air in a manner that maximizes the ability to detect the organisms. Airborne microorganisms can be collected using a number of different techniques (Lundholm 1982; Juozaitis et al. 1994; Grinshpun et al. 1996; Terzieva et al. 1996; Duchaine et al. 2001), but two inertial techniques, surface impaction and liquid impingement, are used in the majority of outdoor aerosol studies. Filtration is a non-inertial technique that separates particles from the airstream when air is passed through a porous medium, such as fibrous filters, membrane filters, or etched membranes (Crook 1995a). For airborne microorganisms, however, filtration poses two major disadvantages: (a) dehydration of cells and therefore loss of viability and/or culturability due to the large volume of air passing over the particle that is deposited on a dry medium, and (b) inconsistent and poor recovery of the deposited material from certain filter types. Two additional techniques, gravity sampling and electrostatic precipitation, have been employed for airborne microorganism collection but are not routinely used due to calibration errors and unknown performance characteristics (Pillai and Ricke 2002).

The most common bioaerosol sampling techniques utilized at cattle, poultry and swine CAFOs are presented in Table 1. Direct impaction of airborne microorganisms on filters was used in ~40% of the studies, while a combination of liquid impingement and multistage or single stage impaction was used in ~33% of the studies. Other sampling techniques included use of a personal slide sampler to measure fungi in a cattle shed (Adhikari et al. 2004) and drag swab for determination of *Salmonella* in a poultry house (Endley et al. 2001). The target organisms in these studies included *Wallemia sebi*, total bacteria and fungi, Gram-negative bacteria, heterotrophs, *E. coli*, enteric bacteria, *Salmonella*, yeast, and molds.

Impaction samplers

The surface impaction method separates particles from the airstream by utilizing the inertia of the particles to force their deposition onto a collection surface (Grinshpun et al. 2007). The collection surface is usually an agar medium for culture-based analysis or an adhesive-coated surface that can be analyzed microscopically. A commonly used impaction system is the multi-stage Andersen viable sampler (Thermo Scientific, Waltham, MA, USA) that concentrates bioaerosols based on their size characteristics. Two-stage and six-stage Andersen models are available. The six-stage Andersen sampler is capable of concentrating particles in the size range of 0.65–7.0 μm in diameter (Grinshpun et al. 2007). Air enters the sampler through an inlet nozzle and heavier particles are deposited on the first stage. Lighter particles not deposited on the first stage are carried by the airstream onto the successive stages.

Single-stage impactors, which use an agar or adhesive-coated impacting surface, are available from a variety of

Table 1 Bioaerosol studies conducted at concentrated animal-feeding operations including the type of operation, the target organism, sampling techniques utilized and the analytical methods used for determination of microorganisms

Operation	Target organisms	Sampling techniques	Analytical methods	References
Cow house	*Wallemia sebi*	Direct impaction on filters	Culture techniques, conventional and real time PCR	Zeng et al. 2004
Duck-fattening unit	Total and aerobic Gram- negative bacteria, fungi, endotoxins	Liquid impingement, multi-stage impaction, and dust sampling	Culture techniques, whole blood assay, ELISA, limulus amebocyte lysate assay	Zucker et al. 2006
Cattle feedlot	Bacteria and fungi	Multi-stage impaction	Culture techniques	Wilson et al. 2002b
Cattle shed	Fungi	Multi-stage impaction and Personal slide sampler	Culture techniques and microscopy	Adhikari et al. 2004
Piggery sheds	Heterotrophs and *E. coli*	Liquid impingement and multi-stage impaction	Culture techniques	Chinivasagam and Blackall 2005
Swine barns	Total and Gram-negative enteric bacteria, total fungi	Multi-stage impaction, liquid impingement, direct impaction on filters	Culture techniques and fluorescence microscopy	Thorne et al. 1992
Swine barns	Cultural bacteria, Gram-negative bacteria, fungi	Liquid impingement, multi-stage impaction	Culture techniques	Chang et al. 2001
Poultry House	*Salmonella*	Drag swab, direct impaction on filters	Culture techniques and PCR	Endley et al. 2001
Swine barns	Heterotrophic bacteria	Direct impaction on filters	Culture techniques	Predicala et al. 2001
Swine barns	Total and respirable microorganisms	Direct impaction on filters, multi-stage impaction	Culture techniques	Predicala et al. 2002
Swine barns	Total bacteria and fungi	Single stage impaction	Culture techniques	Kim et al. 2006, 2007
Poultry House	Total bacteria	Direct impaction on filters, liquid impingement	Culture techniques	Woodward et al. 2004
Swine CAFO	Bacteria	Multi-stage impaction	Culture techniques	Green et al. 2006
Poultry house	Total aerobic bacteria	Single stage impaction	Culture techniques	Venter et al. 2004
Poultry, cow, and swine house	Airborne microorganisms	Direct impaction on filters	Epifluorescence microscopy	Heldal et al. 1996
Swine barns	Total and cultural bacteria	Liquid impingement, impaction on gelatin membranes	Culture techniques, real time PCR, denaturing gradient gel electrophoresis, phylogenetic analysis	Nehme et al. 2008
Dairy barns	Yeasts, molds, mesophilic bacteria, thermophilic bacteria	Liquid impingement	Culture techniques	Lange et al. 1997
Swine CAFO	Viable bacteria	Liquid impingement	Culture techniques	Rule et al. 2005

manufacturers. Adhesive-coated impacting surfaces are used for the detection of total fungal spores and pollen. In addition to the Andersen impactors, there are other impaction-based devices, such as the rotating impactor, slit sampler, and sieve-type sampler (Crook 1995b). Disadvantages associated with culture-based impactors are: (a) detection of microorganisms relies on their ability to grow after sampling and losses of culturability may occur due to sampling stress, (b) multiple particles each containing one or more organisms passing through a single impaction hole may be inaccurately counted as a single colony, and (c) culturable counts account for only 0.0001–10% of the total population within environmental samples, which can severely underestimate the total population of microorganisms in the sample (Parkes and Taylor 1985). This is also a problem when using culture-based techniques with impingement samplers.

Impingement samplers

Impingement samplers remove bioaerosols over a wide range of airborne particle concentrations (Grinshpun et al. 2007). The primary difference between impingement and impaction is that the bioaerosols are trapped in a liquid (e.g., water, mineral oil, buffered solution, or dilute peptone solution). In theory, buffered or dilute peptone solutions are used to maintain the viability of the microbial cells. Most impingers are constructed from glass with a single collection chamber; though multi-stage glass liquid impinges are available (Crook 1995b). The All-Glass Impinger (AGI)-30 (Ace Glass, Inc, Vineland, NJ, USA) is a single chamber design that has been widely used to measure bioaerosols under various conditions (Pillai et al. 1996; Chang et al. 2001; Rule et al. 2005; Tanner et al. 2005; Taha et al. 2006). The SKC BioSampler® (SKC Inc, Eighty-Four, PA, USA) is an improved design over the AGI-30 and can be operated for up to 8 h when mineral oil is used as the collection fluid (Lin et al. 1999). Both the SKC BioSampler® and AGI-30 operate under an airflow rate of 12.5 l min^{-1} through the use of a vacuum pump. During operation of the impinger, the microorganisms are suspended in the collection fluid, but the high airflow velocity required for efficient particle collection also causes re-aerosolization of the biological particles (Grinsphun et al. 1997; Lin et al. 1997) and stress that can lead to viability loss (Lin et al. 1999, 2000). One of the advantages of impingement samplers is the ability to utilize a variety of analytical methods. In addition to culture techniques, samples can also be analyzed via microscopy, flow cytometry, biochemical assays, immunoassays, and molecular techniques such as polymerase chain reaction (PCR) providing better detection of airborne microorganisms which may be non-culturable due to sampling stresses.

High-volume samplers

Another class of bioaerosol samplers that has recently evolved due to bioterrorism and biological warfare concerns is high-volume samplers. Some examples of these units are the SASS® 2300 (Research International, Monroe, WA), BioCapture® 560 (MesoSystems Technology, Inc, Albuquerque, NM), and the Spincon® (Sceptor Industries, Inc, Kansas City, MO). These samplers operate at flow rates of 200–450 l min^{-1} and the bioaerosols are captured in a concentrated liquid sample. While the high-volume samplers are very costly when compared to units such as the AGI-30 and SKC BioSampler®, they are generally more amenable to PCR-based analyses. The ASAP® model 2800 (Thermo Electron Corporation, Greenbush, NY, USA) sampler has an operational flow rate of 200 l min^{-1}, but collects aerosol particles by impaction on polyurethane foam. While the ASAP unit does not use a liquid impingement format like the other high-volume samples, it is currently being marketed as PCR-compatible. At this time, however, a search of the literature reveals a scarcity of peer-reviewed studies with respect to these or comparable units and their operating efficiencies (Bergman et al. 2005). For a comprehensive list of commercially available bioaerosol samplers see Grinshpun et al. (2007).

Sample processing

Once samples have been collected, choosing the appropriate analytical technique is important in order to best answer the question of interest. One of the most popular methods to assess microbial populations in aerosol samples has been the use of culture-based techniques. Culture-based techniques were employed in 89% of the studies reported here (Table 1). As mentioned above, culture-based techniques can drastically underestimate the microbial populations in environmental samples as less than 10% of the populations may be culturable. In order to improve microorganism detection, some studies have combined the use of culture techniques with other methods such as PCR (16%), microscopy (16%), denaturing gradient gel electrophoresis (DGGE, 5%), and immunoassays (5%). Sample preparation is important for all of these techniques, as microorganism populations in bioaerosol samples tend to be small and, therefore, concentration of samples is essential. The most commonly used sample preparation methods compatible with the molecular characterization of bioaerosols can be found below.

Table 2 Filters utilized for preparation of bioaerosol samples for molecular methods including the filter type, type of sample, and the methods used for sample preparation and analysis

Filter	Sample type	Methods	References
Polytetrafluoroethylene, Polyvinylidene difluoride	Bacterial cells in water collected on filters	Freeze thaw lysis of cells from filtered samples, PCR DNA amplification with filters present	Bej et al. 1991a, b
Polycarbonate	Direct impingement of bioaerosols on filter	Filters washed in buffer to remove bacteria, DNA extraction (chemical/enzymatic), RT-PCR	Zeng et al. 2004
Polycarbonate	Bioaerosols collected in liquid impingers and filtered	Impinger solution filtered, DNA extraction, PCR, cloning, sequencing	Paez-Rubio et al. 2005
Track etched polyester	Direct impingement of bioaerosols on filter	Filters washed in buffer to remove bacteria, DNA extraction (physical/chemical/enzymatic), microarray analysis	Wilson et al. 2002a
Mixed cellulose nylon	Bioaerosols collected in liquid impingers and filtered	Cell lysis and DNA extraction (chemical/enzymatic) performed on filters, solid-phase PCR used for amplification	Alvarez et al. 1994
Nitrocellulose	Filtration of bacterial cells in water	Cell lysis and DNA extraction (chemical/enzymatic) performed on filters, solid-phase PCR used for amplification	Toranzos and Alvarez 1992
Polyethersulfone	Direct impingement of bioaerosols on filter	Filters were dried and dissolved in chloroform, DNA extraction (chemical), nested PCR assay	Stärk et al. 1998

Concentration and filter elution

After bioaerosols are collected in a liquid impingement solution, it is necessary to concentrate the microorganisms before molecular methods, such as PCR, can be performed. This is necessary because the impingement solution usually contains a relatively low microbial concentration, which must be maximized to ensure sensitivity and quantification for PCR are achieved. A variety of filter materials have been tested for their compatibility with PCR (Table 2) such as polytetrafluoroethylene (PTFE), polycarbonate, polyvinylidene difluoride, nylon, mixed cellulose ester, and nitrocellulose (Bej et al. 1991a). Bej et al. (1991a) reported that PCR was not inhibited by the presence of PTFE and polyvinylidene difluoride filters, with PTFE giving the greatest sensitivity, but was inhibited by polycarbonate, nitrocellulose, and cellulose acetate filters. Both Nytran (Alvarez et al. 1994) and nitrocellulose (Toranzos and Alvarez 1992) filters have been successfully used in solid-phase PCR, where cell lysis and PCR amplification are performed on the membrane.

Since DNA does bind to some filters, it is recommended that all filters be removed before cell lysis and PCR amplification. Filter materials that have been successfully used in PCR-based bioaerosol studies using liquid samples from glass impingers are Nytran (Alvarez et al. 1994), polycarbonate (Paez-Rubio et al. 2005), nylon (Alvarez et al. 1995), and Teflon (Alvarez et al. 1995). Aerosol samples can also be directly impinged onto filters for subsequent PCR analysis; filters used for this purpose are tracked-etched polyester (Wilson et al. 2002a), polycarbonate (Zeng et al. 2004), and polyethersulfone (Stärk et al. 1998). The filters are added to sterile distilled water (Alvarez et al. 1995) or buffer solution (Wilson et al. 2002a; Zeng et al. 2004; Paez-Rubio et al. 2005) and then the microorganisms are eluted via agitation such as vortexing, shaking, or sonication.

Cell lysis and nucleic acid purification

After elution, the filter is removed and the cells are then prepared for lysis, which can be performed either through physical, chemical, or enzymatic methods. Physical methods include bead beating, sonication, microwave heating, and thermal shock (Roose-Amsaleg et al. 2001), but bead beating and sonication can cause significant DNA shearing (Picard et al. 1992; Miller et al. 1999; Bürgmann et al. 2001). Freeze-thaw lysis has been shown to release 70–75% of DNA in bacterial cells after one cycle with complete lysis within six cycles (Bej et al. 1991b). Chemical lysis, either alone or in combination with enzymatic methods, has been used extensively. The most widely used detergent is sodium dodecyl sulfate (SDS), whose function is to break up and dissolve cell wall lipids. Detergents are used in combination with heat treatments and chelating agents (e.g., EDTA) and various buffers (Tris and phosphate). In addition to a detergent, many protocols include enzymatic lysis. Lysozyme is a commonly used lytic enzyme that breaks the β-1,4-glycosidic bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid in peptidoglycan, thereby weakening the cell wall. Some proteases, like proteinase K, are also used to remove contaminating proteins (e.g., nucleases) that might otherwise degrade nucleic acids during purification. The protease, achromopeptidase, has been used with

lysozyme to increase the lysis of anaerobic Gram-positive cocci (Ezaki and Suzuki 1982) and extraction efficiency of nucleic acids from *Frankia* (Simonet et al. 1984).

Detailed methods on the extraction and purification of nucleic acids can be found in Sambrook and Russell (2001) and Ausubel et al. (2002). Purification of nucleic acids in bacterial lysates is generally accomplished by first mixing with equal volumes of phenol and chloroform. Phenol is used because it removes the proteins from the aqueous phase; chloroform is generally not necessary, but it is used to remove residual phenol from the aqueous phase. The nucleic acids are then precipitated from the aqueous phase by additions of ethanol and collected by centrifugation. The nucleic acids can then be dissolved in buffer (e.g., Tris-EDTA) and stored at −20°C. Alternatively, nucleic acids can be purified using the many commercially available spin column formats that utilize silica-nucleic acid binding (Qiagen, Inc., Fremont, CA, USA; Mo Bio Laboratories, Carlsbad, CA, USA; Promega, Inc., Madison, WI, USA; MP Biomedicals, Solon, OH, USA; Invitrogen, Inc., Carlsbad, CA, USA). As a result, the spin kits require no phenol or chloroform purification or alcohol precipitation. After the silica-based membrane has been loaded with cell lysate, the DNA or RNA is cleaned by rinsing with an ethanol-containing buffer, and then eluted using a small volume of buffer or water.

The characterization of airborne microorganisms

Culture versus molecular-based approaches

Many of the available bioaerosol sampling methods rely on culture-based techniques for the characterization and quantification of airborne microorganisms. Microorganisms (fungi and bacteria) that are collected on a nutrient agar surface by impaction can be cultivated directly. However, only those cells which survive, reproduce, and produce visible colonies under the specified culture conditions will be enumerated. The disadvantage of culture-based techniques is that not all microorganisms are culturable, while they still may be viable (Heidelberg et al. 1997). This could lead to an underestimation of the total microorganism concentration in the aerosol sample. With culture-based techniques, non-culturable microorganisms and their associated byproducts that may cause health effects will go undetected. While liquid samples from impingers are commonly used for culture-based analyses, they can also be analyzed by microscopy to determine total microorganism concentrations or by biochemical, immunological, and molecular assays to detect specific microorganisms, both culturable and non-culturable (Cruz and Buttner 2007).

As an alternative to culture-based techniques, the detection of microorganisms in aerosols by PCR has become increasingly popular over the last two decades (Alvarez et al. 1994; Wakefield 1996; Stärk et al. 1998; Olsson et al. 1998; Williams et al. 2001; Wu et al. 2003; Zeng et al. 2004; Paez-Rubio et al. 2005; An et al. 2006) allowing for the detection of target nucleic acid sequences, thereby eliminating the need to cultivate microorganisms for their detection and identification. This is particularly useful for microorganisms that are difficult to culture, slow growing or have never been cultured before, providing increased sensitivity over traditional culture-based methods (Josephson et al. 1993; Alvarez et al. 1994). A limitation of the PCR assay, however, is the inability to distinguish between non-viable and viable microorganisms. While non-viable pathogenic microorganisms do not present an infectious disease risk, the presence of their DNA in a sample will often produce a positive PCR result. Therefore, one cannot truly determine if the positive result represents a potential disease threat if the viability of the microorganisms in the original sample was unknown. A positive detect for targeted microorganisms only means that a sample contains viable or non-viable cells or both.

Non-quantitative PCR

Traditional PCR involves the separation of DNA (usually a specific gene or portion of a gene) into two strands, the annealing of oligonucleotide primers to the template DNA, and then the primer-template is elongated by use of a DNA polymerase enzyme (e.g., *Taq* polymerase). During PCR, each of the steps is accomplished by regulating the temperature of the reaction and, as a result, multiple copies of the template are produced. Guidance on the optimization of PCR can be found in several laboratory manuals (Weissensteiner et al. 2003; Hughes and Moody 2007). By using carefully designed primers, the genetic sequence of a specific microorganism or microbial function can be targeted and amplified. If ribonucleic acid (RNA) is targeted, then the RNA must be converted into complementary DNA (cDNA) through a reverse transcription process, after which the resultant cDNA is PCR amplified. One advantage of targeting RNA (e.g., mRNA) is that it has a very short half-life and, therefore, it is a good indicator of viable microorganisms (Bej et al. 1991b).

The amplified DNA is visualized most often by running the samples in an electrophoresis gel (e.g., agarose or polyacrylamide), staining the DNA within the gel with ethidium bromide, and viewing the separated DNA under UV light. A standard molecular weight marker is run along side the samples so the size of the DNA can be determined. The amplified DNA can also be processed for genetic fingerprinting, clone library analysis, and microarray

analysis (see subsections immediately below). While these molecular techniques are not quantitative, they are useful in that they can be used to study microorganisms with known health effects in bioaerosols instead of studying indicator organisms.

Denaturing and temperature gradient gel electrophoresis

Popular PCR fingerprinting techniques used to characterize microbial communities are DGGE and temperature gradient gel electrophoresis (TGGE) (Muyser et al. 1993; Muyser and Smalla 1998). These techniques are used to separate amplified DNA that are similar in length but of various sequence compositions. In environmental studies the 16S or 23S ribosomal RNA (rRNA) genes are commonly targeted (Amann et al. 1995; Marchesi et al. 1998; Baker et al. 2003; Chakravorty et al. 2007). Double stranded DNA (dsDNA) is loaded onto a polyacrylamide gel containing an increasing gradient of denaturants (usually urea and formamide) or temperature in the case of TGGE. As the dsDNA migrates, the sequence of the fragment will determine the point in the gel at which denaturation will start to retard mobility. The banding pattern of the separated fragments can then be visualized after staining, photographed, and then analyzed to characterize the microbial community structure and diversity (Dungan et al. 2003; Dilly et al. 2004; Seghers et al. 2004).

The individual bands, which often represent more than one organism, are referred to as operational taxonomic units (OTUs). Afterwards, the DNA bands can be removed from the gel, subject to another round of PCR amplification and then directly sequenced or sequenced after cloning. The sequence information can then be compared to publicly available databases for identification, such as GenBank (http://www.ncbi.nlm.nih.gov), and used to develop taxonomic and/or phylogenetic information about the amplifiable members of the microbial community. Frequency analysis of groups of organisms that constitute OTUs in clone libraries is currently the most widely used approach for studying structures in microbial communities (Rudi et al. 2006), while multivariate statistical analyses is another emerging technique (Mouser et al. 2005; Rudi et al. 2007).

Nehme et al. (2008) utilized DDGE and phylogenetic analysis to characterize the bioaerosol community of swine confinement buildings (Tables 1, 3). Utilizing these techniques they demonstrated that total bacterial concentrations were 100-fold to 1000-fold higher than the total cultural bacteria. The phylogenetic analysis revealed that a large number of the sequences were related to Gram-positive anaerobic bacteria such as *Clostridia* and samples also contained low proportions of *Bateroidetes* and *Lactobacillales-Streptococcales* sequences.

Terminal restriction fragment length polymorphism

Like DGGE and TGGE, terminal restriction fragment length polymorphism (T-RFLP) is a genetic fingerprinting technique (Liu et al. 1997). It also addresses some of the limitations of restriction fragment length polymorphism (RFLP), also known as amplified ribosomal DNA restriction analysis (ARDRA) (Tiedje et al. 1999). The difference between these techniques is that in T-RFLP, primers used to amplify the target DNA are fluorescently labeled at the 5′ end, and then the PCR amplicons are cut with restriction enzymes to create DNA fragments of varying size. The size of the terminal fragments is then determined using an automated DNA sequencer. While T-RFLP does often yield a higher number of OTUs when compared to DGGE, the disadvantage is that the PCR amplicons cannot be recovered and, hence, used to obtain taxonomic or phylogenetic information about the microbial community. While the above mentioned genetic fingerprinting techniques permit rapid analysis of numerous samples, they generate only superficial descriptions of microbial community compositions (Valinsky et al. 2002).

Ribosomal intergenic spacer analysis

Another method of genetic fingerprinting is the use of ribosomal intergenic spacer analysis (RISA), which exploits the variability in the length of the intergenic spacer between the small (16S) and large (23S) subunit rRNA genes in the *rrn* operon (Ranjard et al. 2001). RISA has been used to contrast diversity in soils (Borneman and Triplett 1997), to determine community structure of baterioplankton in lakes (Øvreås et al. 1997), as well as identifying genetic relatedness and origins of airborne clostridia (Pillai et al. 1996). An automated RISA (ARISA) method has been developed to improve both resolution and analysis. ARISA involves the use of a fluorescence-tagged oligonucleotide primer for PCR amplification and for subsequent electrophoresis in an automated system, allowing community structure to be rapidly investigated (Ranjard et al. 2001). ARISA has been used to characterize bacterial and fungal soil communities (Ranjard et al. 2001) as well as freshwater bacterial communities (Fisher and Triplett 1999). While large numbers of samples can be compared with this technique, it may not be easy to make these comparisons as different primer sets have been used which can result in different amplification efficiency and selective amplification of some templates in a mixture of DNA.

Microarray

The recent development of array-based methods, which permits thousands of hybridization events to be examined

Table 3 Prevalent organisms identified at a variety of concentrated animal-feeding operations including the type of facility, the organisms identified and major findings of study

Operation	Prevalent organisms	Notes	References
Duck-fattening unit	*Enterobacteriaceae, Pseudomonadaceae, Vibrionaceae, Legionellaceae*	The inflammation-inducing potential was overestimated by the LAL assay in all samples. This was potentially due to the overestimation of the inflammatory potential of endotoxins originating from *Pseudomonadaceae*	Zucker et al. 2006
Cattle feedlot	*Bacillus, Chrysobacterium, Corynebacterium, Helococcoccus, Micrococcus, Paenibacillus, Alternaria* sp., *Bipolaris* sp., *Chryosporium* sp., *Cladosporium* sp., *Penicillium* sp.	Only non-pathogenic Gram-positive bacteria were recovered. Fungi were recovered in smaller numbers than bacteria, and none were pathogenic	Wilson et al. 2002b
Cattle shed	*Alternaria,* Aspergilli/Penicilli, *Choanephora, Cladosporium, Corynespora, Curvularia, Drechslera, Memnoniella, Nigrospora, Periconia, Torula, Leptosphaeria, Ganoderma*	Average concentration range of total fungal spores was 233–2985 m^{-3} and concentration of viable colony-forming units ranged between 165 and 2225 c.f.u. m^{-3}. Higher concentrations of fungal spores were found during November–February and June–September	Adhikari et al. 2004
Swine barns	*Cladosporium, Cephalosporium, Aspergillus, Alternaria, Penicillium, Fusarium, Curvularia, Sclerotium, Geotrichum, Drechslera, Ulocladium, Diplococcus, Oidium, Aureobasidium, Stemphyllium, Trichoderma, Monilia, Paecilomyces, Zygomyces, Botrytis, Candida, Actinomycetes*	Mean concentrations of cultural bacteria and Gram-negative bacteria were 3.3×10^5 and 144 c.f.u. m^{-3}, respectively. The concentration of airborne culturable fungi was about 10^3 c.f.u. m^{-3}. The highest airborne levels of culturable bacteria and Gram-negative bacteria were identified in the finishing units while the nursery stalls were the least contaminated	Chang et al. 2001
Swine barns	*Staphylococcus, Pseudomonas, Bacillus, Listeria, Enterococcus, Nocardia, Lactobacillus, Penicillium*	The overall mean respirable airborne microorganism concentrations were 9.0×10^3 c.f.u. m^{-3} measured by filtration and 2.8×10^4 c.f.u. m^{-3} by impaction. Total and respirable c.f.u. concentrations measured by impaction were higher than by filtration	Predicala et al. 2002
Swine CAFO	*Staphylococcus aureus*	There was a marked increase in bacterial c.f.u. m^{-3} inside the facility (18,132 c.f.u. m^{-3}) vs. upwind (63 c.f.u. m^{-3}) and a steady downwind decrease out to approximately 150 m	Green et al. 2006
Swine barns	*Eubacterium, Clostridium, Bacillus-Lactobacillus-Streptococcus, Bacteroidetes*	Biodiversity was unchanged during seasons of the year. Total bacterial concentrations were 100–1000 times higher than the total cultural bacteria	Nehme et al. 2008

in parallel, has brought great promise to the field of environmental microbiology (Zhou 2003). Microarray technology is based upon the hybridization of complementary sequences of nucleic acids, where amplified DNA representing individual genes (cDNA microarrays) or oligonucleotide probes (oligonucleotide microarrays) are attached to a solid surface (Lucchini et al. 2001). Microarrays developed for use in environmental microbiology studies are the phylogenetic oligonucleotide array (Loy et al. 2002), functional gene array (Liebich et al. 2006), community genome array (Wu et al. 2004), gene expression array (Dennis et al. 2003), and whole-genome open reading frame array (Murray et al. 2001). While microarray-based detection is potentially quantitative, a drawback of their use in environmental studies is that a high copy number of target DNA/RNA is needed to obtain a sufficient signal (Zhou and Thompson 2002). Techniques to improve the sensitivity of microarrays have been reported (Denef et al. 2003). A microarray study targeting the small-subunit (SSU) rRNA genes of bacteria in an air sample was conducted by Wilson et al. (2002a). In the air sample, the microarray results compared favorably with cloning and sequence analysis of amplicons in determining the presence of phylogenetic groups.

Quantitative PCR

Because the quantity of an etiologic agent in aerosols is also important when assessing health risks to humans and the environment, there is a need for quantitative PCR methodology (Stetzenbach et al. 2004). Real-time PCR (RT-PCR) is a quantitative PCR method that employs fluorescent dyes or probes to quantify the number of copies of a target DNA sequence in a sample. Compared to conventional PCR, the advantages of RT-PCR are species-specific identification and rapid quantification. It also eliminates the need for post-PCR processing, such as gel electrophoresis, which helps increase sample throughput and reduces the chances of carryover contamination (Mackay 2004). The number of copies of a target gene in an aerosol or other environmental sample (e.g., soil, water, food) is determined by monitoring the increase in the amplicon concentration during PCR and then regressing to the original concentration. Standard curves can be prepared by serially diluting genomic DNA that has been isolated from a pure bacterial culture (Ibekwe et al. 2002; Peccia and Hernandez 2006). A relationship between the quantity of DNA and colony forming units (c.f.u.) can then be established. This approach, however, does not take into account the extraction efficiency of the DNA kit, which could then exacerbate potential differences when the kits are used for air samples containing low microorganism concentrations (An et al. 2006). This issue must be addressed by future research if RT-PCR is to be accurately and effectively used to quantify microorganisms in air samples.

The RT-PCR instrument is a thermal cycler with an optical module, which detects the fluorescent signal emitted during each amplification cycle of the target gene. Double-stranded DNA-binding dyes (e.g., SYBR® Green, Molecular Probes, Inc, Eugene, OR, USA) and sequence-specific fluorescently labeled oligonucleotide probes (e.g., *Taq*Man®, Applied Biosystems, Foster City, CA, USA) or molecular beacons (Tyagi and Kramer 1996) are used to monitor amplicon synthesis. Intercalating binding dyes are non-specific and generate fluorescence when bound to dsDNA (Morrison et al. 1998). Therefore, quantitation with DNA-binding dyes is usually less accurate than with sequence-specific probes because all dsDNA is detected, including primer dimmers, resulting in false positive signals (Sharma et al. 2007).

Fluorescently labeled probes (20–60 nucleotides) contain a fluorophore (reporter dye) and quencher at the 5′ and 3′ ends, respectively, which anneal to the target DNA between the primer binding sites. The close proximity of the quencher (e.g., TAMRA: 6-carboxy-tetramethyl-rhodamine) to the fluorophore inhibits fluorescence, but as the DNA is synthesized the fluorophore is cleaved by the *Taq* polymerase, allowing it to fluoresce. Alternatively, in minor groove binding (MGB) probes, the TAMRA quencher is replaced by a non-fluorescent quencher (e.g., BHQ®: Black Hole Quencher, Bioresearch Technologies, Inc, Novato, CA, USA). Compared to TAMRA, a non-fluorescent quencher lacks native fluorescence, thereby increasing signal-to-noise ratios and sensitivity. Probes for distinct target sequences can be labeled with unique reporter dyes (e.g., FAM: 6-carboxyfluorescein; TET: tetrachloro-6-carboxyfluorescein; HEX: 6-carboxyfluorescein; VIC: proprietary dye developed by Applied Biosystems), thus allowing for the quantification of distinct sequences in one reaction tube (technique is known as Multiplex RT-PCR). The fluorescence is tracked by the optical module at the end of each PCR cycle up to a threshold cycle (*Ct*), which is proportional to the starting amount of nucleic acid (Heid et al. 1996). The *Ct* is a point at which the fluorescent intensity is greater than background; the threshold line is set in the exponential phase of the amplification for the most accurate reading.

In addition to the creation of a standard curve, the use of an internal amplification control (IAC) in RT-PCR is gaining acceptance (Hoofer et al. 2003; Klerks et al. 2004; Murphy et al. 2007). A major concern when applying PCR for the detection of pathogens in environmental samples and foods is the reporting of false-negative results. Inhibition of nucleic acid amplification during PCR can occur through the degradation and sequestration of target DNA

and primers, a reduction in polymerase activity, or a number of other possible reasons (Wilson 1997). As a result, it is necessary to include a control strategy so that essential information is available to validate the PCR results (Murphy et al. 2007). The IAC, which consists of a non-target DNA sequence from a known source, is included in the same reaction tube and is co-amplified with the target sequence (Hoofar et al. 2003). In PCR without an IAC, a negative response can mean that no target sequence was present, the PCR thermal cycler has malfunctioned, inhibitory substances are present, or there is poor polymerase activity. When an IAC is implemented, signal will always be produced when no target DNA is present. When no IAC and target signal is produced, then PCR has failed, thus, preventing the reporting of a false-negative result.

RT-PCR has been successfully used in many environmental studies to detect and/or quantify *Escherichia coli* O157:H7 and *Salmonella* spp. in soil (Ibekwe and Grieve 2003; Ibekwe et al. 2004), feces (Bono et al. 2004), wash wastewater (Ibekwe et al. 2002; Malorny et al. 2007; Wolffs et al. 2007), and food (Fortin et al. 2001; Heller et al. 2003; Ellingson et al. 2004). While the detection limit for these pathogens is generally $>10^2$ c.f.u. g^{-1} in food, soils, and feces and $>10^2$ c.f.u. ml^{-1} in aqueous samples, substantially lower detection limits have been achieved after sample enrichment. *Escherichia coli* O157:H7 detection limits in soil were lowered to <10 c.f.u. g^{-1} with a 16-h enrichment (Ibekwe and Grieve 2003) and 1 c.f.u. ml^{-1} in raw milk and apple juice with a 6-h enrichment (Fortin et al. 2001). Although sample enrichment does increase sensitivity, it essentially renders RT-PCR non-quantitative. RT-PCR has also been used to quantify ammonia-oxidizing bacteria in soil (Hermansson and Lindgren 2001), enterococci and human adenovirus in water (He and Jiang 2005), *Vibrio vulnificus* in shellfish and water (Panicker et al. 2004), *Lactobacillus salivarius* in broiler chickens (Harrow et al. 2007) and *Clostridium tyrobutyricum* in milk (López-Enríquez et al. 2007).

Currently there are no accepted protocols for the PCR-based analysis of airborne microorganisms and, to date, only a handful of studies have utilized RT-PCR to quantify airborne fungal spores, viruses, and bacteria (Buttner et al. 2001; Schweigkofler et al. 2004; Zeng et al. 2004; Chen and Li 2005; An et al. 2006; Pyankov et al. 2007; Nehme et al. 2008).

Zeng et al. (2004) utilized RT-PCR to identify and quantify *Wallemia sebi* in bioaerosol samples collected at a cattle feeding operation (Table 1). By utilizing RT-PCR they were able to demonstrate that there are relative high concentrations of this fungus on farms handling hay and grain and in cattle barns, whereas traditional culture techniques often did not detect large concentrations due to the slow growth on culture media. The detection and quantification of *W. sebi* is important as it is suspected to be a causative agent of farmer's lung disease. Nehme et al. (2008) utilized RT-PCR to quantify total bacteria in bioaerosol samples collected from swine confinement buildings and noted that RT-PCR estimated concentrations of total bacteria that were 100-fold to 1000-fold greater than those using culture-based techniques.

Airborne microorganisms found at CAFOs

The prevalent microorganisms identified in bioaerosol samples taken from a variety of CAFOs are presented in Table 3. The mean concentration of cultural bacteria and Gram-negative bacteria reported in swine barns by Chang et al. (2001) were 3.3×10^5 and 144 c.f.u.m^{-3}, respectively, whereas the concentration of airborne culturable fungi was approximately 10^3 c.f.u. m^{-3} (no background concentrations were determined). In this study, the highest airborne levels of culturable bacteria and Gram-negative bacteria were identified in the finishing units while the nursery stalls were the least contaminated. The prevalent organisms identified by Chang et al. (2001) were: *Aspergillus, Alternaria, Penicillium, Fusarium, Curvularia, Sclerotium, Geotrichum, Drechslera, Ulocladium, Diplococcus, Oidium, Aureobasidium, Stemphyllium, Trichoderma, Monilia, Paecilomyces, Zygomyces, Botrytis, Candida,* and *Actinomycetes*. Predicala et al. (2002) reported that the overall mean respirable airborne microorganism concentrations in swine barns were 9.0×10^3 c.f.u. m^{-3} measured by filtration and 2.8×10^4 c.f.u. m^{-3} by impaction and that total and respirable c.f.u. concentrations measured by impaction were higher than by filtration (no background concentrations were determined). The prevalent organisms identified by Predicala et al. (2002) were: *Staphylococcus, Pseudomonas, Bacillus, Listeria, Enterococcus, Nocardia, Lactobacillus,* and *Penicillium*. Green et al. (2006) quantified *Staphylococcus aureus* at a swine CAFO as well as downwind of the facility to determine off site transport of these microorganisms via bioaerosols. They noted that there was a marked increase in bacterial c.f.u. m^{-3} inside the facility (18,132 c.f.u. m^{-3}) vs. upwind (63 c.f.u. m^{-3}) and a steady downwind decrease out to approximately 150 m. Nehme et al. (2008) examined the influence of seasonal variation on microorganism biodiversity and found that biodiversity was unchanged during seasons of the year and consisted mainly of *Eubacterium, Clostridium, Bacillus-Lactobacillus-Streptococcus,* and *Bacteroidetes*.

At cattle feedlots, Wilson et al. (2002b) found only non-pathogenic Gram-positive bacteria such as: *Bacillus, Chrysobacterium, Corynebacterium, Helococcoccus, Micrococcus,* and *Paenibacillus*. They also identified smaller numbers of non-pathogenic fungi such as: *Alternaria* sp.,

Bipolaris sp., *Chryosporium* sp., *Cladosporium* sp., and *Penicillium* sp. Adhikari et al. (2004) reported average concentration ranges of total fungal spores from 233 to 2985 m^{-3} and concentration of viable c.f.u. from 165 to 2225 m^{-3} at cattle sheds (no background concentrations were obtained). Seasonal analysis of bioaerosols determined that higher concentrations of fungal spores were found at the cattle sheds during November–February and June–September. One report from a fattening unit with 15,000 ducks identified *Enterobacteriaceae* (57%), *Pseudomonadaceae* (26%), *Vibrionaceae* (7%), and *Legionellaceae* (1%) as the most abundant airborne Gram-negatives (Zucker et al.Zeng et al. 2004). Maximum airborne concentrations of total aerobic bacteria and Gram-negative bacteria were 1.7×10^6 and 1.8×10^2 c.f.u. m^{-3}, respectively.

Concluding remarks

As animal-rearing practices have shifted towards the use of high density CAFOs over the past several decades, the generation of bioaerosols from these facilities and the impacts on both animal and human health have become concerns. At present, there has been little published data reporting bioaerosol sampling techniques as well as techniques for the characterization and enumeration of microorganisms in aerosol samples collected at CAFOs. The most prevalent sampling techniques employed at a variety of CAFOs have included direct impaction on filters, multistage impaction, and liquid impingement. Each of these methods have their own advantages and disadvantages, with the greatest disadvantage with all sampling techniques being the survivability and viability of the microorganisms, which can impair further identification and enumeration when relying on traditional culture-based techniques. In order to improve microorganism detection and enumeration, some studies have combined the use of traditional culture-based techniques with molecular methods such as RT-PCR, which allows for the identification and quantification of non-culturable microorganisms. Several other molecular techniques which have not yet been utilized for analyzing bioaerosols from CAFOs include T-RFLP, RISA, and microarray analysis. Although not quantitative, a brief discussion of these techniques was included as we believe they are equally suitable for the characterization of airborne microorganisms in terms of broader community dynamics. In the future, the application of molecular-based tools to analyze bioaerosols derived from CAFOs (and other similar environments) will allow individuals to better characterize and enumerate potentially harmful microorganisms found at these facilities and to track the transport of these microorganisms off site.

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