Concentration, size distribution, and control of swine viruses associated with airborne particles

A THESIS

SUBMITTED TO THE FACULTY OF

UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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March, 2016

Acknowledgements

First of all, I would like to thank my advisor **Dr. Montse Torremorell** for her guidance, support and friendship during all these years. Montse, not only you are great researcher and mentor, you are also a working model for all your students and a generous person. I would like to also thank my co-advisor **Dr. Peter Davies** for his support during these years and his guidance in order to do a better job on each of the projects we worked together. Many thanks to both of you for all that I learnt during these 4 intensive years. I also would like to thank my committee member **Dr. Pete Raynor** for being very patient and helpful answering all my doubts and questions about aerobiology and **Dr. Sagar Goyal** for his guidance during nice conversations all these years. Both have had been a great support during the process of developing this thesis.

Also I would like to give special thanks to **Dr. Bernard Olson, Dr. Zuo Zhilli, Dr. Sally Robinson** and, very specially **My Yang** for their support and their unconditional advice. Furthermore, I would like to give my appreciation to the field veterinarians and producers that allowed me to work on their farms while experiencing disease outbreaks. And finally, I would like to thank my graduate school colleagues at the UMN, such a great team to be involved with! Y por último, pero no por ello menos importante, quiero dar las gracias a mis padres **MariCarmen y Martin** por apoyarme en cada una de las decisiones importantes de mi vida y por estar a mi lado cada vez que los he necesitado. Nada hubiera sido posible sin su incondicional ayuda.

Dedication

This work is dedicated to my beloved daughters Martina and Ella, and son-to-be Liam, and very especially to their proud papa and best husband and partner, Tim, because his friendship and love made this journey possible.

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General introduction

In livestock, viruses associated with airborne particles have the potential to be transmitted from infective premises to naïve populations and propagate epidemics that cause substantial economic losses and impact the food supply. Specifically for swine pathogens, foot and mouth disease virus (FMDV), porcine reproductive and respiratory syndrome virus (PRRSV), and influenza A virus (IAV) are examples of airborne pathogens able to cause devastating losses due to their pathogenicity, ability to travel long distances and, for IAV, zoonotic risk. The overall goal of this thesis is to improve the understanding of airborne transmission of swine pathogens and to prevent disease spread within and between farms.

Risk of airborne transmission is most problematic in areas of high farm and animal density where regional spread can be difficult to contain. Over the last few years, swine producers have invested significant funds to improve their biosecurity and protect their investments, yet results have been inconsistent. Therefore, there is a need to characterize infectious aerosols under experimental and field conditions in order to understand within and between-herd transmission and to provide much needed information to develop technologies to mitigate disease spread.

Infectious agents travel associated with particles of various natures. The composition and size distribution of these particles will influence important aspects of disease transmission dynamics including the site of deposition in hosts during inhalation, the time they remain airborne and the distance transported, and survivability and infectivity of the pathogens. Understanding the size of particles with which viruses are associated while airborne will give new insights into specific aspects of airborne virus

transmission. Thus, particle size is central to the epidemiology of airborne pathogens.

In the literature there is limited information regarding the particle sizes with which infectious agents are associated and the information that is available has been derived either from studies with human viruses (i.e. influenza virus, norovirus), animal viruses in artificially generated aerosols or experimentally infected animals. In addition, the information generated has been obtained with air samplers with flow rates between 2 and 22 liters per minute (L/min). These conditions of low air flow and high pathogen load which may not accurately represent field conditions. The limited number of studies of animal viruses conducted under field conditions have not provided insights into the particle size association of viruses, in part due to common use of samplers that do not separate particles based on size. As part of this thesis, a detailed assessment of a high volume, filter- based, size-differentiating air sampler will be performed to assess the sensitivity of virus detection under experimental and field conditions. The main purpose of these studies is to assess and refine methods to study between-farm airborne transmission, taking into consideration the nature of particles with which airborne viruses are associated.

In addition, there have been few studies that characterized the particle concentrations, size distributions, and infectivity of airborne viruses, and in particular for viruses responsible for devastating outbreaks in the contemporary US swine industry. In order to fill these gaps in knowledge, a set of studies with artificially generated infectious aerosols as well as bioaerosols generated by pigs will be performed in experimental and field

settings. We will quantify the amount of viral material using quantitative RNA-based methods and investigate infectivity using cell culture and animal-based bioassay methods. This is crucial information to understand disease transmission and to build risk-based transmission models. In addition, because we are interested in containing and preventing spread of diseases among farms, we will investigate one technology for its potential to mitigate airborne pathogen spread.

In summary, the main goal of this thesis is to expand knowledge on airborne transmission of swine pathogens as well as to investigate technologies aimed at preventing disease spread within and between farms. The specific goals of the thesis chapters are: 1) to review the different aspects related to bioaerosols, factors that affect airborne transmission, and control methods directed at minimizing the risk of airborne transmission in food animal production; 2) to evaluate the distribution and infectivity of particles bearing relevant viruses for the swine industry in bioaerosols emitted from infected pigs under experimental conditions; 3) to characterize the performance of two size-differentiating air samplers, of low and high volume, under experimental conditions to further assess between herd transmission; 4) to measure the concentrations of viruses in the indoor and outdoor air of food animal facilities; and, 5) to evaluate the capabilities of an electrostatic particle ionization technology to prevent airborne pathogen spread.

A better understanding of airborne transmission of swine pathogens and prevention of disease spread should have an important impact on animal and human health, animal well-being and economics of producers within the swine industry. Outcomes from this thesis will allow producers, veterinarians

and health officials to make informed decisions to protect animal and human populations from risk of airborne virus spread.

Chapter 1: Literature review

Animal disease transmission

The spread of infectious diseases in livestock is a global concern due to its impact on animal health, food security and food safety. Diseases affect the productivity of animals and the wellbeing of people. Diseases cost producers significant amounts of money which affects their sustainability and the communities where they live. Specifically for swine, the spread of airborne diseases is concerning because airborne diseases are difficult to control, spread rapidly, and in some instances, represent a risk to public health. The impact on food security is especially relevant for pigs since pork is the most consumed meat worldwide and markets can destabilize compromising production and sales of pork. As a result, strategies to effectively control airborne diseases are needed.

Definitions and classification of disease transmission

The term **disease** is defined by the Dorland's Medical Dictionary as “a particular abnormal condition, a disorder of a structure or function that affects part or all of an organism”. In animals, as well as humans, disease is often constructed as a medical condition associated with specific symptoms and signs and which, for infectious diseases, are caused by factors external to the host. **Infectious diseases** are caused by pathogenic agents such as bacteria, viruses, fungi or parasites. Such pathogens have the ability to spread or transmit from an infected animal host, from an animate or inanimate vector, or from an environmental reservoir to a susceptible host (Neumann, 2012). Successful transmission events lead to the infection of new hosts, and

the efficiency of this process can be affected by several factors (Pantin-Jackwood and Swayne, 2009):

- ✓ Shedding of the agent by the host (titer, duration and source)
- ✓ Environmental stability of the agent (temperature, humidity and organic matter content)
- ✓ Minimal infective dose of the specific agent

Several authors have differentiated between modes of transmission and routes of infection. **Modes of transmission** can be classified broadly as horizontal (from one host to another without any parenteral relationship) or vertical (from one generation to another) (Smith, 2006). **Route of infection** refers to the route by which an etiologic agent gains access to the body of a susceptible individual. As a result, within the horizontal mode, transmission can be distinguished between direct, indirect or airborne (Smith, 2006).

Direct transmission requires an immediate transfer of an agent between an infected and a susceptible host. It may occur by direct contact with animals, via semen, (Maes, et al., 2016); or via touch, scratch, licking or biting (Center for Food Security and Public Health, 2008). Direct contact may also occur by direct projection or “droplet spread” (limited to distances of 1 m or less) where droplets exiting from an infective individual during coughing, sneezing or diarrheic episodes are sprayed onto the conjunctiva or mucus membranes of susceptible animals. This has been extensively described for respiratory diseases such as influenza virus (Fabian, et al., 2008) and *Mycobacterium tuberculosis* (Turner and Bothamley, 2015) and for intestinal pathogens such as norovirus in humans (Bonifait, et al., 2015).

In contrast, **indirect transmission**, requires the passage of infectious agents between individuals through inanimate (vehicle borne-transmission) or animate objects (vector-borne transmission). Mechanical borne-transmission of certain viruses between swine farms has been the focus of the veterinary literature in the last few years. This type of transmission occurs through contaminated objects (fomites) and the agent may or may not have multiplied on the vehicle before being transmitted. Fomites may include transport vehicles (Dee, et al., 2004), coveralls and boots (Otake, et al., 2002a; Allerson, et al., 2013), needles (Otake, et al., 2002b), feed (Dee, et al., 2014; Bottoms, et al., 2015) and meat pork products (Cano, et al., 2007a; De La Torre, et al., 2013). Invertebrate vectors such as flies, mosquitos, or ticks are generally responsible for vector-borne transmission (in some cases vertebrate host can act as vectors) although in many cases those same insects may act as mechanical vectors. “Mechanical” vectors act as carriage of disease agents, and mechanical transmission has been described for diseases such as PRRSV (Otake, et al., 2003) and Salmonella (Wang, et al., 2011). In contrast, “biological” vectors require a period of multiplication, cycle development or both in the vector for the disease to transmit.

Airborne disease transmission

Definitions

Any process that results in the fragmentation of biological material will generate aerosols (Cox, 1995). An **aerosol** consists of material finely divided and suspended in air or other gaseous environment (Hirst, 1995). A

bioaerosol is an aerosol comprising particles of biological origin or activity which may affect living organisms through infectivity, allergenicity, toxicity, pharmacological or other processes, and airborne transmission requires the dissemination of such aerosols. Bioaerosols in animal houses are often rich in both variety and number of particles, especially in intensive livestock production. The main constituents of bioaerosols in animal houses are whole cells or fragments of cells (virus, bacteria, fungal spores, etc.) and non-specific organic and inorganic dust that originates from fecal material, dust, debris, respiratory fluids and, specifically in buildings housing animals, from bedding, feed, and hair particles (Wathes, 1995). Infectious aerosols are the subset of bioaerosols that carry viable pathogenic microorganisms and therefore represent a potential mechanism of disease transmission between individuals (Stark, 1999).

Factors affecting airborne disease transmission

The pathways of airborne disease transmission include three components (Hugh Jones, 1973; Winkler, 1973): aerosolization (or ‘take off’ from source and release); aerosol transport to susceptible animals (or ‘aerial transport’ based on the dispersal of the agent); and inhalation of aerosols to susceptible animals (or ‘landing’ on the target). Disease transmission cannot occur or cannot be detected unless all three components occur, but this process in farm animals is influenced by many factors (e.g. host species, strain of agent, particle size, and air sampling technique) and those factors will be reviewed next:

a. Aerosol production

Aerosol production is influenced by the host species, the period of infectivity, the strain of pathogen, the time of day and the husbandry conditions. All these factors are closely related with the environment in which the particles are generated. In the case of respiratory aerosols, type or frequency of respiratory activities, sites of infection, and pathogen infective load can also influence the production of aerosols (Gralton, et al., 2011). Respiratory processes such as coughing or sneezing produce greater quantities of particles that travel further due to the high velocity of expulsion from the mouth and nose (Morawska, et al., 2009; Johnson, et al., 2011) compared to particles generated through normal breathing. In addition, the distribution of particle sizes generated differs between coughing and sneezing and is highly variable among individuals (Ross, et al., 1955).

The effect of the host species as a factor influencing aerosol production has been well described for foot and mouth disease virus (FMDV). Pigs are considered “amplifiers” of FMD virus since they excrete more virus than cattle and sheep. Pigs can aerosolize up to $1 \times 10^{4.7}$ $TCID_{50}$ /pig/ hr compared to $1 \times 10^{3.2}$ $TCID_{50}$/hr/cow. Excretion totaled 10^6 $TCID_{50}$ per pig over 5 days and from cattle and sheep 3×10^4 $TCID_{50}$ per animal over 4 days (Sellers and Parker, 1969; Alexandersen and Donaldson, 2002).

Infectivity is defined as the time period during which exposed infected animals are able to transmit an infection to any susceptible host. The longer the infectious period the higher the risk of airborne transmission. In the case of pseudorabies disease virus (PRV), viremia and excretion of the virus in the air could last up to 17 days after infection (Donaldson, et al., 1983). In

contrast for swine influenza, nasal shedding and detection of the virus in the air was 7 days (Corzo, et al., 2014).

Type of strain is a main factor influencing pathogen shedding in aerosols, especially for viruses. Shedding of PRRSV in aerosols increased significantly for strain MN-184 compared to strain MN-30100 which was considered of low pathogenicity (Cho, et al., 2006a). For FMDV, airborne shedding was associated to strain transmission characteristics, specifically with regards to quantity shed by the donors (i.e. infectiousness and intensity of contact), length of incubation period, and severity of clinical disease (Pacheco, et al., 2012).

Concentration of infectious agents in aerosols is also related to the number of infected animals in a given location. Time of day, activity of the animals and environmental conditions such as relative humidity, wind speed, ventilation rates in the rooms as well as temperature also appear to be correlated with concentration of pathogens in aerosols (Ellen, et al., 2000).

b. Survival and transport of infectious agents in aerosols

The ability of aerosolized pathogens to initiate disease in recipient hosts depends on how well they survive and maintain infectivity in aerosols. Particle size, air sampling techniques, structural composition of bioaerosols, as well as other important factors such as suspending fluids (as part of the aerosolization process), temperature, relative humidity (RH), topography and meteorological factors (i.e. wind speed, solar effect, and atmospheric gases) have been defined as variables that will influence the infectivity of bioaerosols and their transport.

Particle size

Particle sizes of airborne materials may range from 0.5 to 100µm (Hirst, 1995), and airborne materials can be associated with infectious agents or not.

Particle size is a key component of pathogen carriage, aerosolization, and transmission. Small particles that are aerosolized, termed “droplet nuclei”, have classically been defined as “the vehicle for airborne respiratory disease transmission” (Phair, 1955). Particle size is also used to classify transmission as droplet or airborne. Particles of small size can remain suspended in the air for prolonged periods potentially exposing a large number of individuals including those close to the source and those at a greater distance from the source of emission (Xie, et al., 2007). As an example, a spherical particle of 4 µm in diameter takes 33 minutes to settle 1 m in still air, compared to 8 h for a 1µm particle (Lindsley, et al., 2010).

Particle size studies have been limited. In humans, measures of particle size for influenza A virus (IAV) have been evaluated in controlled laboratory and health care settings, airplanes, daycares and households (Fabian, et al., 2008; Blachere, et al., 2009; Lindsley, et al., 2010; Yang, et al., 2011; Milton, et al., 2013). Distribution of IAV particle size varied between studies and settings, with IAV found in particles 1-4 µm in diameter (49% of particles) and particles > 4 µm (46% of particles) in health care facilities (Blachere, et al., 2009), and particles < 2.5 µm (64% of viral copies) detected in public places (i.e. health care, airplanes and day cares) (Yang, et al., 2011). Furthermore, dispersion models that have incorporated particle size data have

indicated the plausibility of airborne transmission within these settings (Stilianakis and Drossinos, 2010).

For pigs and cattle, quantification and size distribution of FMDV associated particles have been studied with artificially generated aerosols and natural aerosols emitted by inoculated animals. Studies with artificially generated aerosols reported a significant difference in the presence of virus in particles smaller than 3 µm (Donaldson, et al., 1987). However, results from natural aerosols demonstrated an even distribution of the virus across particle sizes of <3, 3 to 6 and > 6 µm (Gloster, et al., 2007). For PRV, pigs sampled on days 2 and 3 after infection excreted particles with similar proportions of infectivity in association with small (<3 µm), medium (3 to 6 µm) and large (>6 µm) particles, whereas on day 4 after infection infectivity was mainly associated with large particles. Specifically for IAV, the information is limited to animal models using ferrets or guinea pigs that assess the risk of IAV airborne transmission to people (Lowen, et al., 2007; Mubareka, et al., 2009; Koster, et al., 2012) and there is no information on size of particles associated with airborne IAV generated by animals raised in agricultural environments.

There is an association between aerosol infectivity, virus survivability and aerosol particle size. The associations among these parameters have been studied on artificially generated aerosols of transmissible gastroenteritis, swine influenza and avian influenza viruses (Zuo, et al., 2013b). Infectivity increased with particle size (max in particles of 0.3-0.45 µm compared to those of 0.1-0.2 µm) and this relationship could be explained by a power law relationship greater than 3 (Zuo, et al., 2013b).

Air sampling technique

Air sampling technique can also be considered one of the most important factors affecting the recovery and apparent infectivity of airborne viruses due to the stress imparted to the microorganisms. Pathogen resistance during sampling, time of sampling, and flow rate are the most important factors that affect microbial collection and survival in bioaerosol samplers (Kesavan and Sagripanti, 2015). Sampling of bioaerosols can be performed by impaction (onto plate or growth media), impingement into liquid, or capture by filtration (Verreault, et al., 2008). Solid impactor methods include Andersen samplers, slit samplers, and cyclone samplers. The efficiency of solid impaction samplers is typically higher for larger particles, however, in the case of the Andersen sampler, the recovery limit can be small (0.4 µm) as a result of the diameter of the holes through which the particles are accelerated. Some of the main advantages of solid samplers against other samplers are their particle sizing capabilities (Andersen cascade impactor) and the time resolution (slit to agar) (Verreault, et al., 2008).

Liquid collection methods have been used for bioaerosol collection since the 1950's, and involve the use of impingers to capture viruses. Impingers are the most commonly used air samplers for collecting airborne viruses and they include the All Glass Impinger (AGI) with a set distance of 4 mm (AGI-4) or 30mm (AGI-30) from the airflow exit to the bottom of a fluid vessel, and the swirling aerosol collectors (Biosamplers, manufactured by SKC, Inc.) with a more gentle impaction into liquid and higher efficiency in the collection of viable microorganisms (Fabian, et al., 2009).

Air filtration sampling is based on the capabilities of the filter media to capture airborne particles when the airstream is forced to pass through the filter. The composition of the microbial population in the air will be determined by the elution and separation of the captured microorganisms from the crustal material. The main advantages of filtration sampling are that it is simple, portable, relatively inexpensive and very sensitive when in association with molecular-based virus identification techniques such a RT-PCR (Farnsworth, et al., 2006). High volume sampling from heating, ventilation, and air conditioning (HVAC) filters has been successfully used for surveillance purposes (Goyal, et al., 2011). Virus elution techniques have been published and recovery efficiency should be considered for virus collection efficiency calculations (Farnsworth, et al., 2006).

Molecular structure and composition of airborne agents

The viable composition of infectious bioaerosols (viable bacterial or virus composition) can be influenced by the sensitivity of microbes to prevailing atmospheric factors, which is a function of their molecular structure and composition (e.g., anthrax spores versus enveloped and non-enveloped viruses) (Cox, 1995). Viruses possessing structural lipids survive best in aerosols maintained at low relative humidities whereas those without structural lipids survive best at high humidities (Akers, 1973; Verreault, et al., 2008).

c. Aerosol inhalation and infection

The particle size distribution of bioaerosols is dynamic and will vary between the sites of generation and deposition. Changes in the constitution of

aerosols will be affected by the nature of the aerosol generated, the prevailing atmospheric conditions, and the distance/time between generation and deposition, which will in turn influence the pattern of deposition of an aerosol in the respiratory tract of recipient hosts (Salem and Gardener, 1984). Particles derived from the respiratory tract are hygroscopic meaning that when they are discharged into the ambient air, they lose their moisture and shrink in size. In contrast, when they are inhaled they take up moisture from the saturated air of the nasopharynx and the lung (Knight, 1973). The size and body shape of the host species determines the morphology of the nasal cavity and respiratory airways influencing the size of the particles that may deposit in corresponding anatomical regions (Thomas, 2013). As a result, in humans droplet nuclei of 2-3μm will have a proportionally higher deposition in the lower respiratory tract than in the upper respiratory tract, while deposition of dust-borne bacteria and droplets is essentially limited to the nasopharyngeal area.

Selected swine viruses transmitted by aerosol

Foot and mouth disease virus (FMDV)

Etiology

FMDV, a member of the genus Aphthovirus of the family Picornaviridae, is a virus that causes a very acute and severe vesicular disease. FMDV is on the World Organization for Animal Health (OIE) list of diseases due to its extensive spread within and between countries and its important economic consequences. With minor exceptions, FMDV affects

cloven-hoofed animals and cattle, pigs, small ruminants (sheep and goats) and water buffalo are the species of greatest significance. Several other species may be infected with FMDV, but typically they do not play an important epidemiologic role. Horses and carnivores are highly resistant but can serve as mechanical vectors for the agent if they become contaminated with the virus and then contact susceptible livestock (Thomson, et al., 2003).

Epidemiology

Pigs usually become infected with FMDV by direct or indirect contact with infected animals, contaminated fomites or via consumption of FMDV-contaminated products (e.g., food waste). When animals are located in close proximity of each other (e.g., in confinement facilities), the movement of the virus via aerosol is probably the predominant form of transmission (Beck and Strohmaier, 1987).

Intradermal or subdermal infection of the virus (damaged skin) is the most efficient route for infection, with a minimum infectious dose of 1×10^2 $TCID_{50}$. By comparison, a single infected animal may excrete 1×10^{10} $TCID_{50}$ or more per day at the peak of excretion, most of it in vesicular fluid, saliva, nasal fluid and other excretions (Alexandersen, et al., 2003).

Airborne transmission of FMDV is a dynamic, complex process affected by the species of animals, the number of animals, topography of the area and the meteorological conditions. Pigs aerosolize up to 1×10^6 $TCID_{50}$ per pig per day for most of the FMDV strains whereas ruminants aerosolize fewer viral particles but are highly susceptible to infection by inhalation. Swine are usually considered the most important species as a source of the virus (or

“amplifiers”) and cattle and sheep as the recipient species (Alexandersen and Donaldson, 2002). Therefore, the pattern of airborne spread of FMVD is most often from infected pigs to cattle and sheep located downwind (Alexandersen, et al., 2012). It has been proposed that apart from infected animals, aerosols could also originate from incineration of infected carcass (Stark, 1999).

Long distance airborne spread requires atmospheric conditions that maintain the infectivity of the virus. FMDV infectivity is dependent on RH above 55%, stable atmospheric conditions and cloud cover. Donaldson et al. (1979) calculated that, given a RH of 60% and a wind speed of 10 m/s, FMDV could survive over 2.7 hours enabling translocation of more 100 km. Topography is important and large tracts of water act to preserve the virus plume and increase the probability of long distance airborne transmission. This information was successfully used to create models that predicted the airborne spread of the virus in the United Kingdom outbreaks in 1981 and 2001, and in Italy in 2003 (Alexandersen, et al., 2003).

In pigs, the airborne viral excretion coincides with the appearance of vesicular lesions during the viremic phase. This pattern is similar in cattle but the amount of virus recovered from blood and breath is higher in pigs. The percentage of ruminants that become carriers of the disease is on average 50% with a maximum reported duration of 3.5 years in cattle and 9 months in sheep (Sutmoller and Olascoaga, 2002). Pigs do not act as long term carriers. FMDV can persist in the environment for long periods of time, for at least 20 weeks in hay and straw and in fecal slurry for 6 months in winter (Hyslop, 1970). Acid or alkaline disinfectants are highly effective.

Pathogenesis and clinical signs

After the initial replication of the virus in the pharynx or in the skin, FMD virus spreads to the regional lymph nodes and into the circulation. Viremia usually last between 4 to 5 days. The main sites for virus replication are cornified epithelia of the skin, tongue and mouth. Normal skin (hairy and hairless) also contains substantial amounts of the virus. Damaged mucosa is always associated with higher severity of the lesions (Alexandersen, et al., 2012).

The incubation period is highly variable (1 to 14 days) and multifactorial based on dose, species and route of infection (Alexandersen, et al., 2003). In experimental conditions, the mean incubation period was 1 to 3 days in pig-to-pig direct contact and 3.5 days in direct cattle-to-cattle contact. FMD is characterized by an acute febrile reaction and the formation of the vesicles in and around the mouth and on the feet. Lameness and lesions may not be consistent findings in all animals.

Prevention and control

Airborne disease transmission of FMDV is problematic and its consequences can be dramatic. In previously unaffected regions, the implementation of a response as soon as possible is essential to control the disease. Extreme measures are required to eradicate the disease. Stamping out has been shown to be effective to control and eradicate the disease in many countries. Vaccination strategies have to be tailored to the strains circulating in the area due to the lack of cross protection between heterologous strains (Alexandersen, et al., 2012).

Porcine reproductive and respiratory disease syndrome virus (PRRSV)

Etiology

PRRSV is a small enveloped, single stranded RNA virus that belongs to the Arteriviridae family in the order Nidovirales. There are two major genetic lineages represented by type 1 (European viruses) and type 2 (North American viruses) genotypes that vary by 44% in nucleotide sequence. The PRRSV genome consists of 2 large open reading frames ORF 1a and ORF 1b that comprise 75% of the viral genome and are translated and processed into 14 non-structural proteins (nsp). The seven structural proteins are encoded by ORFs 2-7 (Snijder and Meulenberg, 1998). The three main structural proteins (N, M and GP5) are required for particle formation and viral infectivity, while minor proteins (GP2a, GP3 and GP4) are only essential for viral infectivity. Phylogenetic analyses are based on ORF5, which encodes the major envelope glycoprotein (GP5) (Zimmerman, et al., 2012).

Epidemiology

PRRSV is present in most swine producing countries in the world with some exceptions being Norway, Finland, New Zealand, Australia, Brazil and Cuba (Zimmerman, et al., 2012). Only domestic and feral pigs are known to be susceptible to PRRSV and the susceptibility of other members of the superfamily Suidae is unknown.

The direct and indirect routes of PRRSV transmission include saliva and nasal secretions from infected pigs (Wills, et al., 1997), contaminated semen (Christopher-Hennings, et al., 1995; Prieto and Castro, 2005), vehicles

(Dee, et al., 2004), insects (Otake, et al., 2002; Otake, et al., 2003), and other fomites such us boots, coveralls and needles (Otake, et al., 2002a; Otake, et al., 2002b). Indirect transmission by aerosols is dependent on the viral variant and environmental factors. Airborne transmission of PRRSV from a point source to an at-risk population has also been demonstrated (Dee, et al., 2010). Risk factors for aerosol transmission included directional winds of low velocity, sporadic wind gusts, low temperatures, high relative humidity and low sunlight levels. Airborne dissemination of PRRSV has been demonstrated out to distances of 9.1km (Otake, et al., 2010).

Vertical transmission of PRRSV occurs transplacentally from viremic dams to fetuses resulting in reabsorption or abortion of fetuses, or birth of stillborn and congenitally infected pigs (Christianson, et al., 1992). If this cycle of transmission is not interrupted, PRRSV tends to circulate in herds indefinitely (endemically) due to the persistence of the infection in naïve animals (Dee and Joo, 1994).

Transmission between herds has also been suspected and it was first defined as ‘neighborhood infection’ by Robertson (1992). In England and Belgium, airborne transmission of PRRSV was considered the cause of many outbreaks (Robertson, 1992). The term of ‘area spread’ was introduced by Lager et al. in 2002 in a study with seven herds within a 40 km^2 area. The high degree of homology between isolates of the different farms suggested a common source of all these viruses (Lager, et al., 2002).

PRRSV is fragile and is quickly inactivated by heat and drying. Many studies have defined the stability of its infectious properties under specific conditions of temperature, moisture and pH. The virus is stable for months at -

7 and -20 °C (Zimmerman, et al., 2012) and the virus can remain infective in manure at low temperatures (Linhares, et al., 2012b).

Pathogenesis and clinical signs

The primary site for PRRSV replication is alveolar macrophages of the lung and other cells of the macrophage lineage located in lymph nodes, spleen, placenta and umbilical cord (Lawson, et al., 1997). The virus can persist in pigs for long periods of time evading the immune system. Using polymerase chain reaction (PCR), PRRSV RNA has been detected in breeding gilts out to 120 days post-infection and up to 210 days in pigs infected in utero as fetuses at 85-90 days of gestation (Benfield, et al., 1997; Batista, et al., 2002a).

Outbreaks of PRRSV involve episodes of reproductive failure (abortions, premature parturition, and elevated levels of fetal losses) as well as a reduced growth performance and elevated mortality, secondary to respiratory disease (Loula, 1991). However, the intensity of the disease appears to vary among isolates with North American type 2 strains being the most pathogenic. Recently, a large scale study evaluated the association between viral load and fetal preservation, and determined the impact of type 2 PRRSV on fetal weights (Ladinig, et al., 2014). A fetal mortality of 41±21.8% and a decrease in viable fetuses' weights of approximately 17% in PRRS infected litters was reported.

Prevention and control

PRRS has proven to be a difficult and frustrating disease to control. The methods for control and elimination of PRRSV leading to initiatives of voluntary regional control and elimination programs of PRRSV in swine dense regions in North America were reviewed by Corzo et al (2010). Methods to control the spread of the disease include semen control (Christopher-Hennings, et al., 1995), gilt acclimation (Dee, et al., 1995), management practices (McCaw, 2000), partial depopulation (Dee and Joo, 1994), live virus exposure (Batista, et al., 2002b), and vaccination programs with modified-live virus (MLV) vaccines (Cano, et al., 2007c; Linhares, et al., 2012a). MLV vaccines have been evaluated by Linhares and others with regards to viral shedding and dynamics of PRRSV infection in pig populations raised under commercial conditions. They reported a significantly lower number of PRRSV positive days in the air compared to the control-challenge group (17 vs 31days) (Linhares, et al., 2012a).

Elimination of PRRSV has been the focus for many practitioners and researchers for the last 10 years. The different methods that have been described to eliminate the virus from the sow herd include test and removal (Dee and Molitor, 1998), whole herd depopulation/repopulation, herd closure (Torremorell, et al., 2003), and herd closure and exposure (Voglmayr, et al., 2006). The use of MLV vaccine during an exposure program compared against the use of live virus inoculation on sow herds demonstrated that sow farms using MLV vaccine recovered production sooner and had less production losses than herds inoculated with live virus (Linhares, et al., 2014).

Despite all of these methods and stringent biosecurity measures, successful elimination on many farms has often been negated by reintroduction of new PRRSV strains leading to the recognition of the important role of aerosol transmission of the virus. As a result, producers are investing significant resources on interventions, such as air filtration, to prevent airborne transmission (Dee, et al., 2012; Alonso, et al., 2013b).

Influenza A virus (IAV)

Etiology

Influenza viruses are members of the Orthomyxoviridae family. The virus is a negative-sense single-stranded RNA virus with a segmented genome. Influenza viruses are classified in types, subtypes and genotypes. Three different types of influenza virus exist: A, B and C, defined by the nucleocapsid protein (NP) and the matrix proteins (M1 and M2). Influenza virus subtypes are defined by the hemagglutinin (HA o H) and neuraminidase (NP) proteins. The combinations of the different HA and NA forms in a virus defines its subtype (i.e H1N1 or H3N2) (Van Reeth, et al., 2012).

Only influenza type A is clinically significant in pigs. Because of the segmented nature of the influenza genome, IAV viruses can exchange RNA segments during viral replication. This is a process known as “reassortment”.

Influenza viruses have important public health significance. Since the disease was first described as a disease of swine in the United States in 1918, the disease has remained unchanged since that time. The first evidence of transmission of swine influenza to farm personnel was in 1976

(Easterday, 1980). Since then, many more data have supported the interspecies transmission of influenza viruses from humans to pigs, pigs to birds, and from birds to pigs.

Epidemiology

Pigs can be infected with swine, human and avian influenza viruses and therefore, transmission of influenza is not only limited to within species events, but also to interspecies events (Kundin, 1970). Different subtypes of influenza have been isolated in different parts of the world showing the results of avian-to-swine transmission in the swine population. Human isolates have also been isolated from pigs, in particular H1 and H3N2 viruses in Asia and also in pigs from Europe and North America (Kitikoon, et al., 2013).

Influenza virus outbreaks occur typically during late fall and early winter in cold climates associated with colder temperatures and cold autumn rains. However, several studies have demonstrated that in swine facilities IAV occurs all year around although seasonality has been demonstrated in pigs (Corzo, et al., 2013; Diaz, et al., 2015).

The primary route of transmission of IAV in farms is pig-to-pig contact with virus titers in nasal secretions in the peak of shedding of $1x10^7$ infectious particles/mL. Airborne transmission of swine IAV was demonstrated by Xie et al. (2007) using a guinea pig model. The distance travelled by particles with an initial diameter of 30-50μm was estimated to be less than a meter during the normal breathing and more than 6 m during a normal sneezing (Xie, et al., 2007). Different animal models using mostly ferrets and guinea pigs have been used to explain IAV airborne transmission. Some of the important

observations from those studies were that different influenza strains differ considerably in their capacity for airborne transmission and that this transmission is dependent on RH and temperature (Lowen, et al., 2007; Koster, et al., 2012). Airborne detection of swine IAV has been shown in aerosols generated under experimental conditions in acutely infected pigs (Corzo, et al., 2013b) and pigs with passive immunity (Corzo, et al., 2014). At the farm level, IAV was detected and isolated in the interior and in the outside air for up to 2.1 km downwind from infected facilities (Corzo, et al., 2013a). Recently, the persistence of IAV for up to 20 days in the air of pig growing facilities experiencing acute outbreaks was quantified (Neira, et al., 2016).

Pathogenesis and clinical signs

In pigs, IAV produces a highly contagious respiratory acute viral disease, it is endemic in most of the swine populations around the world, and it is one of the respiratory pathogens involved in the porcine respiratory disease complex (Thacker, et al., 2001). The virus tends to spread easily in susceptible populations and many pigs become infected with one or more influenza virus subtypes, sometimes without showing clinical signs (Van Reeth, et al., 1996).

Swine IAV causes an acute infection and virus clearance is rapid. The lungs are the major target organ and infection in pigs is limited to the respiratory tract with virus replication demonstrated in epithelial cells of the nasal mucosa, tonsil, trachea, lungs and tracheobronchial lymph nodes (Van Reeth, et al., 2012). Nasal secretions are the primary route of virus excretion. The incubation period for IAV ranges from 1 to 3 days and clinical signs

include pyrexia, anorexia, inactivity and reluctance to rise. The virus can be detected as early as 1 day post infection in nasal secretions and usually last until 5-7 days post infection (Van Reeth, et al., 1996). Morbidity is high (near 100%) but mortality is typically low (usually less than 1%) unless there are concurrent infections with secondary bacteria or viruses.

Prevention and control

Vaccination is the preferred strategy to control influenza in swine farms. Licensed commercial vaccines are based on inactivated whole virus preparation with H1N1, H1N2 and H3N2 viral strains representative of contemporary strains. However, because of the significant genetic diversity of IAV observed in pigs and the limited ability of inactivated vaccines to offer cross-protection, autogenous vaccines (defined as a vaccine prepared from cultures of microorganisms obtained from an individual and then used to immunize that same group of individuals against further spread) are used to immunize pigs against the strain (or strains) circulating in a given farm.

Biosecurity is a key point to protect populations against new strains of swine IAV. Most of the standard biosecurity protocols in place in pig farms to prevent the introduction of pig pathogens through indirect transmission routes, such us personnel and fomites, are also useful for influenza (Otake, et al., 2002a; Dee, et al., 2004; Dee, et al., 2005; Allerson, et al., 2013). Physical measures in humans, including hand washing and mask usage, used to reduce respiratory virus transmission have been analyzed in a meta-analysis study indicated that these measures were effective and should be implemented to prevent interspecies transmission (Jefferson, et al., 2009)

Avian Influenza virus (AIV)-Highly pathogenic avian influenza virus (HPAI)

Etiology

Avian influenza virus (AIV) is a type A influenza isolated and adapted to an avian host. In avian, the HA (H1-H16) and NA (N1-N9) subtypes seem to be able to reassort into any combination and many of the 144 possible combinations have been found in natural reservoir species (Spackman, 2014). However, certain subtypes are more common than others in some species. In gallinaceous (i.e. chicken and turkey) the HA protein is the determinant of pathogenicity towards a systemic disease (i.e. highly pathogenic) or limited to the respiratory/enteric tract (i.e. low pathogenicity) (Rott, 1992). New highly pathogenic avian influenza (HPAI) viruses can emerge from reassortment due to the comingling of migratory birds infected with HPAI strains and low pathogenic avian influenza (LPAI) viruses present in waterfowl. HPAI viruses, either H5 or H7 subtypes, can result in high death rates (up to 100% mortality within 48 hours) in some poultry species (WHO, 2014). Reports of HPAI epidemics in poultry, such as H5N1 and more recently H5N2 in North America, can seriously impact local and global economies and international trade (USDA United States Department of Agricultural) .

Epidemiology

The natural reservoir species for AIV is waterfowl where AIV circulate without causing apparent signs of illness. However, AIV can spread to domestic poultry and cause large-scale outbreaks, specifically by HPAI strains. Transmission occurs through direct contact with body secretions;

however, secondary transmission tends to occur by contact with fecal matter, contaminated feed, water or other fomites such as vehicle tires, footwear and clothing. The virus survives well outside the host in organic material, flesh, water and fecal matter (Baigent and McCauley, 2003).

Transmission of AIV through the air has been demonstrated in the ferret model under research conditions (Sutton, et al., 2004). However, aerosol transmission of AIV under field conditions has been suspected but not demonstrated. Schofield and others detected and isolated HPAI virus from 4 air samples collected inside poultry houses in the Frasey Valley, British Columbia, H7N3 outbreak. The detection for airborne live virus outside poultry houses was also attempted but they were not successful (Schofield and Kournikakis, 2005). The role that dust and other airborne pollutants being exhausted from infected facilities play in the transmission of the disease has been investigated downwind from infected facilities (Chen, et al., 2010) and under experimental settings (Spekreijse, et al., 2013). Under field conditions, concentration of airborne ambient influenza was significantly higher in dust samples collected during “dust storm days” compared to less dusty days. Under research conditions, the transmission between inoculated and recipient birds happened in 2 out of 4 experiments estimating a rate parameter for dust-borne transmission at 0.08 new infections／infectious chicken／day.

AIV can also replicate in mammalian species resulting in cross-species transmission. The effectiveness of this transmission is virus-strain dependent (Beare and Webster, 1991) and, requires a host-virus extensive interaction for it to occur (Spackman, 2014). As a result, infections of AIV in people are of significance to public health. During the past 5 years as a result of H5N1 and

H7N9 infections, the number of human cases with severe pneumonia and mortality (>60%) have increased (Abdel-Ghafar, et al., 2008; Xiao, et al., 2015).

Pathogenesis and clinical signs

In chickens and turkeys LPAI infections may be subclinical; the nasal cavity is the predominant initial site of virus replication with a possible spread to other parts. Secondary infections are needed to produce severe respiratory damage. In the field, LPAI causes a mild-to moderate respiratory disease as a primary presentation. Clinical symptoms are usually seen as a drop in feed and water consumption, and a drop in egg production (Spackman, 2014). In contrast, with HPAI, infection is initiated in the nasal epithelium, it becomes necrotic within 24h, and at that point the virus spreads via capillary endothelial cells towards the vascular and lymphatic systems. After intranasal exposure, replication of most HPAI viruses may be seen within 24 h in visceral organs and within 48 h, the virus titers may be maximal and the lesions severe (Pantin-Jackwood and Swayne, 2009). At that point the virus may disseminate and replicate in multiple organs causing single or multi-organ failure. The differences among types and severity of disease vary greatly with bird species and virus strains (Wood, et al., 1985; Perkins and Swayne, 2003).

Prevention and control

Biosecurity is again a key point to protect populations against the entrance of HPAI. Most of the standard biosecurity protocols for other diseases would prevent the introduction of poultry pathogens through direct transmission,

primarily by avoiding contact with infected wild live animals (Knight-Jones, et al., 2011), as well as indirect transmission routes, such as personnel, bedding material, and other fomites (Conraths, et al., 2016). As an international response, the Food and Agriculture Organization of the United Nations (FAO) together with the International Organization of Epizooties (OIE) updated in 2007 the compendium of global strategies for the control and prevention of HPAI which is a guidance for the different countries on what to do if the disease affects them (FAO, 2007). This compendium of strategies highlighted the following strategies for achieving the prevention and control of the disease: surveillance in domestic and wild life, buildup of laboratory capabilities at the national level, improve strategies for outbreak containment, and vaccination to reduce the clinical signs of the disease (FAO, 2007). As a reportable disease by the OIE in 2016 (OIE, 2016), strategies such us depopulation (i.e. stamping out, mass depopulation), disposal, cleaning, and disinfection procedures on the infected premises have been coordinated at a national level by veterinary services.

Staphylococcus aureus (S. aureus) Methicillin-resistant S. aureus (MRSA)

Etiology

Staphylococcus aureus (*S. aureus*) is a gram-positive *coccal* bacterium member of the *Firmicutes*. *S. aureus* is prevalent in skin of healthy pigs and in the environment of swine facilities. *S. aureus* rarely causes disease but it could be responsible for causing abscesses and other conditions, such as

septicemia, mastitis, vaginitis, metritis, osteomyelitis, and endocarditis (Frana, 2012). However, there is an important public health concern regarding swine as a reservoir for methicillin-resistant *S. aureus* (MRSA) strains which are strains of *S. aureus* that have become resistant to antibiotics commonly used to treat ordinary staphylococcal infections in people. A new lineage of *S. aureus*, named livestock associated MRSA (LA-MRSA) was first discovered in people in the Netherlands with frequent occupational contact with livestock (farmers, veterinarians or abattoir workers) (Armand-Lefevre, et al., 2005; Denis, et al., 2009; Garcia Graells, et al., 2012). LA-MRSA was initially associated with livestock and genotypically differs from strains recovered from health-care and community-associated MRSA cases (HC-MRSA and CA-MRSA respectively).

Epidemiology

Among all the LA-MRSA strains described so far, the lineage ST398 is the most widely disseminated followed by other variants such as ST5 and ST9 lineages, ST9 being more common in Asian countries. Numerous studies have demonstrated that LA-MRSA distribution among pigs and swine veterinarians is similar so direct exposure to pigs is a risk factor for nasal colonization with *S. aureus* of animal origin (Cuny, et al., 2009; Neela, et al., 2009; Cuny, et al., 2013). More specific risk factors associated with the prevalence of LA-MRSA have been extensively analyzed in a recent meta-analysis in fattening pig farms. Growing pigs is the population of animals within a farm with higher prevalence of *S. aureus* positive sites compared to sows and suckling piglets (Linhares, et al., 2015). Prevalence of MRSA in

Dutch finishing farms was estimated at 53.5% and the risk factors associated with prevalence of MRSA infection included herd size, herd type (Broens, et al., 2011), antimicrobial drug usage (OR=1.79) and partially slatted floors (OR=2.39) (Fromm, et al., 2014). Other studies also reported the purchase of gilts, and hygiene measures as possible risk factors. However, no significant associations were demonstrated (Broens, et al., 2011). Despite this high prevalence, a recent study performed in 38 swine farms in 11 North American states demonstrated the relatively low herd prevalence of MRSA in the US swine industry compared to many countries in Europe. However, as expected, *S. aureus* was detected in 97% and 77% of the farms and piglets respectively (Sun, et al., 2015).

Since *S. aureus* is ubiquitous in pig farms, direct transmission is the predominant route for pathogen to spread. However, it is also reported that *S. aureus* is a predominant bacterial species in bioaerosols from swine barns (Gibbs, et al., 2006; Agerso, et al., 2014) and the same spa type has been isolated inside and up to 150 m and 300 m downwind from positive barns on air and soil surfaces samples respectively demonstrating the dissemination of the pathogen through the airborne route specifically around livestock facilities (Schulz, et al., 2012).

Public health considerations

Traditionally, most MRSA disease has been health care and community associated (HC and CA-MRSA). Since a specific lineage of MRSA has been detected in animals and people who are in close contact with livestock (i.e. veterinarians, farmers, abattoir workers) compared to the

general population (Sun, et al., 2015), numerous studies of the prevalence and genetic characterization of LA-MRSA have been published. In Holland it is estimated that swine farm workers are 760 times more likely to be colonized by MRSA than members of the general public (although comparison of infection risks are thus far not reported) (Voss et al., 2005, van Loo et al., 2007). A North American study has recently shown that approximately 65% of swine veterinarians are culture positive (nasal swabs) for *S. aureus*, and that the majority of isolates are variants associated with pigs (Davies, unpublished data). In a Canadian study, isolates from pigs and people were indistinguishable, suggesting inter-species transmission (Khanna, et al., 2008), and another study showed the transmission of ST398 MRSA-positive farm workers to family members without direct contact with livestock (Cuny, et al., 2009; Graveland, et al., 2011). In studies in other species such as calves, persistence of ST398 MRSA in farmers was related to the intensity of animal contact indicating that contamination of nasal mucus is probably more frequent than true colonization of the nasal mucosa (Graveland, et al., 2011).

Despite all these studies, the true zoonotic potential of swine *S. aureus* is still uncertain. The common presence of *S. aureus* in commercial barns and farm workers, and their potential zoonotic significance requires a deeper investigation of bacteria prevention or sanitation strategies at the farm or regional level.

Control methods for airborne transmission

Any measures aimed at reducing the number of airborne particles in the air should directly reduce the risk of airborne disease transmission. Strategies can be classified as those aimed at decreasing disease transmission within a farm or between farms.

Between-farm transmission strategies

Prevention of airborne disease transmission between farms is one of the most challenging, frustrating and costly events for producers and veterinarians. Within those strategies, farm location in low animal density areas, vaccination (Cano, et al., 2007b; Linhares, et al., 2012a), and air filtration (Dee, et al., 2012; Alonso, et al., 2013b) are options that can be used to reduce the risk of disease transmission between farms.

Air filtration technology

Among all potential strategies available to decrease airborne transmission of swine viruses between farms, air filtration is the most common with more than 200,000 sows now housed in air filtered facilities in the US Midwest alone (Dee, S.A 2012 *personal communication*). The concept that air filtration could prevent airborne pathogen transmission to animals raised commercially in confinement was demonstrated 40 years ago with Marek's disease in poultry (Burmester and Witter, 1972; Grunder, et al., 1975). In swine, bioaerosol transmission of PRRSV among farms and the potential for air filtration systems to protect herds at risk was demonstrated (Dee, et al., 2010). Subsequently, numerous farms have implemented air filtration systems

since it demonstrated to reduce the risk of novel PRRSV introductions (Dee, et al., 2012; Alonso, et al., 2013b) . However, the installation of air filtration systems on large sow farms is an expensive intervention, involving initial capital costs of approximately $150 – 200 per sow (or approximately $450,000 to $600,000 for 3,000 sow-herds) (Reicks and Polson, 2011). The fact that the installation of such a system is now common in the Midwestern USA reflects the major economic impact on infectious diseases such as PRRSV (Holtkamp, et al., 2011).

Many filters are needed to equip a swine farm with air filtration (about 1,000 filters in a 3,000 sow-herd facility). Negative pressure conditions force the incoming air to pass through filters which trap particles within the filter media before air enters the animal space. Most common filters used in swine farms are made of non-woven fibrous media that collect particles by a variety of mechanisms (TSI Incorporated, 2012). Particles are captured by “interception” when they follow air around filter fibers and come into contact with the fiber. Large and heavy particles have inertia and can be collected on fibers by “impaction”. Small and light particles move randomly due to Brownian motion and can be captured by “diffusion” on filter fibers. Some other filter media carries electrostatic charges helping capture airborne particles that naturally carry electrical charge.

Due to the mechanisms to capture particles by filters, the efficiency of an individual filter or the system is dependent on particle size and it is measured under laboratory conditions by removing particles of KCl ranging between 0.3 to 10 µm. This information is used to classify the filters on a scale of 1 to 16 as “Minimum Efficiency Reporting Value (MERV)”. Swine facilities

use filters rated as MERV 14 or MERV 16, and they provide 75% and 95% minimum removal efficiency respectively in capturing particles from 0.3 to 1 microns in diameter, and >95% for particles of 1 to 10 microns. However, with these filters a significant proportion of particles (25% of 0.3-1 micron particles in the case of MERV 14 filters) will not be removed from incoming air. Since filters collect bioaerosol particles just as effectively as non-biological particles of the same size and filtration systems tend to reduce rather than eliminate the number of new outbreaks in swine facilities, the relationship between airborne particle size and swine viruses in bioaerosols is important in order to assess the application of filtration and the risk of failure in filtered farms.

Within-farm transmission strategies

All the methods used to decrease dust or particle matter (PM) within a farm will help to reduce aerosol production and disease transmission. Strategies used to reduce PM in livestock systems included the use of “low-dust” feed and feeding techniques (Pedersen, et al., 2000; Takai and Pedersen, 2000), use of feed additives (Takai, et al., 1996), water or oil sprinkling (Senthilselvan, et al., 1997; Takai and Pedersen, 2000; Nonnenmann, et al., 2004), changes in ventilation rates and air distribution (Aarnink and Wagemans, 1997), and electrostatic precipitation and ionization (Stgeorge and Feddes, 1995; Mitchell, et al., 2000; Rosentrater, 2003; Cambra Lopez, et al., 2009; Yao, et al., 2009). Air washers equipped with UV-irradiation systems have been used to specifically kill bacteria and viruses in bioaerosols, but are not commonly used in livestock production due to their limited capacity for handling large volumes of air in livestock buildings

(Schulz, et al., 2013). Other air sanitation interventions will include those chemical treatments applied in contaminated air or surfaces (i.e. chlorine dioxide (Morino, et al., 2011; Akamatsu, et al., 2012), hydrogen peroxide (Bailey, et al., 1996) and formaldehyde (Kim and Kim, 2010)).

Electrostatic particle ionization technology

The electrostatic ionization of airborne particles, due to the effect of releasing negative ions that will charge particles and potentially eliminate them from the air, has been demonstrated to be an effective dust removal technology tested in poultry houses and hatching cabinets (Mitchell, et al., 2000; Mitchell, et al., 2002; Richardson, et al., 2002), pigs (Rosentrater, 2003), cattle (Dolejs, et al., 2006) and rabbits (Chiumenti and Guercini, 1990). Ionization systems for use in swine facilities have not yet been fully developed despite preliminary research indicative of improved air quality (Rosentrater, 2003). USDA studies have shown this technology to remove from 60% to 80% of total particles suspended in the air (Mitchell, et al., 2004; Ritz, et al., 2004). The use of ion emissions combined with photocatalytic oxidation (due to the incorporation of photons and ultraviolet energy activating a catalyst) demonstrated significant pathogen removal efficiency and biocidal capabilities (Grinshpun, et al., 2007).

An electrostatic particle ionization (EPI) technology, with agricultural application in livestock, has become commercially available recently. This technology consists of a long ionizer bar with sharp point electrodes connected to a power supply of high voltage (-30 KV) that generates a high negative ion output which charges airborne particles electrically. The ionized

airborne particles are attracted towards opposite charges and in an enclosed space, like a confinement livestock rearing facility, may be cleared from the air by adhesion to the walls or other charged surfaces (Mitchell, 1997). Furthermore, the EPI technology resulted in a 12.2% average daily gain improvement in nursery pigs and a reduction of 1.2% in nursery mortality in a commercial system that used the EPI system throughout a 12 month period (Rademacher, et al., 2012). Overall, this technology seems promising to improve air quality in barns, and there are factors such as ion density (related to the distance to the ion source and the number of sources), size of particles, PM concentrations, ventilation rates and humidity levels that are known to influence the performance of ionization in livestock facilities (Cambra-Lopez, et al., 2010).

However, there is limited information on the effect of ionization in the reduction of swine airborne pathogens. Most previous studies have evaluated the effect of ionization in poultry agents such as *Salmonella enteritidis* (Seo, et al., 2001), Newcastle disease virus (Mitchell and King, 1994), as well as other pathogens such us *Bacterium tuberculosis* in humans(Escombe, et al., 2009). However, there is limited information on the effect of ionization on viruses that affect livestock. Overall, more research is needed to evaluate the effectiveness of ionization to reduce particles, pathogen load and viability of infectious agents important to livestock. This is particularly relevant for food animals raised in confinement conditions (i.e. pigs and poultry) where animals are housed in enclosed environments and, specifically for pigs where the importance of airborne transmission for swine and zoonotic pathogens such as PRRSV, PEDV, IAV, and MRSA is well documented.

Chapter 2: Concentration, size distribution, and infectivity of airborne particles carrying swine viruses

Chapter published as followed:

Alonso, C; Goede, DP; Morrison. RB; Davies, PR; Marthaler, DG; Torremorell, M; Evidence of long-distance transport and infectivity of airborne porcine epidemic diarrhea virus. Vet Res. (2014) July 14, 45(1):73

Alonso, C; Raynor, PC; Davies, PR; Torremorell, M; Concentration, size distribution, and infectivity of airborne particles carrying swine-associated viruses. PLoS One (2015) Aug 19;10(8):e0135675. doi: 10.1371/journal.pone.0135675

Summary

When pathogens become airborne, they travel associated with particles of different size and composition. Particle size determines the distance across which pathogens can be transported, as well as the site of deposition and the survivability of the pathogen. Despite the importance of this information, the size distribution of particles bearing viruses emitted by infectious animals remains unknown. In this study we characterized the concentration and size distribution of inhalable particles that transport influenza A virus (IAV), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine epidemic diarrhea virus (PEDV) generated by acutely infected pigs, and assessed virus viability for each particle size range. Aerosols from experimentally infected pigs were sampled for 24 days using an Andersen cascade impactor able to separate particles by size (ranging from0.4 to 10 micrometer (µm) in diameter). Air samples collected for the first 9, 20 and the last 3 days of the study were analyzed for IAV, PRRSV and PEDV respectively using quantitative reverse transcription polymerase chain reaction (RT-PCR) and quantified as geometric mean copies/m^3 within each size range. IAV was detected in all particle size ranges in quantities ranging from $5.5x10^2$ (in particles ranging from 1.1 to 2.1µm) to $4.3x10^5$ RNA copies/m^3 in the largest particles (9.0-10.0µm). PRRSV was detected in all size ranges except particles between 0.7 and 2.1µm in quantities ranging from $6x10^2$ (0.4-0.7µm) to $5.1x10^4$ RNA copies/m^3 (9.0-10.0µm). PEDV, an enteric virus, was detected in all particle sizes and in higher quantities than IAV and PRRSV ($p < 0.0001$) ranging from $1.3x10^6$ (0.4-0.7µm) to $3.5x10^8$ RNA copies/m^3 (9.0-10.0µm). Infectious status was demonstrated for the 3 viruses,

and in the case of IAV and PRRSV, viruses were isolated from particles larger than 2.1µm. In summary, our results indicated that airborne PEDV, IAV and PRRSV can be found in a wide range of particle sizes. However, virus viability is particle size dependent.

Introduction

Among all infectious agents, those transmitted through aerosols are the most difficult to control (Hyslop, 1971). The speed of dispersion of airborne infectious agents makes them hard to contain and protect against, and the wide reach of susceptible hosts makes the control of airborne pathogens a priority for public and animal health officials.

Infectious agents travel associated with particles of various natures including fecal material, dust, debris, water, respiratory fluids and, specifically in buildings housing animals, with bedding and hair particles. The composition and size distribution of these particles will determine the location of deposition in the susceptible host, and influence the time the infectious agents can remain suspended in the air, the distance across which they can be transported, and the survivability and infectivity of the pathogens (Austin, et al., 1979; Stilianakis and Drossinos, 2010; Zuo, et al., 2013b). Thus, particle size is central to the epidemiology of airborne pathogens.

Particles of small size can remain suspended in the air for long periods, potentially exposing a large number of susceptible individuals, including those close to the source and those at greater distances (Xie, et al., 2007). A spherical particle of 4 µm in diameter takes 33 min to settle 1 m in still air,

compared to a 1µm particle that will take 8 h (Lindsley, et al., 2010). Relative humidity, temperature and wind currents are the most important environmental factors that will determine the settling time of airborne particles that contain volatile components.

There is limited information in regards to the particle sizes with which infectious agents are associated. In humans, measures of particle size for influenza A virus (IAV) have been evaluated in controlled laboratory and health care settings, airplanes, daycares and households (Fabian, et al., 2008; Blachere, et al., 2009; Lindsley, et al., 2010; Yang, et al., 2011; Milton, et al., 2013). Distribution of IAV particle size varied between studies and settings, with IAV found in particles 1-4 µm in diameter (49% of particles) and particles > 4 µm (46% of particles) in health care facilities (Blachere, et al., 2009), and particles < 2.5 µm (64% of viral copies) detected in public places (i.e. health care, airplanes and day cares) (Yang, et al., 2011). Furthermore, dispersion models that have incorporated particle size information have indicated the plausibility of airborne transmission within these settings (Stilianakis and Drossinos, 2010).

For pigs and cattle, the size distribution of particles associated with foot and mouth disease virus (FMDV) depended on whether aerosols were generated artificially or by experimentally infected animals (Donaldson, et al., 1987; Gloster, et al., 2007). In the case of pigs affected by Pseudorabies disease virus (PSV), particle size distribution varied based on days of infection (Donaldson, et al., 1983). Specifically for IAV, no information was found for animals raised in agricultural environments, and the information is limited to animal models using ferrets or guinea pigs that assess the risk of

IAV airborne transmission to people (Lowen, et al., 2007; Mubareka, et al., 2009; Koster, et al., 2012). Overall, there is a lack of information on particle size distribution for pathogens affecting humans and animals, including zoonotic viruses. Furthermore, studies have been limited to viruses affecting the respiratory tract even though there is evidence that enteric viruses [i.e porcine epidemic diarrhea virus (PEDV), human-noroviruses, adenoviruses and enteroviruses can also be transmitted through the air (Miller and Artenstein, 1967; Tseng, et al., 2010; Alonso, et al., 2014; Bonifait, et al., 2015).

In this study we characterized the particle concentrations, size distributions and infectivity of three viruses that affect swine. These viruses were selected because of their differences in pathogenesis and modes of transmission. IAV, important because of its zoonotic potential, is shed only in respiratory secretions of pigs, and it is well established that it can be transmitted through aerosols. Porcine reproductive respiratory syndrome virus (PRRSV) is a systemic virus that can be shed in many body secretions (Rossow, et al., 1994), is exhaled in air, and has been detected in air as far as 9.1 km from swine herds (Otake, et al., 2010). Lastly, PEDV, an emerging virus in North America (Stevenson, et al., 2013), is an enteric virus that primarily replicates in large quantities in the small intestine, is largely concentrated in feces of diarrheic pigs and can replicate in alveolar macrophages (Park and Shin, 2014). The information from this study may contribute to the understanding of airborne transmission of viruses of different pathogenesis and routes of transmission, which is necessary to fully prevent the spread of infectious diseases.

Materials and methods

Ethics statement

Protocols and procedures followed throughout the study were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC1110A05802), and the Institutional Biosafety Committee (IBC1208H18341).

Housing and experimental animals

The study was performed at the University of Minnesota BSL-2 research animal units, St. Paul, MN. The room has a total air space of 35.1 m^3 and the air entering the isolation room was filtered. Relative humidity and temperature were monitored continually using a data logger (ThermaData Logger-Model HTD, ThermoWorks, Lindon, UT, US). A group of twelve, 5-week-old pigs were purchased from a herd that tested negative for IAV, PRRSV, PEDV and *Mycoplasma hyopneumoniae* on routine serologic and antigenic testing. The negative status of the experimental pigs was confirmed prior to inoculation. Serum samples were tested for IAV and PRRSV via enzyme linked immunosorbent assays (ELISA) (IDEXX AI Ab Test and HerdChek X3 ELISA, IDEXX, ME, US) (Vincent, et al., 2010; Sattler, et al., 2014). Nasal swabs and rectal swabs were tested for IAV and PEDV respectively using real-time reverse transcriptase PCR (RT-PCR) (Slomka, et al., 2010; Alonso, et al., 2014). Microchips were subcutaneously implanted to record body temperature following manufacturer specifications (LifeChip,

Destron Fearing, MN, US). Pigs were housed in solid floors without bedding and fed once a day on the floor. Water was provided *ad libitum*.

Virus inoculation

Ten pigs out of 12 were inoculated with PRRSV and IAV 48 h after arrival at the isolation units. Two pigs were removed from the room prior to inoculation and commingled back with the rest of the pigs 6 hours after to serve as contact infected controls. Pigs were sedated using an intramuscular injection of Telazol (Fort Dodge Animal Health, Fort Dodge, IA, USA) at 6mg/Kg, and experimentally inoculated intra-nasally and intra-tracheally with 1 ml/each of 4.4×10^6 tissue culture infective dose ($TCID_{50}$/ml) of IAV/Swine/Iowa/00239/2004 H1N1. Following that, pigs were inoculated intra-muscularly and intra-nasally with 1 ml/each of 1.13×10^5 $TCID_{50}$/ml of PRRSV strain MN-1-8-4 (Dee, et al., 2009; Corzo, et al., 2014).

At day 21 of the study, all pigs were intra-gastrically inoculated with a suspension of 20 ml of PEDV-material obtained from mucosal scrapings from PEDV infected pigs following published procedures (Alonso, et al., 2014). The inoculation material was confirmed positive by PEDV RT-PCR and diluted to a cycle threshold (Ct) value of 15 to 16. The inoculation material was prepared and kept refrigerated for 24h at 4ºC prior to inoculation. The total duration of the study was 24 days.

Clinical scores and body temperature

Body temperature and clinical signs were recorded twice daily starting 72 hours pre-inoculation. Fever was defined as rectal temperature higher than 40℃ for 2 or more consecutive days. Clinical scores included lethargy and signs of respiratory disease (i.e. coughing and sneezing episodes). Lethargy score was defined *a priori* on a 1 to 5 scale and measured based on the response to clapping or flight reaction, and curiosity of the pigs towards the investigator and sampling rope (Table 2.1). This procedure was repeated 3 times each day and final scores were averaged. The respiratory disease score was defined as the number of pigs with coughing and/or sneezing episodes (i.e. one or several episodes in a sequence by an individual pig) registered in 3 minutes. The respiratory score was calculated as a percent by dividing the number of animals with coughing and/or sneezing episodes by the total number of animals observed and multiplying the result by 100 (Bahnson, 1994).

Sampling procedures

Nasal swabs were collected for IAV testing from all pigs on -2, 3, 5, 7, 9 and 13 days post infection (DPI) using rayon-tipped swab applicators with Stuart's medium (BBL CultureSwabs liquid Stuart single plastic applicator/Becton, Dickinson and Com., Sparks, Maryland, USA). After sample collection, swabs were transported to the laboratory, suspended in 2 ml of minimal essential medium (MEM Mediatech Inc., Manassas, VA, USA) supplemented with 4% of bovine serum albumin (BSA) and stored at -80℃.

Serum samples for PRRSV viremia were collected using venipuncture of the jugular vein at days -2, 4, 12 and 20 DPI. After collection, serum was separated, divided into 2 aliquots and stored at -80℃.

Oral fluids for PRRSV and IAV were collected daily at the same time when air samples were collected by hanging 0.4 m of cotton rope from a convenient metallic post following published procedures (Detmer, et al., 2011; Romagosa, et al., 2012). Oral fluid samples were then refrigerated, transported to the laboratory and stored at -80℃. For PEDV, at the termination of the study, intestines were removed from all animals and sections from 6 different areas of the jejunum and ileum were collected for histopathology.

Total particle concentrations and size distributions were measured using an optical particle counter (OPC) (AeroTrak 9306 Handheld Particle Sizer, TSI, Inc., St. Paul, MN) able to separate particles from 0.3 to >10 μm into 6 size intervals. To measure airborne virus concentrations, samples were collected twice daily (9:00 a.m. and 3:00 p.m.) for 17 days and once (9:00 a.m.) for 6 days using both a liquid cyclonic collector (Midwest Micro-tek, Brookings, SD, USA)(Corzo, et al., 2014) and an Andersen cascade impactor (ACI; Thermo Electron Corporation, Waltham, MA, USA) (Appert, et al., 2012), which is able to separate particles according to size. The capability of both air collectors for quantifying the presence of virus was compared. Briefly, the 2 air samplers were positioned approximately 1.3 m height from the floor and the OPC was positioned 1.7 m from the floor. The distance between the air samplers was 0.9 m. The pigs did not have direct contact or access to the devices.

Sample collection using the cyclonic air collector (which was demonstrated to process 200 l of air per minute) was carried out for 30 min. Ten milliliters of MEM supplemented with 4 % of bovine albumin serum were used as collection media. After collection, an average of 4 ml of sample was recovered, divided into 2 aliquots, and stored at -80℃. The collector was then disinfected with 70% ethanol (or 10% chlorine during PEDV air sampling), rinsed with distilled water and dried with paper towels. After disinfection, the collection vessel and the turbine were swabbed, and samples stored at -80℃ until analysis. The ACI sampled air at 28.3 l/min for 1 hour, and separated particles into 8 size intervals: 0.4-0.7, 0.7-1.1, 1.1-2.1, 2.1-3.3, 3.3-4.7, 4.7-5.8, 5.8-9.0 and >9.0 µm. Samples from this device were eluted from every plate stage using a cell scraper and 1 ml of MEM (Appert, et al., 2012). All samples were transferred into 1.5 ml sterile plastic tubes, placed on ice and stored at -80℃ until testing. The ACI was then disassembled and plates and stages were scrubbed and disinfected with alkyl dimethyl benzyl ammonium chloride soap (Lysol, Reckitt Benckiser) and finally rinsed and dried with paper towels. After disinfection, a minimum of 4 collection plates and individual ACI stages were swabbed, and samples stored at -80℃. For each air sampling event, there were 8 stages assayed for the ACI and 1 sample for the cyclonic air collector.

Laboratory procedures

Oral fluid and air samples were tested using quantitative PRRSV, IAV and PEDV RT-PCRs as previously described (Cho, et al., 2006b; Slomka, et al., 2010; Alonso, et al., 2014). The samples from day 1 to day 9 DPI were

tested for IAV quantitative RT-PCR; for PRRSV from day 1 to day 20 DPI; and for PEDV from DPI 22 to 24 of the study. Nasal swabs, serum samples and intestinal/fecal swab samples were tested for IAV, PRRSV, and PEDV semiquantitative RT-PCRs respectively. For all three viruses, RT-PCR Ct values <35 were considered positive, 35 – 40 suspect, and > 40 negative.

To assess the infectivity of the air samples, virus isolation was attempted from all RT-PCR positive and suspect air samples in Madin-Darby canine kidney (MDCK) cells for IAV, MARC145 for PRRSV and VERO cells for PEDV using published procedures (Hofmann and Wyler, 1988; Mengeling, et al., 1996; Brookes, et al., 2010). Furthermore, because virus isolation is typically unsuccessful for North American variants of PEDV, a bioassay was performed by inoculating three susceptible piglets with air samples that tested PEDV RT-PCR positive. Briefly, four 10-day-old pigs from a PEDV-negative farm were purchased and each pig allocated to a separate isolation room. Each pig was intra-gastrically inoculated as described earlier in methods with PBS only (negative control) or 2 ml of pooled air samples containing the collection media of the cyclonic air collector diluted 1:10 with PBS to obtain a total of 20 ml of inoculation material per pig. All pigs were euthanized 4 days post-exposure by injection of 2 ml of pentobarbital (Fatal-Plus, 100 mg/kg IV) into the external jugular vein. Histomorphology analysis was then performed.

Statistical analysis

Data from the quantitative RT-PCR results, OPC by sampling day, type of sample and pathogen were consolidated in a spreadsheet (Microsoft EXCEL; Microsoft Corporation, Redmond, Washington, USA) and organized

for analysis. Means, standard deviations, and minimum and maximum values for quantitative variables, and frequency counts and percentages for qualitative variables were calculated for descriptive analysis. Considering day as a repeated measure and particle size as an independent variable, the quantity of virus (RNA copies) for each particle size per m^3 of air and mean concentration difference across all three viruses per size interval were assessed for significance using a repeated measures regression model in SAS 9.1 (SAS Institute, Cary, North Carolina, USA). Negative days (considered those of which results from both collectors were negative) were not included in the analysis. An ACI sample was included in the analysis when one or more samples from the eight stages of the sampler had a Ct value within the positive or suspect ranges. Tukey's method was also used in conjunction with ANOVA for pairwise comparison of virus concentrations among all particle size intervals.

Results

Environmental conditions, clinical signs and pig infection results

All inoculated and contact pigs became infected. For IAV all inoculated pigs, and contact controls became positive by 3 and 7 DPI (Ct values 24.6 ± 2.21 and 21.1 ± 2.79) respectively. For PRRSV all inoculated pigs and 1 of the 2 contact pigs were viremic at 7 DPI, and all pigs remained positive until 20 DPI.

Animals had an average body temperature of 39.3 ±0.6℃ (mean ± standard deviation) and, an average lethargy and respiratory score of 1.98 ± 0.98 and 3.5% ± 0.05% respectively across the entire study. For the first 4 days after infection with IAV and PRRSV, animals had the maximum lethargy and respiratory scores of 4 and 16.7%, respectively (Figure 2.1). Animals recovered from any respiratory clinical signs by 16 DPI. Lethargy scores ranged between 0 and 1 for the remainder of the study, except for the day after PEDV infection that increased to 2.5. All pigs had diarrhea 48h after PEDV inoculation.

The mean indoor temperature (mean ± standard deviation) was 23.2℃±0.7℃ with a mean relative humidity of 32.6% ±7.4% and a mean air pressure of -27.4 Pa ± 0.15 Pa relative to the hallway pressure.

Viral load and viral viability in air samples

IAV RNA was detected by RT-PCR in air samples collected at 36h post-infection and until 9 DPI. A total of 58.3 % (70/120) ACI stages and 93.3% (14/15) samples from the cyclonic collector tested IAV RT-PCR positive. All negative control samples tested negative. IAV RNA was detected in particles of all size ranges tested (Figure 2.2). However, there was higher viral load of IAV in larger particles (> 9 μm) compared to smaller ones (≤9 μm) ($p < 0.001$) (Table 2.2).

Using the cyclonic air collector, IAV RNA was detected in the air for 8 DPI and the total geometric mean (and geometric standard deviation) viral concentration was similar [$9.1x10^4$ ($1.3x10^1$)] to the ACI [$4.77x10^4$(4.07)] (p = 0.403). IAV was isolated in cell culture from 28.6% (20/70) of ACI samples

and 35.7 % (5/14) of air cyclonic samples, and from particles of all sizes > 2.1 microns (Table 2.3).

PRRSV RNA was detected for the first time in air samples collected at 7DPI and until 17 DPI, on a total of 8 days. A total of 5.1% (14/272) samples from the ACI stages and 23.5% (8/34) from the cyclonic collector tested RT-PCR positive. Negative results from ACI stages collected during 12 days of the study were not considered for the viral load analysis. All negative control samples tested negative.

PRRSV RNA was detected in particle sizes ranging from 0.3 to 0.7 µm and in particles ranging from 2.1 to 10 µm (Figure 2.2). However, there was higher viral load of PRRSV in larger particles (0.9-10 µm) compared to smaller ones (<9 µm) (p = 0.015) (Table 2.2).

There was no difference in geometric mean concentration of PRRSV RNA in air samples collected with the cyclonic collector [$5x10^3$ ($3.8x10^1$)] versus the ACI [$1.5x10^4$ ($1.1x10^1$)] (p=0.353). PRRSV was isolated in cell culture from 78.6% (11/14) ACI samples and 75 % (6/8) air cyclonic samples, but only in samples from particles > 2.1 microns (Table 2.3).

PEDV RNA was detected in all air samples collected from 24 hours post infection to the termination of the study (Figure 2.2). All negative control samples tested negative.

PEDV RNA was detected in particles of all size ranges tested but there was higher concentration in particles >3.3 to 10 µm compared to those in ranges 0.4 to 3.3 µm. No difference in geometric mean viral concentration was observed between samples from the cyclonic air collector [$9.1x10^7$(2.7)] versus the ACI [$4.5x10^7$(1.8)] (p = 0.1601). PEDV could not be isolated by

standard cell culture techniques. However, all bioassay pigs infected with air samples experienced moderate to severe diarrhea, shed high quantities of PEDV in feces ranging from 3.96 x 10^{10} to 7.57 x 10^{10} (RNA copies/ml, Ct 15-16), and had histopathological lesions of moderate to marked atrophic enteritis compatible with PEDV infection. The negative control pig showed no clinical signs, tested negative by PCR and had normal intestinal histomorphology.

Overall there were higher concentrations of airborne PEDV compared to PRRSV and IAV ($p < 0.0001$) associated with each particle size, and higher concentrations of IAV compared to PRRSV in large particle sizes ranging from 4.7 to 9 µm ($p < 0.05$) (Figure 2.2).

Total particles by size

An optical particle counter was used to measure the size distribution of total particles in the air for the 24 days of the study (Figure 2.3). There were more total particles within the first submicrometer size (0.3-0.5 µm) compared to particles larger than 0.5 µm ($p < 0.05$). There were no statistically significant differences in the distribution of particles sizes among days throughout the study (results not shown).

Discussion

We investigated the particle concentration, size distribution, and infectivity of three animal viruses with different pathogenesis and transmission routes. IAV, PRRSV and PEDV emitted by infected pigs were found

associated with a wide range of particle sizes that can deposit throughout the respiratory tract and later be swallowed (Bonifait, et al., 2015). However, virus viability was particle size dependent with IAV and PRRSV isolated only from particles larger than 2.1 µm. PEDV, primarily an enteric virus, was found in larger concentrations than PRRSV and IAV while suspended in the air. Our results support the relevance of the aerosol route in the transmission of IAV, PRRS and PED viruses.

Viruses transmit differently based on the routes of excretion and ports of entry in susceptible hosts. The viruses investigated in this study have been shown to be infectious while found airborne (Dee, et al., 2010; Alonso, et al., 2014; Corzo, et al., 2014). Our study indicated that virus-associated particles disperse simultaneously across a wide range of particle sizes. This is important because it shows that viruses in airborne particles emitted or generated by animals can be transmitted simultaneously across both short and long distances. Although various conditions, including environmental temperature and relative humidity can affect the size distribution of aerosols, the findings from this study are relevant given that the viruses were emitted by pigs housed under environmental conditions similar to those found in environments where pigs and people interact.

Higher quantities of IAV, PRRSV and PEDV were found associated with larger particles, and in the case of IAV and PRRSV virus viability was also associated with larger particle size. Viability of PEDV based on particle size could not be assessed because of the lack of protocols to grow PEDV in cell culture at the time of the study, although viability in air samples was confirmed by bioassay. The association of increasing virus infectivity and

concentration, with increasing particle size, has been reported before, and it has been shown to follow a power law relationship greater than 3 (Zuo, et al., 2013b). The bigger the particle, the higher the probability that it will carry virus and be infectious. In this study, viability was only shown for PRRSV and IAV in particles bigger than 2.1 μm in diameter, indicating that measures should be considered to mitigate transmission across both short and long distances.

Determining the particle size distribution for both respiratory and enteric viruses has important implications for the control of animal and human diseases and the use of droplet and airborne infection control measures. In general, it is considered that only respiratory viruses are airborne despite some limited evidence that enteric viruses, such as adenovirus, norovirus or enteroviruses, can be airborne (Miller and Artenstein, 1967; Tseng, et al., 2010; Alonso, et al., 2014; Bonifait, et al., 2015). Infectious particles sized less than 10 μm tend to have more serious health implications as they are able to penetrate into the lower respiratory tract to establish infection, and in the case of enteric viruses they may deposit in the upper respiratory tract (i.e. tonsils) or the enteric tract by inhalation.

Interestingly, the quantity of PEDV in airborne particles was higher than that for PRRSV and IAV indicating the complexity of airborne transmission and that other factors such as quantity shed (i.e. course of infection), volume of secretions or excretions (i.e. mucus, feces, vomits), age of the animals, type and severity of clinical signs, and type of housing and air flows need to be taken into consideration when the risk of airborne transmission is being evaluated. In this case, we speculate that both, the quantity of PEDV shed per gram of feces in acutely infected pigs, as well as the volume of liquid

found in the diarrheic material may explain the higher quantities of RNA copies of PEDV compared to IAV or PRRSV. Furthermore, comprehensive personal protective equipment including respiratory protection should be considered for potential exposures to both respiratory and enteric viruses, in particular in settings where animals and people interact. In addition, other biosecurity measurements such as air filtration could be considered to protect nearby at risk populations as previously demonstrated (Alonso, et al., 2013b).

The information generated in this study is especially important to design effective airborne disease control programs for both enteric and respiratory viruses, including mitigation of occupational exposure of zoonotic pathogens. Changes in recommendations to protect from airborne viruses should be considered based on exposure to particles of different sizes.

Table 2.1 Lethargy score classification. Scores based on the combination of two parameters: the response to clapping or flight reaction, and the curiosity of the pigs towards the investigator and sampling rope.

	Lethargy scores				
	1	2	3	4	5
Response to clapping and flight reaction	Rapid reaction and fleeing	Rapid reaction, no fleeing	Slow reaction	Very slow reaction	No reaction after clapping
Curiosity (i.e. towards investigator, rope)	Very curious and chewing rope	Moderate curiosity and chewing rope	Low curiosity and delayed attention to rope	Low curiosity and no chewing rope	No curiosity and no chewing rope

Table 2.2 Distribution of virus quantity by particle size**.** Quantity distribution [geometric mean of RNA copies/m^3 (geometric standard deviation)] of influenza (IAV), porcine reproductive and respiratory syndrome (PRRSV) and porcine epidemic diarrhea (PEDV) viruses in air samples.

Particle size range (µm)	IAV	PRRSV	PEDV
0.4-0.7	$8x10^2$ (3.2) [a]	$6x10^2$ (4.1) [a]	$1.3x10^6$ (3.4) [a]
0.7-1.1	$6.1x10^2$ (1.7) [a]	[†]≤$5.1x10^2$ (1) [a]	$3x10^6$ (4.7) [a]
1.1-2.1	$5.5x10^2$ (2.5) [a]	[†]≤$3.6x10^2$ (1) [a]	$1.6x10^6$ (2.4) [a]
2.1-3.3	$1.4x10^3$ (4) [a,b]	$7.8x10^2$ (4.82) [a]	$5.2x10^6$ (3.3) [a,b]
3.3-4.7	$7.8x10^3$ (5.1) [b,c]	$1.8x10^3$ ($1.26x10^1$) [a]	$4.5x10^7$ (1.8) [c]
4.7-5.8	$2.3x10^4$ (7.4) [c]	$1.7x10^3$ (4.8) [a]	$5.5x10^7$ (2.2) [c,d]
5.8-9.0	$1.5x10^4$ (6) [c]	$1.1x10^3$ (7) [a]	$3.1x10^7$ (2) [b,c]
> 9.0	$4.3x10^5$ ($1.25x10^1$) [d]	$5.1x10^4$ ($2.8x10^1$) [b]	$3.5x10^8$ (2.9) [d]

[a, b, c, d] Different superscripts between rows of the same column indicate statistically significant differences (Tukey's test, $p < 0.05$)

[†]LOD: Limit of q-PCR detection

Table 2.3 RT-PCR and virus isolation results by particle size and air sampler type. Number of positive RT-PCR and virus isolation (VI) air samples for influenza (IAV), porcine reproductive and respiratory syndrome (PRRSV) and bioassay for porcine epidemic diarrhea (PEDV) viruses collected from acutely infected animals. Results are presented by air sampler, and in the case of the Andersen cascade impactor, by particle size rage.

Virus		Andersen cascade impactor (ACI) (particle size ranges in µm)									Total cyclonic collector
		0.4-0.7	1.1-0.7	1.1-2.1	2.1-3.3	3.3-4.7	4.7-5.8	5.8-9.0	9.0-10.0	Total ACI	
IAV	[1]PCR	*6/15 (40.0%)	3/15 (20.0%)	5/15 (33.3%)	6/15 (40.0%)	12/15 (80.0%)	12/15 (80.0%)	13/15 (86.7%)	13/15 (86.7%)	70/120 (58.3%)	14/15 (93.3%)
	[2]VI	0/6	0/3	0/5	1/6 (16.7%)	1/12 (8.33%)	6/12 (50.0%)	4/13 (30.8%)	8/13 (61.5%)	20/70 (28.6%)	5/14 (35.7%)
PRRSV	PCR	1/34 (2.9%)	0/34	0/34	1/34 (2.9%)	2/34 (5.9%)	1/34 (2.9%)	2/34 (5.9%)	7/34 (20.6%)	14/272 (5.1%)	8/34 (23.5%)
	VI	0/1	0	0	1/1 (100%)	2/2 (100%)	1/1 (100%)	0/2 (0%)	7/7 (100%)	11/13 (84.6%)	6/8 (75%)
PEDV	PCR	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	6/6 (100%)	48/48 (100%)	6/6 (100%)
	Bioassay	[†]NA	NA	NA	NA	NA	NA	NA	NA	NA	[§]3/3 (100%)

[1]PCR: Reverse transcription polymerase chain reaction results (includes samples with cycle threshold (Ct) value less than 40)
[2]VI: Virus isolation results *Number of positive samples out of total number of samples tested (%)
[†]NA: Not applicable [§]Bioassay results were obtained from the inoculation of susceptible pigs with RT-PCR positive air samples using the air cyclonic collector

Figure 2.1 Representation of clinical scores. Clinical scores of lethargy (1 to 5 scale) and percentage of respiratory disease (number of pigs with coughing and/or sneezing episodes) throughout the study.

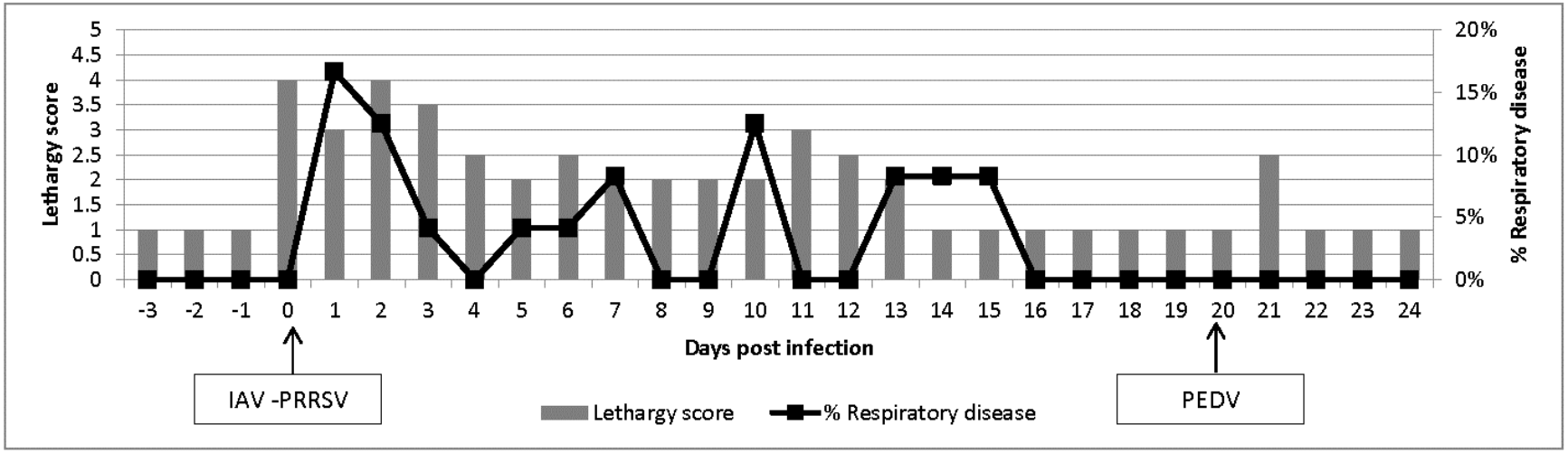


Figure 2.2 Particle size distributions of airborne viruses. Particle size distribution (Least Squares Means of log10 RNA copies/m^3 of air and 95% confident interval) for influenza, porcine reproductive and respiratory syndrome and porcine epidemic diarrhea viruses detected by the Andersen cascade impactor from aerosols generated by infected pigs.

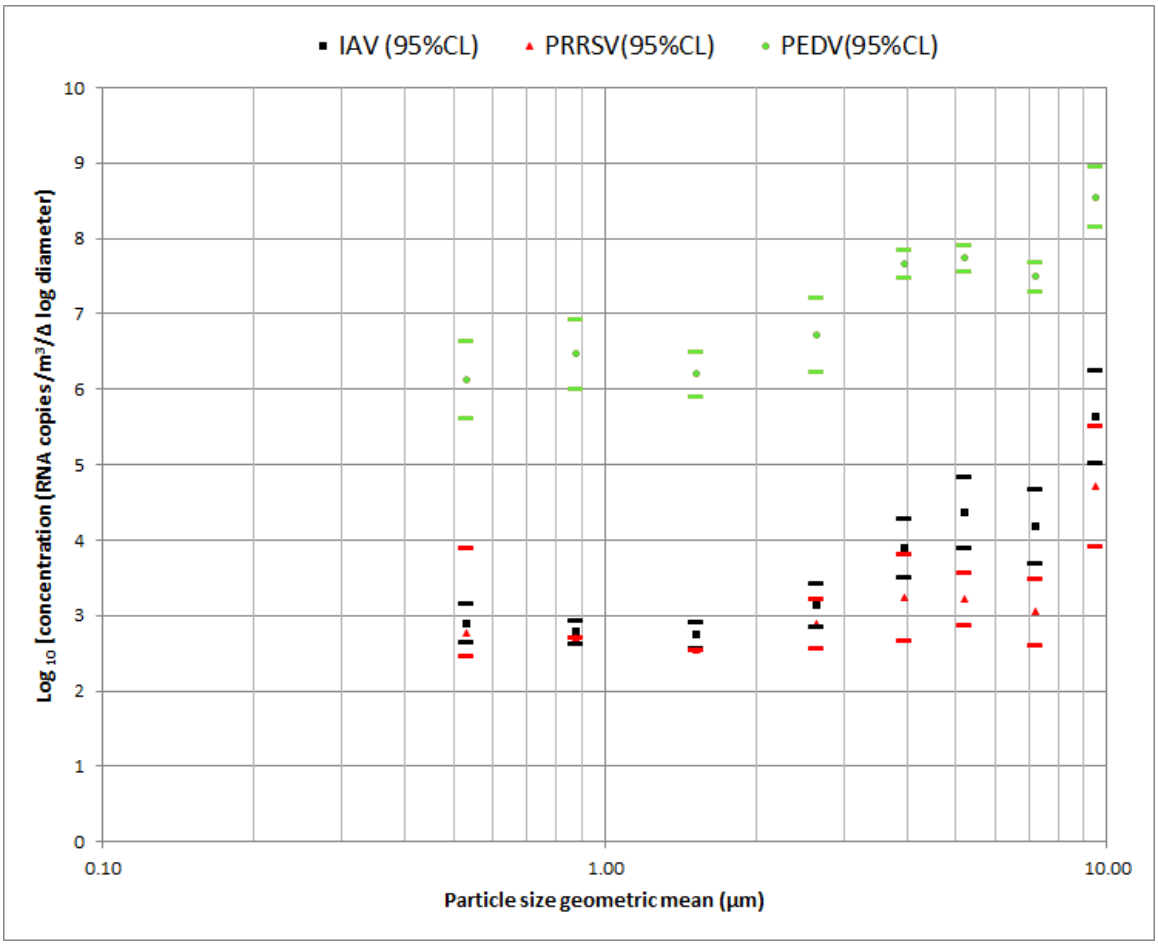
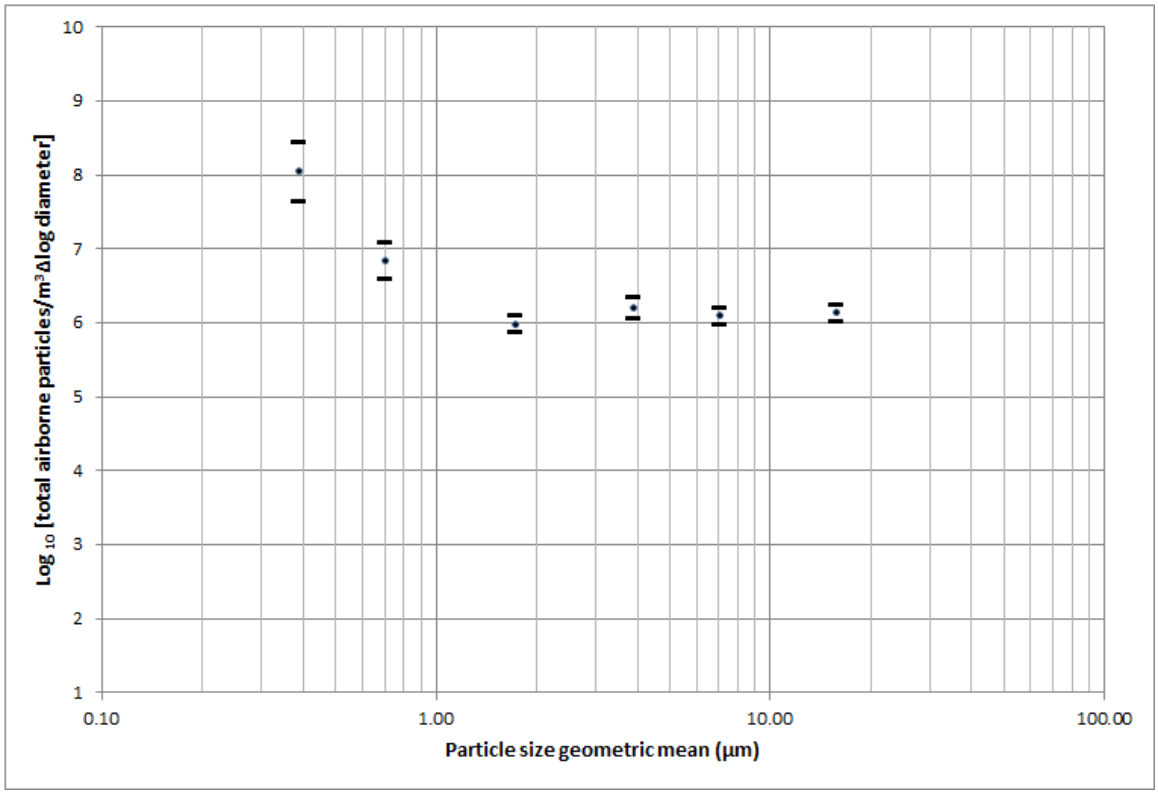


Figure 2.3 Total airborne particles distribution. Distribution of total airborne particles (geometric mean of number of particles/m^3 and 95% confident interval) measured using an optical particle counter.



Chapter 3: Comparison of two size-differentiating air samplers for detecting airborne swine viruses under experimental conditions

Summary

Quantification of dilute viral aerosols, as encountered outside animal housing facilities, requires methods able to detect low pathogen concentrations in large volumes of air. This study compared the performance of two size-differentiating cascade impactors, an Andersen 8-stage (ACI; 28.3 L/min) and a high volume Tisch (TCI; 1,133 L/min) to assess sampling efficiency for detecting influenza A virus (IAV) and porcine reproductive and respiratory syndrome virus (PRRSV). Samples of particles sorted by aerodynamic diameter were analyzed by quantitative polymerase chain reaction (qPCR) and collection efficiency assessed by particle size. Collection media [minimum essential medium (MEM) and beef extract (BE)], elution technique (active vs passive), and sampling time (10, 20, 30 min) were variables assessed for the TCI sampler. Extraction efficiency was 35% higher with BE compared to MEM ($p = 0.0007$), active extraction technique was 19% more efficient than the passive technique ($p = 0.03$), and time of sampling did not affect the virus concentration significantly. The ACI sampler was more efficient at IAV and PRRSV detection from small and medium size airborne particles (≤3μm) compared with the TCI sampler ($p < 0.001$). The TCI sampler was more efficient at IAV detection from large airborne particles (> 3μm) ($p=0.0025$). Under the conditions of this study the TCI air sampler was superior in the recovery efficiency of viruses associated with

particles > 3μm. There is potential in the use of the TCI air sampler for field conditions to predict the presence of small amounts of viruses in aerosols.

Introduction

In livestock, viruses associated with airborne particles have the potential to be transmitted from infective premises to naïve populations and propagate epidemics that cause substantial economic losses and impact the food supply (Stark, 1999). Infectious diseases affecting swine are of particular importance since pork is the most consumed protein worldwide. Foot and mouth disease virus (FMDV), porcine reproductive and respiratory syndrome virus (PRRSV), and influenza A virus (IAV) are examples of airborne pathogens able to cause devastating losses due to their pathogenicity, ability to travel long distances and, for IAV, zoonotic risk (Gloster, et al., 2010; Otake, et al., 2010; Corzo, et al., 2013a).

To determine the relative contribution of the aerosol route to the overall transmission of viruses among herds, sensitive and precise methods for quantifying virus in air samples are required. Knowledge of the concentration of pathogens associated with airborne particles of different aerodynamic diameters at different distances from a point source should help predict the risk of airborne transmission events in the vicinity of infected herds. This is complicated by the complex dynamics of dilution of contaminated air plumes that exit infected

facilities (Cooper, et al., 2014) and the reliability of sampling methods to determine pathogen concentrations. High-volume size differentiating air samplers have been commonly used because they are able to sample large volumes of air and, in conjunction with quantitative polymerase chain reaction (PCR) techniques, detect low quantities of infectious agents relatively rapidly, which should aid in the face of an emergency response (Cooper, et al., 2014). However, the sensitivity of high-volume air sampling equipment for detecting low virus concentrations has not been rigorously evaluated. In contrast, commercially available low-volume air samplers (i.e. liquid impingers, swirling aerosol collection and solid impactors) have been commonly used to collect viral aerosols (Verreault, et al., 2008) and they can be used as a reference to evaluate high-volume collection air samplers' efficiency and sensitivity.

In this study, we first compared the recovery efficiency of PRRSV and IAV using different eluents, extraction techniques, and three types of collection substrates of glass fiber. Following that, we further investigated the recovery efficiency sensitivity using different concentrations of viral PRRSV and IAV solutions. Finally, and based on previous results, we compared the performance of filter based high-volume size-differentiating air sampler (TCI) versus a lower-volume but well-characterized 8-staged impactor (ACI) (Appert, et al., 2012; Zuo, et al., 2013b; Ge, et al., 2014) for detecting two swine viruses, PRRSV and IAV.

Materials and methods

Virus propagation and titration

IAV/Swine/Iowa/00239/2004 H1N1 and PRRS MN 1-8-4 strains were propagated and titrated in Madin-Darby canine kidney (MDCK) and in a subline of the African monkey kidney cell line (MARC-145 cells) respectively (Mengeling, et al., 1996; Brookes, et al., 2010). Titers of live virus were determined by the Karber method (Hamilton, et al., 1977) resulting in a 4.4×10^6 $TCID_{50}$/mL (50% tissue culture infective dose) in the case of IAV and 1.13×10^5 $TCID_{50}$/mL for PRRSV. The stocks of virus were then aliquoted and stored at -80°C until use. Both viruses, provided by the University of Minnesota Veterinary Diagnostic Laboratory, are known to be shed in aerosols by infected pigs (Cho, et al., 2007; Corzo, et al., 2014).

Spike test and virus elution techniques for substrate processing

In order to quantify the virus elution efficiency from the different filters or substrates, 300µL applied as a single drop containing the 2 previously described viruses in minimum essential media (MEM, Mediatech Inc., Manassas, VA, USA) supplemented with 4% of bovine serum albumin was spiked onto 3 types of glass fiber substrates from the 2 air samplers: 1) TCI substrates (slotted 15.2 cm x 17.8 cm, Model P/N TE-230-GF, New Star Environmental, Inc. Roswell, GA, USA); 2) final TCI substrate (Model G653, 20.3 cm x 25.4 cm, Whatman, GE Healthcare,

UK); and 3) final ACI substrate (Model 934-AH, 80mm, Whatman, GE Healthcare, UK). The spike test was performed in triplicate. Two elution techniques (active and passive) were evaluated with the 2 types of elution media [MEM and 3% beef extract (BE) in 0.05 M glycine at pH 9.5]. A total of 36 filters were evaluated (3 filter types x 2 elution techniques x 2 elution media x 3 replicates). BE at alkaline pH was selected based on the capabilities of reducing the virus absorption to surfaces demonstrated in previous studies ((Zuo, et al., 2013a). MEM was also selected due to the known capabilities to preserve swine viruses, specifically influenza, from surfaces and other materials (Tiwari, et al., 2006; Thomas, et al., 2008b).

Prior to elution, all filters were placed in a biosafety cabinet to air dry (90 ± 15 min). For the passive technique 20 mL of eluent was added to each of the Petri dishes, and after 3 min, the filter was hand moved 20 times within the Petri dish using sterile forceps. The recovered eluent solution was frozen at -80°C until testing with reverse transcription polymerase chain reaction (RT-PCR) assay. For the active technique, filters were cut in 1 cm^2 squares using sterile scissors and eluted in 50 Falcon tubes with 20 mL of the eluent. Each tube was inverted ten times to mix the content. The mixing step was followed by a 5 minute period; another mixing step of 10 inversions; and finally each tube was vortexed for a 5 minute period at 1,500 rpm. All eluted virus samples performed with the BE were transferred to a 15 mL Falcon tube and the pH was adjusted to 7 using hydrogen chloride (HCl). One ml of viral solution was kept as a positive control. Spiked

MEM solution onto the 3 types of filters was used as negative control and samples eluted at the same time as the contaminated filters.

Sensitivity study of virus recovery efficiency by virus type

Based on previous results, a sensitivity analysis was performed to determine the minimum detection dose using the active virus elution technique with BE at basic pH. Three glass fiber filters were spiked with 300µL of the 2 different viruses (PRRSV and IAV) at 3 different concentrations (10 fold serial dilutions of IAV and PRRSV with starting concentrations of $3.7x10^5$ and $1.1x10^7$ RNA copies/mL respectively, see total virus spiked in Table 1) and placed in a biosafety cabinet to air dry (90 ± 15 min). A total of 18 filters were analyzed (3 concentrations x 2 virus x 3 replicates) and recovery efficiency calculated.

Comparison of air samplers

The experiment was carried out in one room at the University of Minnesota BSL-2 research animal units, St. Paul, MN. The room was mechanically ventilated with negative pressure of 0.11 inches of water, included incoming and outgoing filtered air, and had a total air space of 35.1 m^3. Relative humidity and temperature were monitored continually using a data logger (ThermaData Logger-Model HTD, ThermoWorks, Lindon, UT, US). Stock solutions of viruses were suspended in MEM with a fluorescent tracer dye (fluorescein sodium salt, Fluka, Buchs, Switzerland) and then aerosolized

continuously at 20 psi using a 6-jet Collison- nebulizer (BGI Inc., Waltham, MA, USA). A series of air samples were obtained in order to compare the 2 air samplers: the ACI, an Andersen 8-stage nonviable cascade impactor with 80 mm aluminum impaction plates and a flow rate of 28.3L/min (Thermo Scientific, Franklin, MA, USA), and the TCI, a high volume Tisch cascade impactor (Model 230, Tisch Environmental, Inc. Ohio, USA) with slotted 15.2 cm x 17.8 cm aluminum plates covered with 4 slotted collection substrates of glass fiber, as previously described, and a flow rate of 1,130 L/min. Both air samplers were provided with final filters. Air samplers were compared over different sample times (10, 20 and 30 min) on 3 occasions (replicates). For each sampling event, samples were collected first using the ACI and then the TCI. There was a 15 min interval between each sampling event to allow the aerosol to reach steady state. The top part of both samplers was located at 70 cm from the floor. Sample collection started after 15 min of aerosol generation which was the time needed to reach steady state.

The ACI sampler separates airborne particles into 8 size stages with aerodynamic diameter cut-points of 0.4, 0.7, 1.1, 2.1, 3.3, 4.7, 5.6, and 9.0 µm. Particles smaller than 0.4 µm are captured by a final filter. After each sampling period, the ACI was removed from the room, and samples were eluted from the plate on each of the 8 stages using a cell scraper and 1.5 mL of MEM (Appert, et al., 2012). All samples were transferred into 1.5 mL sterile plastic tubes, placed on ice and stored at -80℃ until testing. Between replicates, all plates and stages

were scrubbed and disinfected with alkyl dimethyl benzyl ammonium chloride soap (Lysol, Reckitt Benckiser) and finally rinsed and dried with paper towels. After disinfection and drying, a minimum of 4 collection plates and individual ACI stages were swabbed, and samples stored at -80℃ to serve as negative controls.

The TCI sampler separates particles into 4 stages with aerodynamic particle diameter cut points of 0.95, 1.5, 3 and 7.2 μm. Particles from these 4 stages are captured by slotted glass fiber collection substrates and particles smaller than 0.95 μm are captured by a final filter. After each sampling period, collection substrates and filters were removed in the laboratory using sterile gloves, folded and placed into individual labeled Petri dishes, and refrigerated at 4°C until sample elution processing. Between replicates, all stages were disinfected with 70% alcohol, air dried, and finally rinsed and dried with paper towels. After disinfection and drying, all stages were swabbed, and samples stored at -80℃. For each replicate, there were 8 stages assayed for the ACI, 4 stages for the TCI and 2 backup filter samples.

Total airborne particle counts by particle size were collected using an optical particle counter (OPC) (AeroTrak 9306 Handheld Particle Sizer, TSI Inc., St. Paul, MN) for 10, 20 and 30 min during each sampling event of each of the air samplers.

Real-time quantification PCR

All air samples from the ACI, the TCI substrates, and the eluted samples originating from the spiked assessments were tested using a quantitative IAV and PRRSV reverse transcription polymerase chain reaction (RT-PCR) as previously described (Cho, et al., 2006b; Corzo, et al., 2013a).

Fluorometric quantification

For the purpose of quantifying the amount of total particles in the initial suspension (before aerosolization) as well as in the final suspension (after air sampling), a fluorescent dye was added to the nebulizer suspension. A fluorescence-based method was used to understand the physical collection efficiency of both air samplers as previously described (Agranovski, et al., 2005; Appert, et al., 2012; Ge, et al., 2014). Fluorescein levels in the nebulizer suspension (before and after sampling), as well as those from substrates and impactor stage eluates were assessed using a spectrofluorometer (Model Synergy H1, BioTek, Winoosk, VT, US). All samples were transferred in triplicate into 96-black well plates (Thermo Fisher Scientific, Roskilde, Denmark) and fluorescein intensity was measured at λ = 515nm and at λ = 485nm after the transition of the eluent to a high-energy electronic state or excitation. Results were expressed as fluorescein intensity per mL or volume of air (m^3) depending on the type of sample. To quantify the fluorescein in the eluted samples, linear regression trend lines were used to calculate the calibration curves for the 2

types of media; slope (m) and intercept (c) from the linear regression equation were obtained (Microsoft EXCEL; Microsoft Corporation, Redmond, Washington, USA). Unknown concentrations of fluorescein (y) were then calculated substituting the fluorescein intensity reading (x) in the equation y = mx+c.

Statistical analysis

Data from the qRT-PCR results, replicate number, virus, type of air collector, time of sampling and fluorescein tracer concentrations were consolidated in a spreadsheet (Microsoft EXCEL; Microsoft Corporation, Redmond, Washington, USA) and organized for analysis. Means, standard deviations, and minimum and maximum values for quantitative variables, and frequency counts and percentages for qualitative variables were calculated for descriptive analysis. Negative PCR results in the stages of both air samplers were included in the analysis when one or more stages of the sampler had a Ct value within the positive or suspect range (Ct value <40). Negative results included in the analysis were assigned a value of 100 RNA copies/mL according to the limit of detection of the qRT-PCR technique.

In regards to the efficiency comparison between both air samplers for different particle size ranges, a new variable called “size” was created. If the geometric mean point of a specific interval (square root of both ends of the interval product) was < 1 µm, ≥1 and ≤ 3 µm, or > 3 µm then “size” was equal to “small”, “medium” or “large”, respectively. Due to the number of variables in the

study, a multivariate modeling analysis was performed. The variable “replicate” was considered as a random variable. Under these premises, the total quantity of virus (RNA copies/m^3) adjusted by type of air sampler, size of particles, virus and time of sampling was assessed for significance using a mixed linear regression model in SAS 9.1 (SAS Institute, Cary, North Carolina, USA). P-values of the multiple comparisons were adjusted by Tukey-Kramer’s test and considered significant at $p<0.05$.

Results

Spiked test recovery efficiency results by extraction technique, type of substrates, and elution media

Results from the qPCR analysis demonstrated that “type of eluent media”, “type of substrates”, and “extraction technique” were the only significant variables in the model. Briefly, the BE eluent yielded a 35% higher extraction efficiency compared to MEM ($p = 0.0007$); the active extraction technique was 19% more efficient than the passive technique ($p = 0.03$); and, filter type ACI final filter yielded the highest efficiency (73%), followed by the TCI filter (25%) and ultimately the TCI-backup filter (26%). No difference was observed between the 2 TCI types of filters.

Sensitivity of recovery efficiency by virus type

Results from the sensitivity analysis are shown in Table 3.1. The recovery efficiency using the active technique and BE as elution media decreased with the decrease in virus concentration. Virus recovery efficiency ranged between 0% to 22% and, 12% to 22% for PRRSV and IAV respectively from the least to the most concentrated samples.

Comparison of air samplers

- *Comparison by viral load*

Raw data of all sample points collected by the 2 air samplers during different sampling periods are represented in Figure 3.1 for both IAV (Figure 3.1A) and PRRSV (Figure 3.1B). For the comparisons between air samplers based on the multivariable analysis, the variable “time” was removed from the final model due to lack of significance. For both IAV and PRRSV, the ACI sampler was more efficient than the TCI sampler ($p < 0.001$) at recovering virus from small and medium size particles when virus concentration in the air was high (Figure 3.2). In contrast, the TCI sampler appeared to be more efficient at recovering virus from larger particles, although the difference was statistically significant only for IAV ($p=0.0025$) but not PRRSV ($p=0.258$).

- *Comparison using fluorescein tracer*

Results of fluorescein intensity for the different filters were expressed as µg/mL of air sample and then transformed to volume of air (m^3) using the following calibration curves: $y = 0.0072x + 0.0263$ ($R^2 = 0.9623$) and $y = 0.0039x + 0.0188$ ($R^2 = 0.9699$) for DMEM and BE respectively. The variable "time" was again removed from the final analysis due to lack of significance in the model. Overall, the ACI sampler was more efficient ($p<0.001$) at recovering fluorescein associated with small and medium airborne particles at higher concentrations of fluorescein compared with the TCI sampler (Figure 3.3). However no difference between samplers was seen for recovery of fluorescein from larger airborne particles ($p=0.62$).

Distribution of airborne particles

An optical particle counter was used to measure the size distribution of total particles in the air at the same time when the ACI (Figure 3.4A) and TCI samplers (Figure 3.4B) were collecting air samples. Overall there were more total particles within the smallest sizes (0.5-3 µm) than larger particles (> 3 µm) ($p < 0.05$) during all air sampling events. There were no statistically significant differences in the distribution of particle sizes among different sampling times (10, 20, 30 min) when ACI and TCI samplers were operated throughout the study (results not shown).

Discussion

Bioaerosol research is constrained by the limits of measurement tools to obtain accurate, reliable and comparable exposure estimates for airborne microorganisms. Error in estimates of bioaerosols may originate in any step of an air sampling, collection and processing protocol. Our specific interest was to advance understanding of methodologic factors that may influence observations of virus concentration in air, with a view to the particular challenge of sampling of aerosols from outside of animal housing facilities. Our data indicate that the higher airflow and collection principles of the high volume TCI sampler overall did not improve the efficiency of detection of airborne viruses compared to the conventional ACI sampler. However the TCI sampler was superior in the recovery efficiency of viruses associated with particles > 3μm demonstrating a potential in the use of the TCI air sampler for field conditions to predict the presence of viruses associated to larger particles.

The primary limitation to the use of filters for viral air sampling is the extraction of the sample from the filter. Based on a spike test, we calculated the recovery efficiency using MEM and BE at basic pH as eluents and tested different extraction techniques. Difference in sampling efficiency between both collectors could be due to lower recovery efficiency after the aerosols test when compared to the spike test as demonstrated by other authors (Zuo, et al., 2013a). The increase in recovery efficiency after increasing the concentration of virus solution used for challenge the substrates has been also demonstrated

previously in a filter based study comparing two large volume air samplers (EPA., 2009). Further research of additional substrates should be performed to improve virus recovery efficiency in large volume air samplers.

The methodology described in our study only allowed us to calculate recovery efficiency of virus based on physical penetration of RNA particles. Future studies should incorporate cell culture methods to measure live virus recovery (infectivity penetration) associated with different size ranges, giving the fact that both air samplers are using different methods for collecting particles that may affect the infectivity of the virus. Infectivity penetration was calculated in a filtering face piece study to be of a magnitude of 2 orders lower than the physical penetration (Zuo, et al., 2013a). Fiber glass substrate and high air flow could be responsible for structural damage and desiccation of the virus particles (Verreault, et al., 2008), as well as the impaction of particles in aluminum plates as part of the sampling process (Appert, et al., 2012). Differences in both air sampling methods (especially in smaller particles) could be responsible for the different efficiency of virus recovery obtained between air samplers.

Fluorescein tracers have been previously applied to calculate physical and infectivity losses of viruses during the nebulization process in terms of fluorescein intensity as a reflection of the concentration of fluorescein in the virus solution (Appert, et al., 2012; Ge, et al., 2014). Since previous studies demonstrated no significant changes in the virus titer after the nebulization and virus viability was not part of the design of this study, the use of the fluorescein tracer was preferred

as a confirmatory test to our molecular analysis based on concentration of viral RNA copies.

Results from this study demonstrate the difficulty of estimating the viral concentration in bioaerosols, specifically those from outside of animal housing facilities, due to air sampling, collection and processing challenges. Further research is needed to overcome these challenges in order to use these size differentiating air samplers for field conditions to better understand between-farm disease transmission risks of such important diseases for food animals and more specifically for the swine industry.

Table 3.1 Spiked test recovery efficiency results. Mean quantity (RNA copies ± SD) of porcine reproductive and respiratory syndrome virus (PRRSV) and influenza A virus (IAV) spiked and eluted from the filters as well as recovery efficiency (%) of the viruses as a function of virus concentration after the active technique was applied.

PRRSV			IAV		
Total spiked	Total eluted	RE* (%)	Total spiked	Total eluted	RE (%)
$3.5x10^8$	$2.3x10^7 \pm 1x10^6$	22	$1.2x10^7$	$7.8x10^5 \pm 1x10^5$	22
$3.1x10^7$	$2.3x10^5 \pm 1x10^5$	1.6	$1.1x10^6$	$4.6x10^4 \pm 1x10^4$	14
$3.3x10^6$	†ND	§NA	$1.1x10^5$	$5.7x10^3 \pm 8x10^2$	12

*RE: Recovery efficiency

†ND: Non detectable by qRT-PCR

§NA: Not applicable

Figure 3.1 Raw data of particle size distribution associated to viruses. Particle size distribution (concentration of $\log_{10}$ RNA copies / m^3 of air) of influenza A virus (IAV) and porcine reproductive and respiratory syndrome virus (PRRSV) detected on different sampling periods by the Andersen cascade impactor (ACI) and the high volume Tisch cascade impactor (TCI) from mechanically generated aerosols .

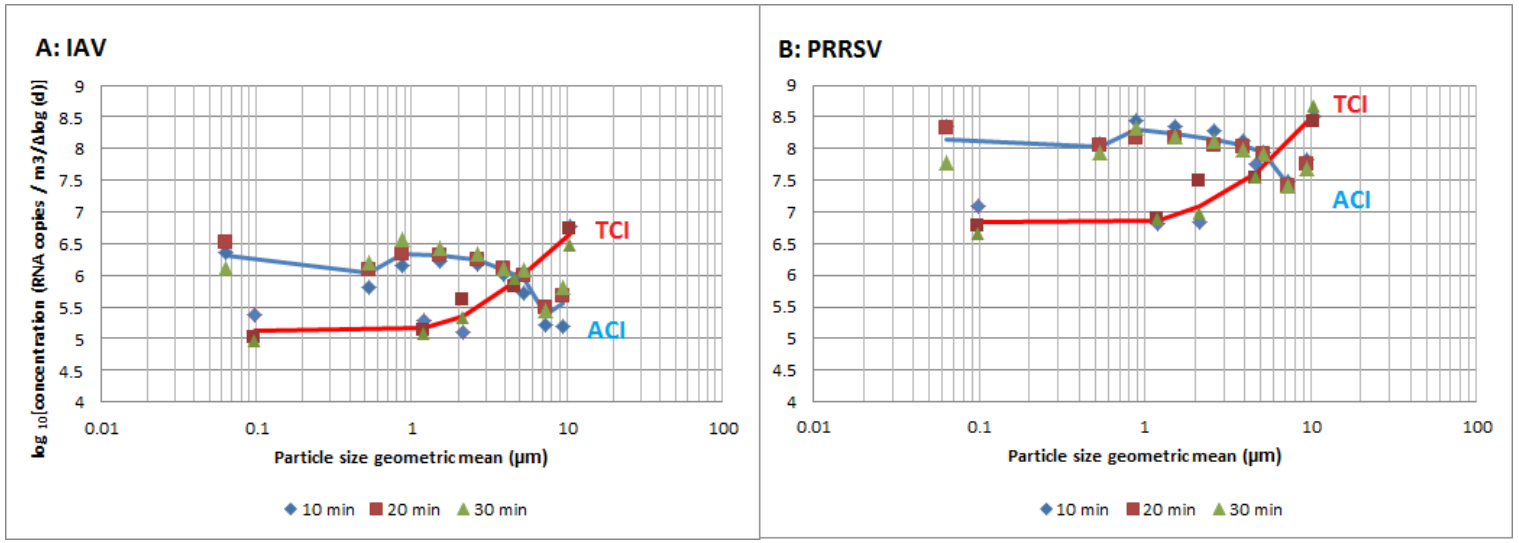


Figure 3.2 Particle size distributions associated to virus, modelling analysis. Particle size distribution (least squares means of $\log_{10}$ RNA copies/m^3 of air and standard errors) of influenza A virus (IAV) and porcine reproductive and respiratory syndrome virus (PRRSV) detected by the Andersen cascade impactor (ACI) and the high volume Tisch cascade impactor (TCI) from mechanically generated aerosols.

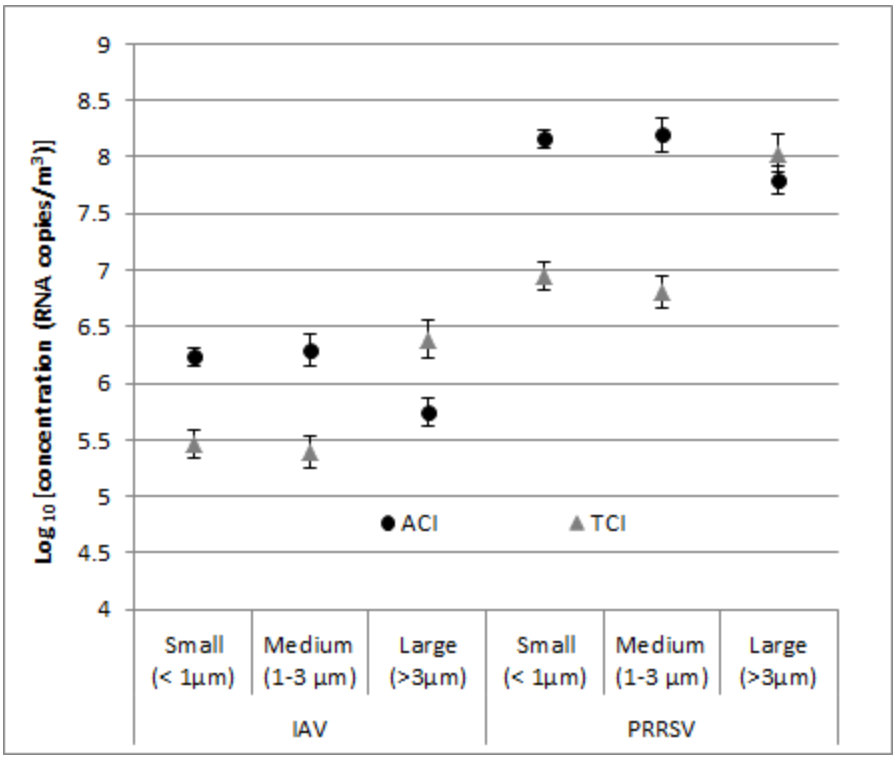


Figure 3.3 Particle size distributions of fluorescein particles. Particle size distributions (least squares means of $\log_{10}$ RNA copies/m^3 of air and standard errors) of fluorescein particles collected by the Andersen cascade impactor (ACI) and the high volume Tisch cascade impactor (TCI) from aerosols mechanically generated.

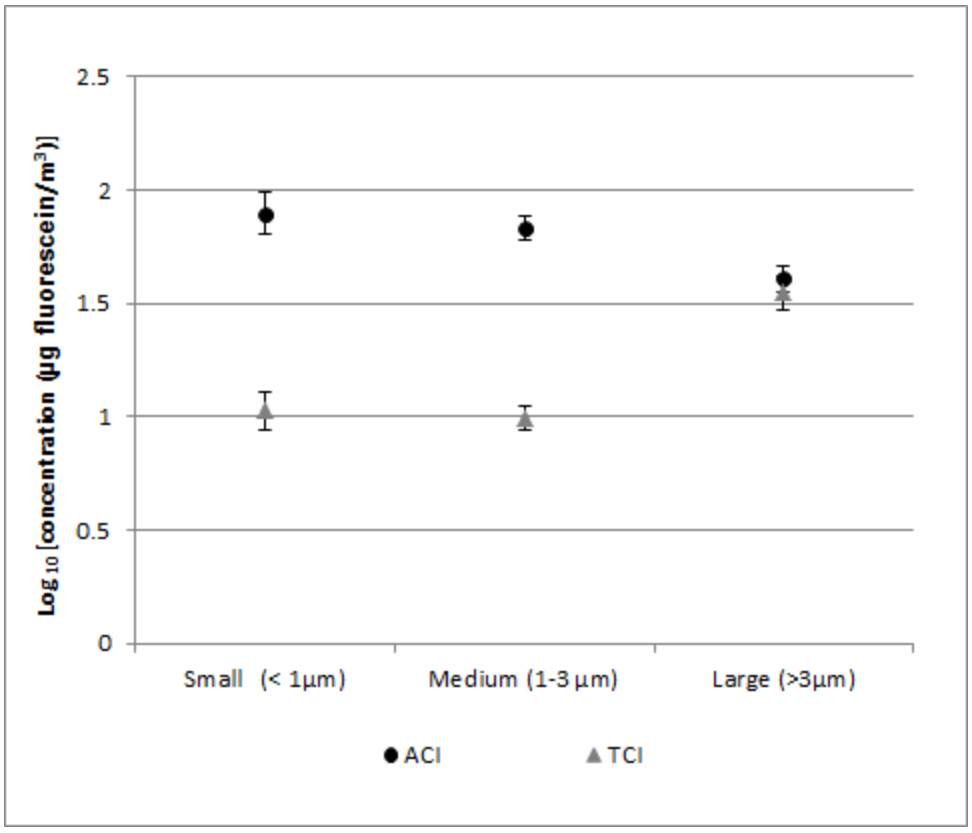
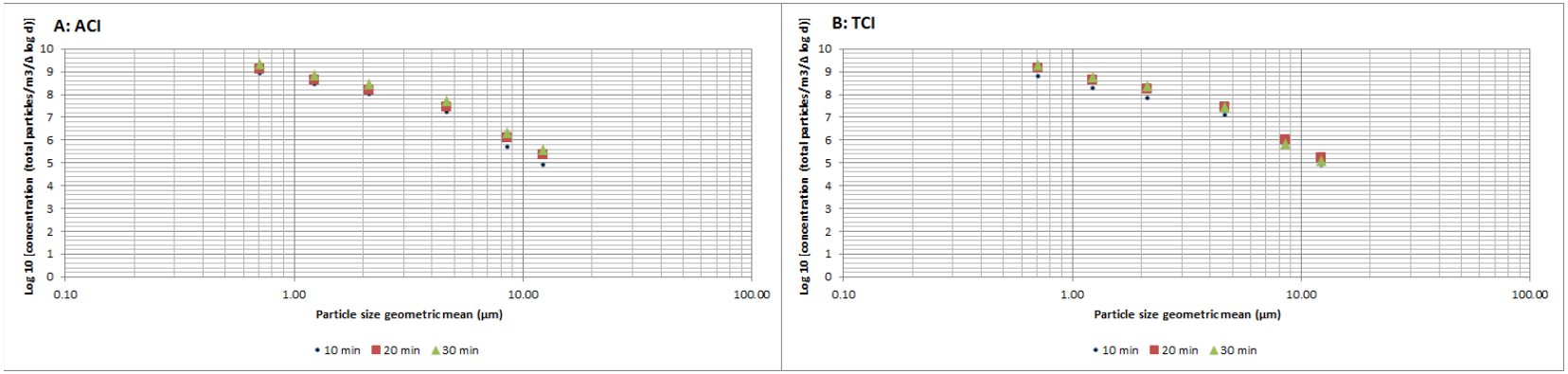


Figure 3.4 Distribution of total airborne particles. Distribution of total particles (geometric mean of number of particles/m^3) measured using an optical particle counter during sampling periods of 10, 20 or 30 minutes using the Andersen cascade impactor (ACI) (A) and the high volume Tisch cascade impactor (TCI) samplers (B) .



Chapter 4: Size distribution of aerosolized particles emitted from animals naturally infected with PRRS, PED or HPAI viruses

Summary

Pathogens that spread via aerosols represent a major threat to animal and human health. Porcine reproductive and respiratory syndrome (PRRSV), porcine epidemic diarrhea (PEDV) and highly pathogenic avian influenza (HPAIV) viruses are economically significant viruses that can spread via aerosol. The size distribution of aerosolized particles bearing viruses and the reliability of methods for quantifying particle-associated viruses under field conditions are not well documented. We compared the performance of 2 air samplers [the Andersen (ACI) and the high volume Tisch (TCI) cascade impactors] that quantify particles by size, and measured concentrations of PRRS, PED and HPAI viruses stratified by particle size both within and outside swine and poultry facilities. All 3 viruses were detectable associated with aerosolized particles, with proportions of positive sampling events being 69%, 61% and 8% for PEDV, HPAIV and PRRSV, respectively. Highest virus concentrations were found with PEDV, followed by HPAI and then PRRSV. Overall, both air collectors performed equally with regards to total virus concentration. Some differences were observed between collectors associated to different viruses and particle sizes ranges. For all 3 viruses, higher concentrations of RNA copies were associated with larger particles, however a biomodal distribution of particles was observed in the case of PEDV and HPAI.

Introduction

Infectious agents that can be transmitted via air include some of the most economically important pathogens affecting intensively reared animals. These include foot and mouth disease virus (FMDV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV) and influenza A virus (IAV) in pigs, and Newcastle disease virus, Marek's disease virus and avian influenza virus (AIV) in poultry. These pathogens cause significant economic losses due to their pathogenicity and ease of transmission, including the ability to travel long distance (Otake, et al., 2010; Holtkamp, et al., 2011; Alonso, et al., 2014) and in some instances also present a zoonotic risk (Choi, et al., 2015).

Risk of airborne transmission is most problematic in areas of high farm and animal density where regional spread can be difficult to contain. In such scenarios, biocontainment and biosecurity to limit herd to herd (or flock to flock) transmission is a major challenge. As a result producers are investing significant resources to prevent airborne transmission of infectious agents such as PRRSV and AIV (Alonso, et al., 2013a; Alonso, et al., 2015).

One of the outstanding questions about airborne transmission relates to the nature and sizes of particles with which airborne viruses are associated. The size of particles in air influences the distances the particles (with their associated viruses) are transported, the survivability and infectivity of the viruses in aerosols, and the locations at which they are deposited in the respiratory tract after

inhalation. There is limited information on particle size associations of pathogens with aerosols, and much existing information has been generated in humans or with animals, but under experimental conditions that simulate natural infection processes rather than from natural infections in field settings (Donaldson, et al., 1983; Donaldson, et al., 1987; Gloster, et al., 2007; Alonso, et al., 2015). Furthermore, this information has not provided insights into the particle size association of viruses transported between herds, in part due to the limitations of air sampling methods. Sampling of bioaerosols can be performed by impaction (onto plate, membranes, or growth media), impingement into liquid, or capture by filtration. In addition, most air samplers have been tested under experimental conditions using flow rates between 2 and 22 liters per minute (L/min) which are most suitable for conditions of low air flow and high pathogen load. More recently, a cyclonic air collector (Midwest Microtek, LLC) has been used in field studies to assess airborne dissemination of swine viruses (i.e. PRRSV and IAV) (Otake, et al., 2010; Corzo, et al., 2013a). However, this apparatus does not separate particles by size.

Mathematical modeling of airborne viruses provides a complementary tool for using empirical data to interpret and predict the risk of airborne transmission events. However, current models do not incorporate information on particle size. For example, atmospheric dispersion models for FMDV are based on dates of disease onset, virus strain, number and type of animals involved and stage of the disease (Mikkelsen, et al., 2003; Valarcher, et al., 2008; Gloster, et al., 2010;

Gloster, et al., 2011; Sanson, et al., 2011). Since infectivity is linked to particle size (Zuo, et al., 2013b; Alonso, et al., 2015), characterization of pathogen-associated particle size in aerosols is essential to understand and predict the likelihood of transmission of infective viruses among farms. Furthermore, the efficiency of air sanitation methods such as air filtration and electrostatic ionization technology are particle size dependent (Alonso, et al., 2013b; Alonso, et al., 2015). Therefore, knowledge about sizes of particles harboring viruses should help to identify the most cost effective biocontainment and biosecurity measures to prevent the dissemination of infectious viruses within and among farms.

In this study we measured the concentrations of viruses in the indoor and outdoor air at swine and poultry rearing facilities experiencing active disease outbreaks with PRRS, PED or HPAI viruses, and characterized the performance of two size-differentiating air samplers in the classification of particles associated with airborne viruses.

Materials and methods

All procedures were conducted in accordance with the University of Minnesota Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC). Permission to sample the air of the HPAI affected flocks was obtained via USDA/APHIS veterinarians as part of the regulatory

response to the HPAI outbreak in the US Midwest in 2015 (USDA United States Department of Agricultural,).

Farm selection /description

Swine and poultry farms were selected during the months of March through May of 2015 (farms 1 through 9, Table 4.1). Veterinarians serving swine herds in Southern Minnesota were contacted and asked to alert the investigators when acute outbreaks of PRRSV or PEDV were suspected on client farms. Four nursery farms were included in the study (1 and 2 for PRRSV, and 3 and 4 for PEDV) and diagnoses were confirmed as described below. Recruitment of poultry flocks was achieved via the USDA/APHIS veterinarians engaged in the emergency response to the HPAI outbreak in the US Midwest. Three turkey flocks located in Minnesota, 1 layer flock in Iowa, and 1 layer flock in Nebraska were selected. Chosen flocks had a confirmed diagnosis of HPAI at the time of sampling, and air sampling was conducted within 2 to 11 days of the diagnostic confirmation.

Sampling procedures

To measure concentration and particle size distribution of viruses inside and outside affected facilities, air samples were collected using 2 air samplers : a) Andersen cascade impactor (ACI; Thermo Electron Corporation, Waltham, MA, US) (Appert, et al., 2012) and b) Tisch cascade impactor (TCI; Model 230,

Tisch Environmental, Inc. Ohio, USA)]. The TCI is a high volume air sampler and both the ACI and the TCI are size-differentiating air samplers that distribute captured particles into several size categories.

Air samples were first collected using the TCI air sampler immediately outside of affected facilities (approximately 5m from the exhaust fan). Additionally, the ACI sampler was used to collect air samples outside of poultry facilities. Subsequently, both air samplers were dissembled, disinfected, and brought inside. Air samplers inside were located in strategic locations chosen based on secure/stable areas close to the animals (within a 1 m range) but out of their reach to avoid any direct contact.

Total airborne particle counts by particle size were also collected using an optical particle counter (OPC) (AeroTrak 9306 Handheld Particle Sizer, TSI Inc., St. Paul, MN) able to separate particles from 0.3 to >10 µm into 6 size intervals. Total airborne particles were counted during each sampling event and 2 replicates of 30 min where performed at the respective locations. Relative humidity (%) and temperature (°C) were recorded (Kestrel 3000, Nielsen-Kellerman, Broothwyn, PA, US) during each sampling event to document environmental conditions.

The ACI sampled air at 28.3 L/min for 1 hour, and separated particles into 8 size intervals based on their aerodynamic diameter: 0.4-0.7, 0.7-1.1, 1.1-2.1, 2.1-3.3, 3.3-4.7, 4.7-5.8, 5.8 -9.0 and >9.0 µm. Particles smaller than 0.4 µm were captured by a backup filter. Samples from stages of this device were eluted

from each aluminum plate using a cell scraper and 1 mL of MEM {{304 Appert, J. 2012}}. All samples were transferred into 1.5 mL sterile plastic tubes, placed on ice and stored at -80℃ until testing. The ACI was then disassembled and plates and stages were scrubbed and disinfected with alkyl dimethyl benzyl ammonium chloride soap (Lysol, Reckitt Benckiser) and finally rinsed and dried with paper towels. After disinfection, a randomly selected collection of plates and individual ACI stages were swabbed to serve as negative controls, and samples stored at -80℃.

The TCI sampler sampled air at 1,130 L/min for 15 min separating particles into 4 stages with aerodynamic particle diameter cut points of 0.95, 1.5, 3 and 7.2 μm. Particles were captured using slotted glass fiber collection substrates (Model P/N TE-230-GF, New Star Environmental, Inc. Roswell, GA, USA) and particles smaller than 0.95 μm were captured by a backup filter. After each sampling period, the substrates and filters were removed using sterile gloves, folded, placed into individual labeled Petri dishes, and refrigerated at 4°C prior to sample elution and processing which took place within 24 h of collection. Between collection events, all stages were disinfected with 70% alcohol, air dried, and finally rinsed and dried with paper towels. After disinfection and drying, all stages were swabbed, and samples stored at -80℃. To extract the viruses from substrates and filters, samples were removed from the Petri dishes, cut individually in 1 cm^2 squares using sterile scissors, and eluted in 50 mL Falcon tubes with 20 mL of the eluent solution (3% beef extract 0.05 M glycine solution

(BE) at pH 9.1). As part of the elution process, each tube was inverted ten times to mix the contents, followed by a 5 minute set period; another mixing step of 10 inversions; and finally each tube was vortexed for a 5 minute period at 1,500 rpm. Volumes eluted from filters were measured, transferred to a 15 mL Falcon tube and the pH returned to 7. All samples were transferred into 1.5 mL sterile plastic tubes, and stored at -80℃ until testing.

For each sampling event, there were 8 stages from the ACI, 4 stages from the TCI and 2 backup filter samples. At each sampling location, 2 samples were collected with the ACI sampler and 3 with the TCI sampler.

Laboratory analysis

Air samples collected in swine facilities were tested using a quantitative PRRSV or PEDV reverse transcription polymerase chain reaction (RT-PCR) in order to estimate the number of RNA viral copies per m^3 of air (Cho, et al., 2006b; Slomka, et al., 2010; Alonso, et al., 2014). Quantitatively, Ct values < 35 were considered positive, 35-40 suspect, and >40 negative. Air samples from poultry facilities were screened using the reverse transcription polymerase chain reaction (RT-PCR) matrix for influenza viruses at the University of Minnesota Veterinary Diagnostic Laboratory (Spackman, et al., 2002). If samples tested influenza positive, they were re-tested using specific H5 and N2 RT-PCRs. Positive or suspect samples were also tested using a quantitative RT-PCR (qRT-PCR) (Corzo, et al., 2013a).

Oral fluids for PRRSV and PEDV infectious status confirmation were collected at the same time as air sampling by hanging 0.4 m of cotton rope from pen railings following published procedures (Detmer, et al., 2011; Romagosa, et al., 2012). Samples were then refrigerated, transported to the laboratory and stored at -80℃.

Statistical analyses

Data from the qRT-PCR results, replicate, virus, location, type of air collector and particle size were consolidated in a spreadsheet (Microsoft EXCEL; Microsoft Corporation, Redmond, Washington, USA) and organized for analysis. Means, standard deviations, and minimum and maximum values for quantitative variables, and frequency counts and percentages for qualitative variables were calculated. The difference in the proportion of PCR positive, suspect and negative results of air samples between the different impactors at the different locations was analyzed by a two-tailed Fisher's exact test. For the quantitative analysis, negative stages of both air samplers were included in the analysis when one or more stages of the sampler had a Ct value within the positive or suspect range (Ct value <40). Negative results included in the analysis were assigned a value of 100 RNA copies/mL according to the limit of detection of the qRT-PCR technique. In order to compare the efficiency of both air samplers collecting different particle size ranges, a new variable called “size” was created. If the geometric mean point of a specific size interval (square root of the product of the

two ends of the interval) was < 1μm, ≥ 1 and ≤ 3μm , or > 3 μm then “size” was equal to “small”, “medium” or “large”, respectively. Due to the number of variables in the study, a multivariate modeling analysis was performed for each of the specific size intervals (small, medium and large). The variables “replicate” and “farm” were considered as random variables. The total quantity of virus (RNA copies/m^3), adjusted by type of air sampler, virus, and location of sampling, was assessed for significance using a mixed linear regression model in SAS 9.1 (SAS Institute, Cary, North Carolina, USA). P-values of the multiple comparisons were calculated according to the Tukey-Kramer’s method.

Results

Sample description, total viral load and air sampler comparison

Table 4.1 summarizes the populations tested according to farm type and species, animals per barn, number of room tested, sampling date, location of sampling, mortality (%), relative humidity (%) and temperature (C°).

A total of 444 qRT-PCR samples from 68 air sampling events were obtained for the analysis. From those sampling events, 35% (24/68), 24% (16/68) and 41% (28/68) were collected and analyzed for HPAI, PEDV and PRRSV respectively (Table 4.2). There were 69% (11/16), 61% (17/28) and 8% (2/24) positive sampling events for PEDV, HPAI and PRRSV respectively. A total of 65% (44/68) of all sampling events were collected inside and 35% (24/68) were

collected outside the study facilities (Table 4.2). Inside samples were more often positive (23 of 44, 52%) than air samples collected outside facilities (7 of 24, 29%).

Results of the sampling events collected for PRRSV inside swine facilities with the ACI sampler differed significantly from those collected with the TCI sampler ($p=0.001$). No other statistically significant differences were observed between collectors (Table 4.2).

Overall, we found no differences in the virus concentration between the ACI (5.09 ± 1.22 $\log_{10}$ RNA copies/m^3 of air) and the TCI air sampler (4.47 ± 1.35 $\log_{10}$ RNA copies/m^3 of air) (Figure 4.1A).

The highest airborne virus concentrations were found for PEDV (6.04 ± 0.72 $\log_{10}$ RNA copies/m^3 of air), followed by HPAI (4.53 ± 0.97 $\log_{10}$ RNA copies/m^3 of air) and then PRRSV (3.79 ± 1.35 $\log_{10}$ RNA copies/m^3 of air) (Figure 4.1B).

Particle size distribution by virus type, sampling location and air sampler type

All three viruses were detected inside barns with the ACI sampler, and 2 out of 3 (PED and HPAI viruses) were detected inside and immediately outside by the TCI sampler.

All viruses were associated with a wide range of particle sizes. Higher concentrations of viral RNA copies were detected in association with large

particles and these associations were different with the 3 viruses tested (Figure 4.2). In the case of PRRSV, there were higher concentrations of viral RNA in particles > 5.8μm compared to those < 5.8μm ($p \leq 0.0302$). For PEDV, there were higher concentrations of viral RNA in particles > 3.3μm diameter compared to those ranging between 0.4 and 1.1μm ($p \leq 0.0212$); and for HPAI, a significantly higher viral RNA concentration was associated to the largest particles (> 9μm) compared to the smallest particles (< 0.7μm) ($p \leq 0.017$). Viral RNA was detected in the smallest size range (particles < 0.4μm) only for PEDV and HPAI viruses. No PRRSV RNA copies were detected in the smallest size ranges (< 2.1μm), and values were imputed as the limit of qPCR detection values (Figure 4.2).

The particle size distribution analysis by air sampler, type of virus and location is summarized in Figure 4.3. Variable “replicate” was removed from the final model due to lack of significance and total concentration of viral particles was obtained as an average of all replicates. Three models were created, one for each particle size range (“small”, “medium” and “large”). For PRRSV, in small and medium particle sizes there only low quantities of RNA (ranging from 2.5 and 2.6 $\log_{10}$ RNA copies /m^3) detected with the ACI and no samples were positive with the TCI sampler , however for the purposes of the model the minimum detectable concentration value was imputed. For HPAI virus, the ACI sampler yielded significantly higher concentrations of viral RNA associated with small and medium size particles inside (4.2 vs 4.4 $\log_{10}$ RNA copies/m^3) compared to the

TCI sampler (2.2 and 3.5 $\log_{10}$ RNA copies /m^3). For large particle sizes (> 3µm) collected inside, the ACI sampler yielded significantly higher concentrations of HPAI and PRRSV (5 and 4.8 $\log_{10}$ RNA copies/m^3, respectively) than the TCI sampler (3.8 and 2.3 $\log_{10}$ RNA copies of HPAI and PRRS viruses, respectively). No difference between samplers was observed with regards to concentration of PEDV RNA in samples collected inside associated with any of the 3 particle sizes measured.

A significantly lower concentration of RNA copies associated with particles ranging between 1-3 µm was observed outside compared to inside and measured with the TCI sampler. HPAI virus was the only virus detected with both air samplers in outside air samples, but we observed no significant differences between air collectors. All samples collected outside swine facilities with the TCI sampler were negative for PRRSV RNA.

Total airborne particles

The distribution of total particles in the air measured with the optical particle counter at the time of virus sampling inside (Figure 4.4A) and outside barns (Figure 4.4B) was different. There was higher concentration of total particles within the first submicrometer size (0.3-0.5 µm) compared to particles larger than 0.5 µm ($p < 0.001$) for both locations; however this difference was more evident in samples collected outside.

Discussion

The cost-effective implementation of biosecurity interventions at a farm level to prevent herd to herd transmission of pathogens remains a significant challenge for farmers raising food animals. A particular challenge is presented by pathogens with attributes that enable airborne transmission, which facilitates rapid spread between farms and frustrates efforts to contain transmission within a region. To meet this challenge, there is a need to obtain a better understanding of the aerobiology of pathogen transmission under field conditions and to develop better methods to study these phenomena. We found that the observed concentrations of viral RNA, and the observed associations between viruses and particles, were influenced by the choice of measurement device. Overall, both devices performed similarly. However, the ACI sampler yielded higher concentrations of PRRSV and HPAIV RNA copies compared to the TCI sampler. Using two size-differentiating air samplers we demonstrated that these important infectious agents can be associated with a wide range of particle sizes under field conditions and get spread by different modes (airborne as well as droplet transmission) from infected premises.

PEDV, primarily an enteric virus, was found in larger quantities in air samples than HPAIV (primarily a systemic infection in birds but with an important enteric component) and PRRSV (primarily a respiratory infection with a systemic component). Since different farms were measured and tested for different viruses, there is need to highlight possible difference between premises. The

stage of clinical progression of outbreaks, the percentage of actively infected animals at the time of sampling, the difference in mechanisms of spread among the 3 virus, and environmental conditions inside and outside of barns would be expected to influence the variability in positive yields observed between farms and viruses.

Determining the particle size associations for these viruses has important implications for the control of the diseases, and the use of droplet and airborne infection control measures. While results from the ACI sampler from inside the facilities demonstrated the presence of virus across all size ranges studied, higher quantities were associated with larger size particles (Figure 4.2). Similar results on virus particle size distribution have been shown for swine influenza, PRRSV and PEDV under experimental conditions (Alonso, et al., 2015). Furthermore, our results showed that PEDV and HPAIV RNA copies are associated with the smallest particles (<0.4 μm) demonstrating a possible bimodal distribution of viral copies that could suggest potential for transmission via multiple mechanisms or under varying environmental conditions. Smaller particles tend to remain airborne for longer periods of time compared to bigger particles. For example, a 4 μm particle takes 33 min to settle 1 m in still air, while a 1 μm particle requires 8 h (Lindsley, et al., 2010). In addition, infectious particles with a small aerodynamic diameter tend to have more serious health implications than larger particles as they are inhaled into the lower respiratory tract (Thomas, 2013). Overall, our results indicate that PEDV, PRRSV, and HPAI

RNA can be associated with particles that are small enough to remain airborne for a long time and, in the cases of HPAI and PED viruses, disperse inside as well as outside infected premises. Our PRRSV data suggest that long distance airborne transmission is less likely which is in contrast to previously published studies (Brito, et al., 2014). In addition, due to the potential zoonotic risk of some of HPAI viruses, these results underline the importance of using proper personal protection equipment by people working in and near affected flocks.

Difference in the air sampling process between the collectors could have influence sampling efficiency. The ACI sampler impacts the airborne particles directly onto aluminum plates while the TCI sampler is a filter based air sampler, and as a result, airborne particles are captured in filter media when the airstream is forced to pass through the filter. However, the elution and separation of the captured virus from the crustal material is a critical step in pathogen detection (Farnsworth, et al., 2006). Low efficiency of the virus elution process from the filter media, effects of environmental factors, and desiccation of the virus particles due to high airstream are variables that warrant further investigation before considering the use of the large volume air sampler under field conditions.

Despite a growing body of evidence, the relative contribution of the airborne route to the overall transmission of these viruses among farms remains controversial. Understanding the capabilities of available air samplers that can characterize aerosols of such important pathogens by size, specifically under

field conditions, will fill a knowledge gap about airborne transmission that should greatly enhance understanding of this phenomenon.

Table 4.1 Descriptive data of farms/ flocks/locations included in the study. NR- not register

Farm ID	US State	Species/Type	Barn/flock size	Targeted virus	Sampling date (dd/mm/yy)	Barn (Room) ID sampled	Air samplers	Sampling location/ distance (m)	Average mortality (%)	Average RH (%)	Average Temp (C°)
1	MN	Swine/Nursery	4,700	PEDV	13/03/15	1	ACI TCI	Inside	2-5	40.2	25.1
1	MN	Swine/Nursery	4,700	PEDV	13/03/15	2	ACI TCI	Inside	2-5	36	29.7
2	MN	Swine/Nursery	4,000	PEDV	18/3/15	1 (1)	TCI	5	2-5	38.4	6.6
2	MN	Swine/Nursery	4,000	PEDV	18/3/15	1 (2)	TCI	5	2-5	40.1	7.2
2	MN	Swine/Nursery	4,000	PEDV	18/3/15	1 (1)	ACI TCI	Inside	2-5	39.9	23.8
2	MN	Swine/Nursery	4,000	PEDV	18/3/15	1 (2)	ACI TCI	Inside	2-5	38.8	27.8
3	MN	Swine/Nursery	6,500	PRRSV	8/4/15	1 (1)	TCI	5	3	55	6
3	MN	Swine/Nursery	6,500	PRRSV	8/4/15	1 (2)	TCI	5	3	NR	NR
3	MN	Swine/Nursery	6,500	PRRSV	8/4/15	1 (1)	ACI TCI	Inside	3	28	77
3	MN	Swine/Nursery	6,500	PRRSV	8/4/15	1 (2)	ACI TCI	Inside	3	25.1	75.5

Farm ID	US State	Species/Type	Barn/flock size	Targeted virus	Sampling date (dd/mm/yy)	Barn (Room) ID sampled	Air samplers	Sampling location/ distance (m)	Average mortality (%)	Average RH (%)	Average Temp (C°)
4	MN	Swine/Nursery	6,800	PRRSV	13/4/15	1 (1)	TCI	5	5	13.9	40
4	MN	Swine/Nursery	6,800	PRRSV	13/4/15	1 (2)	TCI	5	5	15	31.1
4	MN	Swine/Nursery	6,800	PRRSV	13/4/15	1 (1)	ACI TCI	Inside	5	28.7	47.2
4	MN	Swine/Nursery	6,800	PRRSV	13/4/15	1 (2)	ACI TCI	Inside	5	24.3	39.8
5	MN	Turkey/Layer	28,000	HPAIV	24/04/15	2	TCI	5	70-80	NR	NR
5	MN	Turkey/Layer	28,000	HPAIV	24/04/15	1	ACI TCI	Inside	0.4	50.6	18.7
5	MN	Turkey/Layer	28,000	HPAIV	24/04/15	2	ACI TCI	Inside	70-80	66.6	16.7
6	MN	Turkey/Grow	70,000	HPAIV	27/04/15	3	TCI	5	30-40	32.5	19.7
6	MN	Turkey/Grow	70,000	HPAIV	27/04/15	3	ACI TCI	Inside	30-40	40	23.3
7	MN	Turkey/Breeder	4,205	HPAIV	28/04/15	1	TCI	5	30	94.7	10.4
7	MN	Turkey/Breeder	4,205	HPAIV	28/04/15	1	ACI TCI	Inside	30	92.8	13
8	IA	Chickens/Layers	575,000	HPAIV	12/05/15	2	ACI TCI	5	10	36	19.1
9	NE	Chickens/Layers	1.8M	HPAIV	05/23/15	21	ACI TCI	5	14	82.4	19.3
9	NE	Chickens/Layers	1.8M	HPAIV	05/23/15	21	ACI TCI	Inside	14	81.4	21.8

Table 4.2 Number (%) of air sampling events tested by reverse transcription polymerase chain reaction (RT-PCR) for porcine reproductive respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV) and highly pathogenic avian influenza (HPAI) virus collected by the Andersen cascade impactor (ACI) and high volume Tisch cascade impactor (TCI) from inside and outside (5m) infected swine and poultry facilities.

		Inside			Outside (5m)			Total
	RT-PCR[*]	ACI	TCI	[†]p-value	ACI	TCI	p-value	
PRRSV	Positive	2 (25%)	0 (0%)	[a] p=0.001	[§]NT	0 (0%)	NT	2 (8%)
	Suspect	5 (62.5%)	0 (0%)		NT	0 (0%)		5 (21%)
	Negative	1 (12.5%)	8 (100%)		NT	8 (100%)		17 (71%)
PEDV	Positive	6 (100%)	4 (66.7%)	p=0.45	NT	1 (25%)	NT	11 (69%)
	Suspect	0 (0%)	2 (33.3%)		NT	3 (75%)		5 (31%)
	Negative	0 (0%)	0 (0%)		NT	0 (0%)		0 (0%)
HPAI	Positive	6 (75%)	5 (62.5%)	p=1	2 (50%)	4 (50%)	p=1	17 (61%)
	Suspect	1 (12.5%)	2 (25%)		1 (25%)	3 (37.5%)		7 (25%)
	Negative	1 (12.5%)	1 (12.5%)		1 (25%)	1 (12.5%)		4 (14%)
Sub total		8 (50%)	8 (50%)		4 (33.3%)	8 (66.7%)		28 (100%)

		Inside			Outside (5m)			Total
	RT-PCR*	ACI	TCI	†p-value	ACI	TCI	p-value	
Total	Positive	14 (63.6%)	9 (40.9%)	p=0.051	2 (50%)	5 (25%)	p=0.51	30 (44%)
	Suspect	6 (27.3%)	4 (18.2%)		1 (25%)	4 (20%)		15 (22%)
	Negative	2 (9.1%)	9 (40.9%)		1 (25%)	11 (55%)		23 (34%)
	Total	22 (100%)	22 (100%)		4 (100%)	20 (100%)		68 (100%)

*: Positive Ct values <35; suspect Ct 35-<40 c: negative Ct >40

†: Exact (two-tailed) probability calculated from 2x3 contingent tables (Fisher exact test)

a: Significant difference observed between air collectors during an specific air virus sampling

§: Not tested

Figure 4.1 Box plots of virus concentration by virus type (Figure 4.1A) and air sampler (Figure 4.1B)**:** maximum and minimum values, first and third quartiles, median and mean indicated with horizontal lines and diamond symbols respectively.

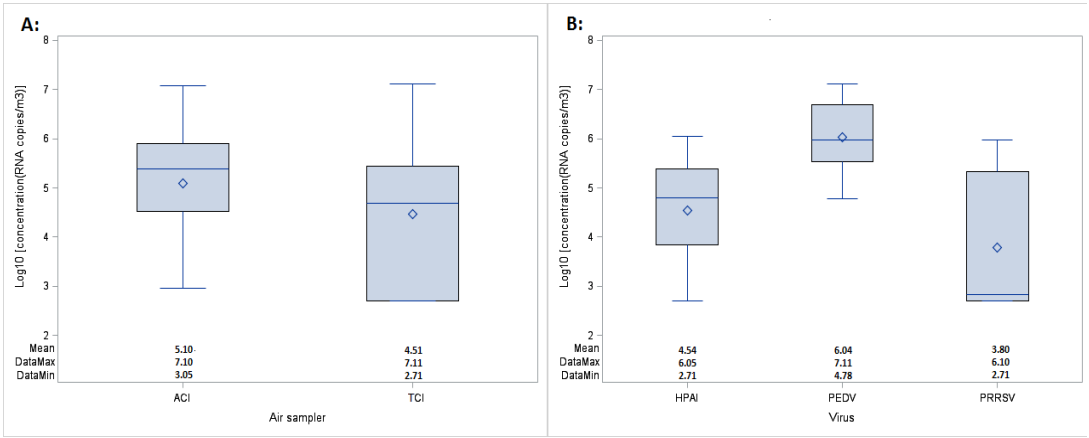


Figure 4.2 Distribution of viral RNA concentration (Least Squares Means of log_{10} RNA copies/m^3 of air and standard error) of porcine reproductive and respiratory syndrome (PRRS), porcine epidemic diarrhea (PED) and highly pathogenic avian influenza (HPAI) viruses by particle size as detected by the Andersen cascade impactor from aerosols generated by infected animals inside infected premises.

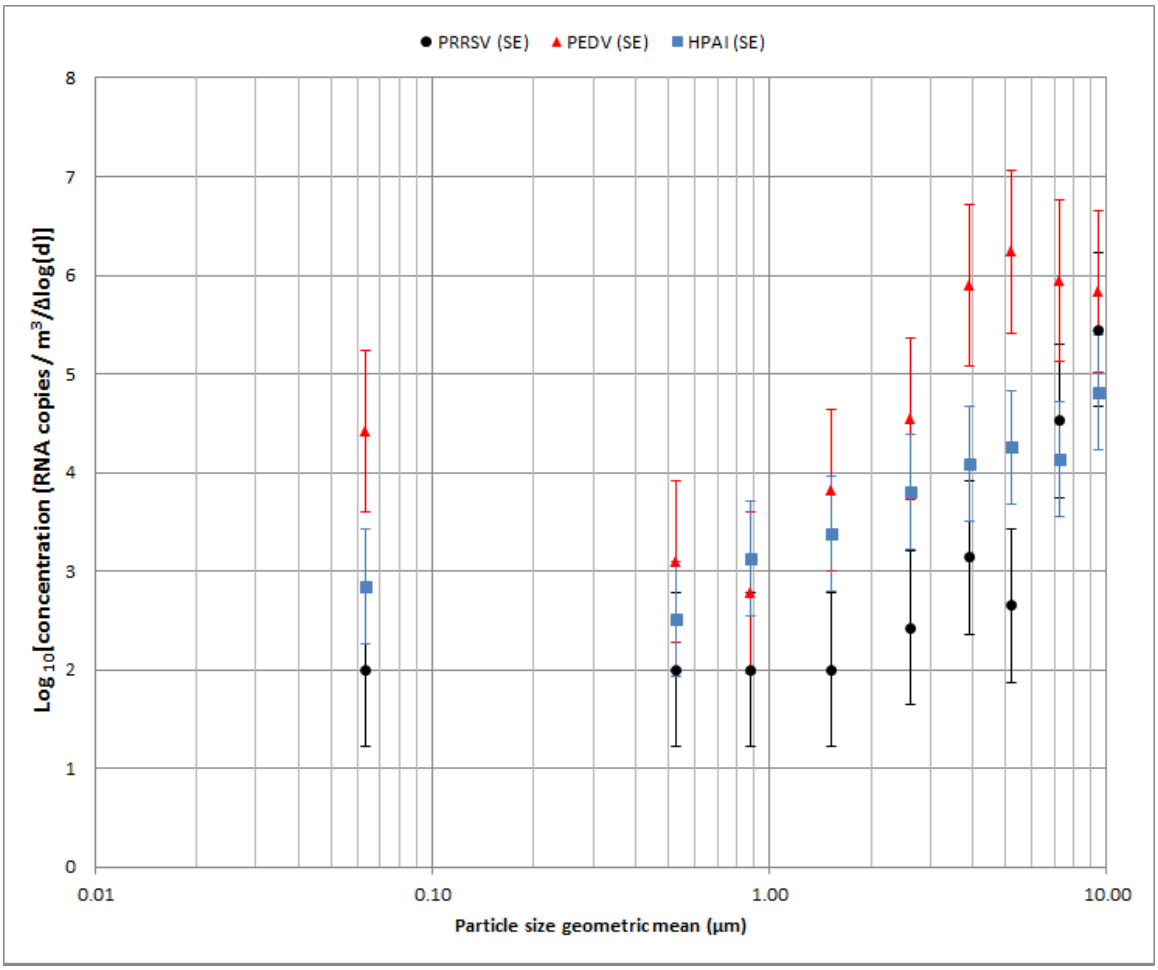


Figure 4.3 Distribution of viral RNA concentration (Least Squares Means of log_{10} RNA copies/m^3 of air and standard error) of porcine reproductive and respiratory syndrome (PRRSV) viruses, porcine epidemic diarrhea virus (PEDV) and highly pathogenic avian influenza virus (HPAI) by particle size categories (small, medium and large) detected by the Andersen cascade impactor (ACI) and the Tisch cascade impactor (TCI) from inside and outside (5m from exhaust fan) swine and poultry facilities.

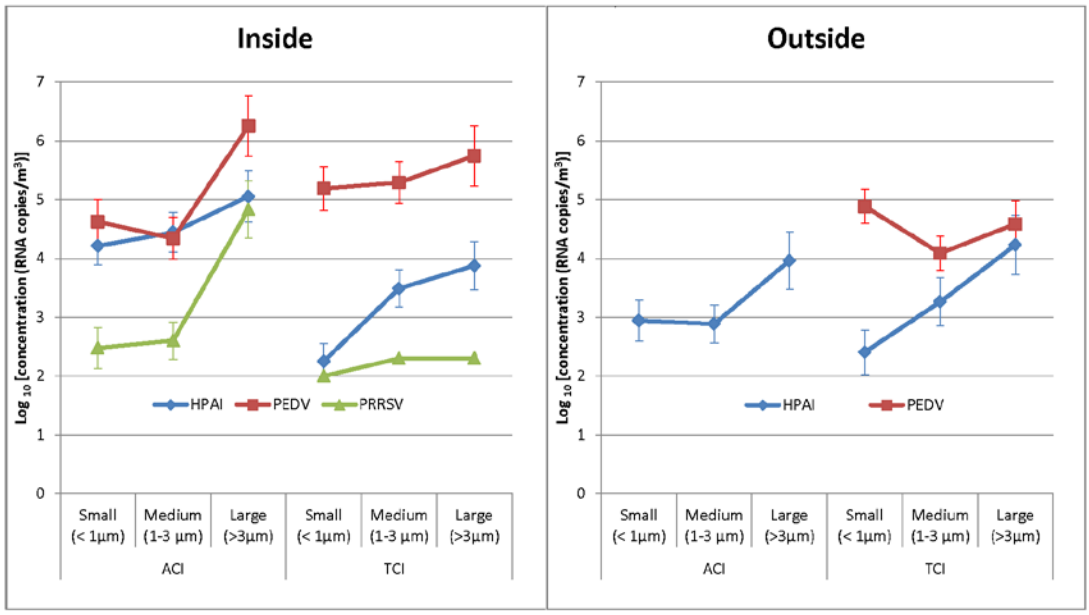
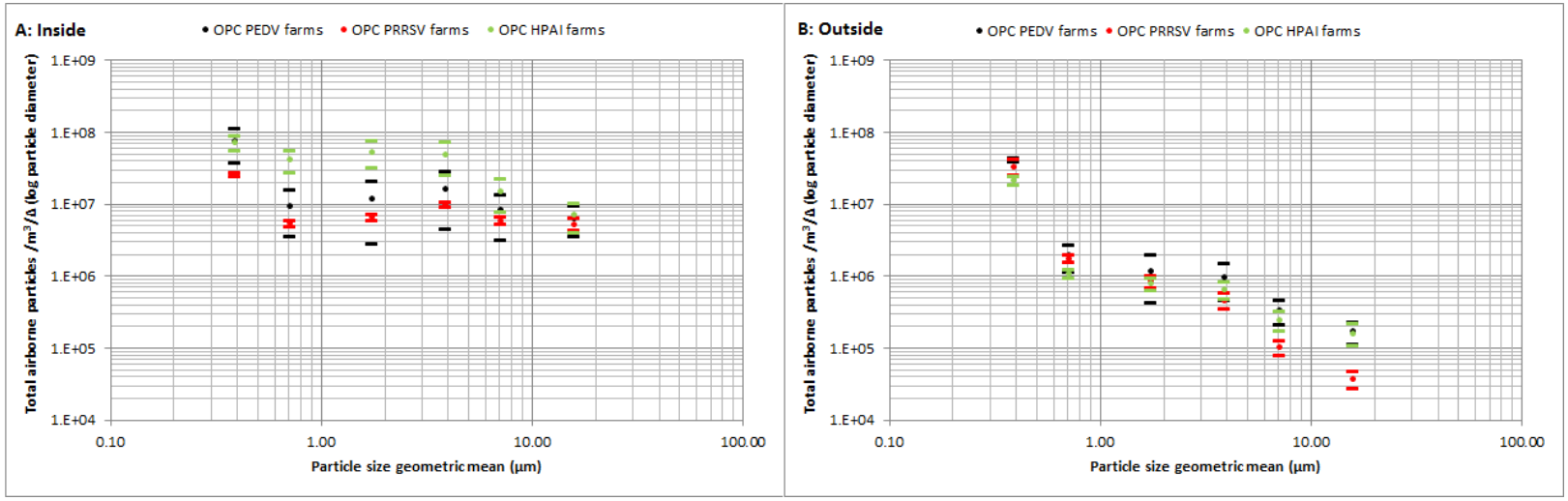


Figure 4.4 Distribution of total airborne particles inside (4.4A) and outside (4.4B) of farms. Distribution of total airborne particles [number of total particles/m^3/Δlog (diameter) and 95% confidence interval] inside (A) and outside (B) of farms experiencing outbreaks of porcine reproductive and respiratory syndrome (PRRS), porcine epidemic diarrhea (PED) and highly pathogenic avian influenza (HPAI) viruses using an optical particle counter.



Chapter 5: Evaluation of an electrostatic particle ionization technology for decreasing airborne pathogens in pigs

Chapter published as followed:

Alonso, C; Raynor, PC; Davies, PR; Torremorell, M; Evaluation of an electrostatic particle ionization technology for decreasing airborne pathogens in pigs. Aerobiology (2015) Nov doi:10.1007/s10453-015-9413-3

Summary

Influenza A virus (IAV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine epidemic diarrhea virus (PEDV) and *Staphylococcus aureus* (*S. aureus*) are important swine pathogens capable of being transmitted via aerosols. The electrostatic particle ionization system (EPI) consists of a conductive line that emits negative ions that charge particles electrically resulting in the settling of airborne particles onto surfaces and potentially decreasing the risk of pathogen dissemination. The objectives of this study were to determine the effect of the EPI system on the quantity and viability of IAV, PRRSV, PEDV and *S. aureus* in experimentally generated aerosols and in aerosols generated by infected animals. Efficiency at removing airborne particles was evaluated as a function of particle size (ranging from 0.4 to 10µm), distance from the source of ions (1, 2 and 3 meters) and relative air humidity (RH30% vs. 70%). Aerosols were sampled with the EPI system “off” and “on”. Removal efficiency was significantly greater for all pathogens when the EPI line was the closest to the source of aerosols. There was a greater reduction for larger particles ranging between 3.3 and 9 microns, which varied by pathogen. Overall airborne pathogen reduction ranged between 0.5 and 1.9 logs. Viable pathogens were detected with the EPI system on but there was a trend to reducing the quantity of viable PRRSV and IAV. There was not a significant effect on the pathogens removal efficiency based on the RH conditions tested. In summary, distance to the source of ions, type of pathogen and particle size influenced the removal

efficiency of the EPI system. The reduction of infectious agents in the air by the EPI technology could potentially decrease the microbial exposure for pigs and people in confinement livestock facilities.

Introduction

Among all infectious agents affecting swine, airborne pathogens are the most costly and difficult to control (Hyslop, 1971). Porcine reproductive respiratory syndrome (PRRS), influenza A (IA), foot and mouth disease (FMD), classical swine fever (CSF) and porcine epidemic diarrhea (PED) viruses are important swine pathogens that spread via aerosols (Stark, 1999). All these pathogens are responsible for causing devastating losses in pig farms, specifically those located in high swine dense regions, due to their ability to spread rapidly and, in some instances, cause zoonotic infections. Unfortunately there are limited options to limit the spread of airborne pathogens.

Viruses and bacteria that become airborne travel as part of particulate matter (PM) of various origins and sizes (Dutkiewicz, et al., 1994). PM from livestock houses includes indoor airborne pollutants (viable and non-viable) that may be detrimental for animal performance and the health and well-being of animals and farmers (Donham and Leininger, 1984; Donham, 1991; Spencer, et al., 2004). PM is considered a health hazard due to irritant effects on the respiratory tract, increased susceptibility to respiratory diseases, and its role as a vehicle of transmission of viruses and bacteria from livestock (Harry, 1978). PM

concentration and size-distribution depends on factors related to animal housing and feeding, animal type, season and sampling period within a day (Ellen, et al., 2000).

A first step in decreasing infectious aerosol concentrations is PM reduction. Proposed strategies to reduce PM in livestock systems included the use of “low-dust” feed and feeding techniques (Pedersen, et al., 2000; Takai and Pedersen, 2000), use of feed additives (Takai, et al., 1996), water or oil sprinkling (Senthilselvan, et al., 1997; Takai and Pedersen, 2000; Nonnenmann, et al., 2004), changes in ventilation rates and air distribution (Aarnink and Wagemans, 1997), and electrostatic precipitation and ionization (Mitchell and King, 1994; Stgeorge and Feddes, 1995; Rosentrater, 2003; Cambra Lopez, et al., 2009; Yao, et al., 2009). Air washers equipped with UV-irradiation systems have been used to specifically kill bacteria and viruses in bioaerosols, but are not commonly used in livestock production due to their limited capacity for handling large volumes of air in livestock buildings (Schulz, et al., 2013).

The concept of utilizing ionization as a means to reduce or eliminate airborne particles or microbial levels has been reported previously. The use of ion emissions combined with photocatalytic oxidation demonstrated significant pathogen removal efficiency and biocidal capabilities (Grinshpun, et al., 2007). Ionization has been tested in poultry houses and hatching cabinets (Mitchell, et al., 2000; Mitchell, et al., 2002; Richardson, et al., 2002), pigs (Rosentrater, 2003), cattle (Dolejs, et al., 2006) and rabbits (Chiumenti and Guercini, 1990).

Ionization is considered more efficient in removing particles from the air than conventional techniques such as water or oil sprinkling, changes in ventilation rates or changes in air distribution (Daniels, 2001). An electrostatic particle ionization (EPI) technology, with agricultural application in livestock, became commercially available recently. This technology consists of a long ionizer bar with sharp point electrodes connected to a power supply of high voltage (-30 KV) that generates a high negative ion output which charges airborne particles electrically. The ionized airborne particles are attracted towards opposite charges and in an enclosed space, like a confinement rearing facility, may be cleared from the air by adhesion to the walls or other charged surfaces (Mitchell, 1997).

Ionization systems for use in swine facilities have not yet been fully developed despite preliminary research indicative of improved air quality (Rosentrater, 2003). Ion density (related to the distance to the ion source and the number of sources), size of particles, PM concentrations, ventilation rates and humidity levels are factors that influence the performance of ionization in livestock facilities (Cambra-Lopez, et al., 2010). More research is needed to evaluate the effectiveness of ionization to reduce particles, pathogen load and viability of infectious agents important to livestock. This is particularly relevant for food animals raised in confinement conditions such as pigs and poultry where animals are housed in enclosed environments and, specifically for pigs where the importance of airborne transmission for swine and zoonotic pathogens such as PRRSV, PEDV, IAV, and MRSA (methicillin resistant *S. aureus)* is well

documented. For zoonotic agents such as influenza, airborne spread from animals to people is of great concern as pigs can serve as “mixing vessels” that generate new viruses (Ito 2000), and large populations of people can be put at risk very quickly, as occurred during 2013 with H3N2 variant influenza infections associated with agricultural fairs (Wong, et al., 2012; Bowman, et al., 2014). In addition, *S. aureus* is the most common bacteria readily found in the air of swine barns, and personnel working in swine facilities are frequently colonized with *S. aureus* in their noses (Denis, et al., 2009; Van Cleef, et al., 2010; Garcia Graells, et al., 2012), emphasizing the need to develop strategies to inactivate infectious aerosols.

The objectives of this study were to evaluate the efficiency of the electrostatic particle ionization technology at reducing the quantity and viability of IAV, PRRSV, PEDV and *S. aureus* in mechanically generated aerosols (study 1) and in aerosols emitted by infected animals (study 2). We evaluated the impact of size of the airborne particles, relative humidity (RH) levels and distance to the source of ions on removal efficiency. Understanding the capabilities of this technology as a potential strategy for mitigating airborne pathogen spread is important for assessing the opportunity to improve both animal and human health.

Materials and methods

System design and testing protocol

All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC).

Studies were primarily performed in the BSL-2 research animal units at the University of Minnesota campus in St. Paul. The BSL-2 animal unit was mechanically ventilated with negative pressure of 0.11 inches of water, included incoming and outgoing filtered air, and had a total air space of 35.1 m^3. Environmental conditions of RH (%) and temperature (Cº) were monitored continually.

For the specific aim of evaluating the effect of RH, an environmentally-controlled chamber located in the Department of Mechanical Engineering at the University of Minnesota Minneapolis campus was utilized (Song Ge, 2014). The chamber measured 1.95 m wide by 1.95 m deep by 1.45 m tall and was equipped with clear acrylic door and hypalon gloves to safely manipulate and retrieve instruments. To control the temperature inside the chamber, the walls and floor were constructed using plate heat exchangers attached to aluminum plates. A water heater and a water chiller were connected to adjust the temperature of the heat exchanger fluid. The chamber was insulated and HEPA-

filtered, and air flow, negative pressure, temperature and RH were monitored and recorded at all times.

All variables and factors are described in Table 5.1. For study 1, the total number of tests was 27 (3 pathogens x 3 heights of the EPI line x 3 replicates). For the naturally-generated particles or study 2, the total number of tests was 40 [(17 days x 2 times) + (6 days x 1 time)].

The methods for each of the studies are described in the following sections.

Aerosol generation

- *Study 1. Experimental pathogen aerosolization*

Suspensions of 10^6 and 10^5 tissue culture infective dose per mL (TCID50/mL) of IAV A/Swine/Iowa/00239/2004 H1N1 and PRRSV strain MN-1-8-4, respectively, were suspended in phosphate-buffered saline (PBS) and used to generate aerosols. Both viruses, provided by the University of Minnesota Veterinary Diagnostic Laboratory, are known to be shed in aerosols by infected pigs (Cho, et al., 2007; Corzo, et al., 2014). For S. *aureus*, a 10^8 colony forming unit (CFU)/mL suspension of a methicillin susceptible strain (MLST 398; spa type t034) isolated from a commercial pig was used.

Aerosols were generated continuously at 20 psi using a 6-jet Collison nebulizer (CN60, BGI, Inc, Waltham, MA) at a constant rate for a total of 60 minutes per test. The nebulizer was located on a wooden platform attached to

the north wall of the room, beside the air inlet, 2.8 m from the floor. Aerosols were produced at a rate of 1.1 mL/min and, according to the nebulizer manufacturer specifications, created aerosol particles with a mass median aerodynamic diameter of 1 to 3 µm (Thomas, et al., 2008a).

- *Study 2. Experimental virus infection in pigs*

A group of twelve, 5-week-old pigs were purchased from an IAV, PRRSV, PEDV and *Mycoplasma hyopneumoniae* negative herd based on routine serologic and antigenic testing, and clinical herd history. Ten pigs of 12 were sedated using an intramuscular injection of Telazol® (Fort Dodge Animal Health, Fort Dodge, IA, USA) at the recommended dose of 6mg/kg, and inoculated with PRRSV strain 1-8-4 and influenza A/Swine/Iowa/00239/2004 H1N1at 48 h post arrival. Pigs were inoculated intra-nasally and intra-tracheally (IAV), and intra-muscularly and intra-nasally (PRRSV) with 2 mL inoculum of each pathogen. Two of the pigs were removed from the room prior to inoculation and commingled back with the rest of the pigs at 6 hours after inoculation to serve as contact controls. At day 21 of the study, all pigs were intra-gastrically inoculated with a 20 mL solution of PEDV-positive material obtained from PEDV infected pigs. The inoculation material was confirmed positive by PEDV RT-PCR and diluted to a cycle threshold (Ct) value of 15 to 16. The inoculation material was prepared and kept refrigerated for 24h at 4ºC prior to inoculation.

Electrostatic particle ionization (EPI) system

The Electrostatic Particle Ionization system (EPI air, Baumgartner Environics, Inc , Olivia, MN, USA), consisting of stainless steel corona points attached to a stainless steel cable, was installed along the length of the animal isolation room. Corona points were suspended by a wooden frame and energized with 30,000 V of electricity at a low current of 2mA by a specially designed corrosion resistant power supply. The power supply was mounted inside the room and operated at 110 V and 60 Hz.

Specifically for study 1, the EPI line was located at heights of 1, 2 and 3 m from the floor (Figure 5.1). During each test, the system operated in “off” position for 30 min for the collection of the first sample, then it was turned “on” for 15 min for the aerosols to reach steady state. It then remained “on” for another 30 min for the next air sample collection. The time to reach steady state with the system “off” and “on” in mechanically generated aerosols had been determined in a set of previous studies (unpublished data).

During study 2, the EPI line was placed at a height of 1.3 m from the floor (Figure 5.2). During each test, the system operated in “off” position for 60 min as air samples were taken; then was turned “on” for 15 min; and then remained “on” for another 60 min for additional air sampling.

Sampling procedures

Pathogen sampling

Aerosols were collected using a liquid cyclonic collector (Midwest Micro-tek, Brookings, SD, USA)(Corzo, et al., 2013b), an Anderson Cascade Impactor (ACI) (Thermo Electron Corporation, Waltham, MA, USA)(Appert, et al., 2012), as well as a viable ACI. The two latter collectors separate aerosolized virus or bacteria by associated particle sizes. For study 1, the air samplers were located 0.2 m above from the floor and kept 0.9 m apart (Figure 5.1). For study 2, air samples were collected twice daily (9 a.m. and 3 p.m.) for 17 days and once (9 a.m.) for 6 days. Samplers were hung 1.2 m from the floor and were also 0.9 m apart (Figure 5.2). The pigs did not have direct contact or access to the devices.

Sample collection, using the cyclonic air collector, which draws air at 200 L/min, was carried out for 30 minutes. Collection media during sampling were 10 mL of PBS, or 10 mL of minimum essential media (MEM) supplemented with 4 % of bovine serum albumin for the mechanically generated aerosols (study 1) and the aerosols generated by infected animals (study 2), respectively. After collection, an average of 4 mL of sample was recovered, divided into 2 aliquots, and stored at -80℃. The collector was then disinfected with 70 % ethanol, rinsed with distilled water and dried with paper towels. After disinfection, the collection vessel and the turbine were swabbed to serve as controls, and samples were processed and stored in sterile plastic tubes at -80℃ until analysis.

The ACI samples air at 28.3 L/min and separates particles into 8 stages with particle size diameter cut-points of 0.4, 0.7, 1.1, 2.1, 3.3, 4.7, 5.6 and 9.0 µm. Air samples were collected for 30 min in study 1 and 60 min in study 2. After each sampling period, samples from the ACI were eluted from the plate on each stage using a cell scraper and 1 mL of PBS or MEM (Appert, et al., 2012). All samples were transferred into 1.5 mL sterile plastic tubes, placed on ice and stored at -80℃ until testing.

Specifically for *S. aureus*, air samples were taken using a 6-stage viable ACI with Columbia-CNA agar plates able to capture bacteria associated with particles with size diameter cut-points of 0.7, 1.1, 2.1, 3.3, 4.7 and 5.6 µm. After each sampling period, plates were removed, inverted in their covers, incubated for 12-18 hours at 37℃ and CFUs were counted as previously described (Andersen, 1958; Butera, et al., 1991). After each sampling, the ACI was disassembled and collection plates were scrubbed and disinfected with alkyl dimethyl benzyl ammonium chloride soap (Lysol, Reckitt Benckiser) and finally rinsed and dried with paper towels. After disinfection, a random number of collection plates and individual ACI stages were swabbed, and samples stored at -80℃.

In order to monitor for infection status in pigs challenged in study 2, oral fluids (Detmer, et al., 2011; Romagosa, et al., 2012), serum samples and fecal swabs were collected to test them by RT-PCR for IAV, PRRSV and PEDV as described below.

Total particle testing

Total airborne particle counts by particle size were collected using an optical particle counter (OPC) (AeroTrak 9306 Handheld Particle Sizer, TSI Inc., St. Paul, MN) for 30 min at each sampling event of study 2.

Ion decay testing

In order to ascertain the ion density released by the EPI system, ion decay readings were measured in triplicate at a location between both air samplers using an ionizer performance analyzer (Model 287, Monroe Electronics, Inc., NY, US). The ionizer performance analyzer measures the time in seconds it takes for the positive ions accumulated on a voltage plate to decay from 1050 V to zero after interacting with the negative ions released in the room. The smaller the decay time, the faster the charge releases from the voltage plate, and the higher the ion concentration.

Relative humidity test

In order to generate IAV aerosols, a 6-jet Collison nebulizer was utilized as described in methods earlier, and aerosols were sampled using an ACI for 15 minutes with the EPI system “off” and “on”. RH conditions were set at 30% and 70% (± 2%) and three replicates were performed at each RH level.

Diagnostic assays

Oral fluid samples, nasal swabs, serum samples and fecal swabs were tested for quantitative PRRSV, IAV and PEDV RT-PCRs as previously described (Cho, et al., 2006a; Slomka, et al., 2010; Alonso, et al., 2014). After incubation, *S. aureus* colonies on the agar plates were counted as previously described (Andersen, 1958; Butera, et al., 1991). To assess the infectivity of the air samples collected using the air cyclonic collector, virus isolation was attempted from RT-PCR positive samples in Madin-Darby canine kidney (MDCK) cells and a subline of the African monkey kidney cell line named MARC-145 cells for IAV and PRRSV, respectively. In the case of PEDV, a bioassay consisting of inoculating susceptible piglets with air samples testing RT-PCR positive was performed because of the difficulty to grow the virus in cell culture. Briefly, four 10-day-old pigs from a PEDV-negative farm were purchased and each pig allocated to a separate isolation room. On arrival, pigs were rectal swabbed and confirmed negative by PEDV RT-PCR. Inoculation material for each pig consisted of a pool of 3 air samples containing the collection media of the cyclonic air collector with the system “off” (2 pigs) and another pool of 3 samples collected with the system “on” (1 pig). The fourth pig was kept as a negative control. Each pig was intragastrically inoculated as previously described (Alonso, et al., 2014) with 2 ml of the pooled air samples diluted 1:10 with PBS to obtain a total of 20 mL of inoculation material per pig. All pigs were euthanized 4 days

post-inoculation by injection of 2 mL of pentobarbital (Fatal-Plus®, 100 mg/kg IV) into the external jugular vein.

Statistical analysis

Data of RNA copies/m^3 of air for PRRSV, IAV and PEDV, and number of *S. aureus* CFUs/m^3, ion decay time, type of air sampler, replicate, distance of the EPI system to the ground and type of pathogen were consolidated in a spreadsheet (Microsoft EXCEL; Microsoft Corporation, Redmond, Washington, USA) and organized for analysis. Means, standard deviations, minimum and maximum values for quantitative variables, and frequency counts and percentages for categorical variables were calculated for descriptive analysis. For each of the studies, differences in the total pathogen concentrations in air and particle size specific concentrations with the EPI system “on” and “off” were assessed for significance using PROC MIXED in SAS 9.3 (SAS Institute, Cary, North Carolina, USA) a mixed linear regression model for random and fixed effects. Results from all stages of the ACI were considered in the analysis when at least one of the eight stages that integrate the air sampler had a Ct value within the positive or suspect ranges. Negative results included in the analysis had a value of 100 RNA copies according to the limit of detection of the RT-PCR technique. Removal efficiency by the EPI system was calculated for each of the agents and it was defined as the initial concentration of aerosolized virus or

bacteria with the EPI system “off” minus final concentration with the EPI system “on” divided by initial concentration (Wu, et al., 2006).

Results

Study 1. Mechanically generated aerosols

Virus quantification in air samples

A total of 144 ACI stages and 18 cyclonic collector air samples were analyzed by IAV and PRRSV RT-PCR with the system “off” and “on”. Viral suspensions in the nebulizer had a mean RNA copies/mL of $1.53x10^8$ for IAV and $1.41x10^9$ for PRRSV.

The estimated mean of IAV RNA copies/ m^3 of air measured by the ACI ranged from $1.33x10^2$ to $1.21x10^4$ with the EPI system “off”, and from 0 to $3.0x10^2$ with the EPI system “on”. The measurements using the cyclonic air collector ranged from $6.7x10^3$ to $2.6x10^4$ with the EPI system “off” and from $7.8x10^3$ to $3.0x10^4$ with the system “on”.

The estimated mean of PRRSV RNA copies/m^3 of air measured by the ACI ranged from $8.30x10^2$ to $2.48x10^6$ with the EPI system “off” and from 0 to $4.98x10^5$ with the EPI system “on”. The values using the cyclonic collector ranged from $3.0x10^4$ to $7.3x10^5$ with the EPI system “off” and from $3.9x10^4$ to $2.8x10^5$ with the system “on”. For both viruses, negative controls tested negative.

In study 1, IAV was found in all particle size ranges with the system “off” and in most of the size ranges with the system “on” except for particle sizes of 5.8 to 10µm at 2 m distance, and for particles of 4.7-5.8 and >9.0 µm at 3 m distance. There was a reduction in the number of RNA copies/m^3 with the system “on” and this reduction was greater at 3 m distance of the EPI line to the ground. Results from the reduction difference predicted by the model indicated a total reduction between 0.56 logs (for particles between 2.1 and 3.3 µm) and 2.58 logs (for particles between 3.3 and 4.7µm) at 3 m distance of the EPI line to the ground.

PRRSV was found in all particle size ranges with the system “off” and in most of the sizes with the system “on”, except for the largest particle of >9.0µm at 2 m distance. There was an overall reduction in the number of RNA copies/m^3 with the system “on” and this reduction was greater and significant for virus associated with larger particles (3.3 to 10.0 µm) at 3 m distance of the EPI line to the ground. The results indicated a total reduction between 1 log (for particles between 0.4 and 2.1 µm) and 3.8 logs (for particles between 4.7 and 5.8µm) at 3 m distance of the EPI line to the ground.

Bacterial quantification in air samples

A total of 108 plates collected using the viable ACI were incubated and analyzed. The estimated mean of *S. aureus* CFUs/ m^3 of air measured by the ACI ranged between $1.88x10^1$ and $2.87x10^3$ with the EPI system “off”, and from 0

to 1.43x10^3 with the system “on”. Plates incubated with the air samples collected with the cyclonic collector had too many CFUs to count and results are not reported. Negative controls tested negative.

S. aureus was found in all particle size ranges with both the system “off” and “on”. There was a reduction in the number of CFUs/m^3 with the system “on” and this reduction was greater at 3 m distance of the EPI line to the ground. These results indicated a total predicted reduction difference of 0.62 logs (for particles between 0.7 and 1.1 µm) to 1.35 logs (for particles between 4.7 and 5.8 µm) at 3 m distance of the EPI line to the ground.

Virus and bacteria removal efficiency

Removal efficiency was greater for all pathogens when the EPI line was located at 3 m from the ground and closer to the source of aerosols. Removal efficiency measured at 3 m, 2 m, and 1 m from the floor varied from 87.6 to 99%, 22.4 to 65% and -10.7 to 51.7% for IAV, PRRSV, and *S. aureus* respectively. The EPI system significantly reduced airborne IAV and PRRSV when the EPI line was located at 2 and 3 m from the floor, and at 1 and 3 m for aerosols containing *S. aureus* (Figure 5.3).

Ion decay time

A total of 186 readings were taken during the study. Average ion decay time was 1.18 sec (± 0.02), 9.4 sec (± 0.76), and 44.18 sec (± 17.08) at 1, 2 and

3 m respectively. Distance from the ion analyzer to the EPI lines, which is directly related with electrostatic field strength, played a statistically significant role after adjusting by replicate (p-value < 0.0001).

Relative humidity test

Results from the relative humidity performance test of the EPI system demonstrated a difference in the number of IAV RNA copies/m^3 with the system "on" under the 2 different RH scenarios. A greater removal efficiency of 89% was observed at 70% RH compared to 47% at 30% RH. However, this difference was not statistically significant (*p*-value=0.26).

Study 2: Aerosols released by infectious animals

Virus quantification in air samples

All pigs were confirmed positive by RT-PCR for IAV, PRRSV and PEDV after inoculation. All pigs tested positive for IAV in nasal swabs on day 3 (inoculated pigs) and on day 7 (contact pigs) after infection (RT-PCR Ct 24.6 ± 2.2 and 21.1 ± 2.8 respectively). All inoculated pigs experienced the peak of PRRSV viremia ($1.25x10^9 \pm 8.93x10^8$ ORF6 RNA /mL) on day 7, and contact pigs experienced the peak on day 13 ($4.57x10^8 \pm 2.96x10^8$ ORF6 RNA /mL).

The total concentrations of IAV, PRRSV and PEDV associated with airborne particles of different sizes measured with the ACI with the EPI system "off" and "on" are shown in Fig 5.4. Higher concentrations of viral particles were

associated with larger particle sizes. There was a reduction in the number of viral particles/m^3 for IAV, PRRSV and PEDV across all particle size intervals with the system “on”. However, in the case of PRRSV, RNA copies of the virus were not detected associated with particle sizes ranging from 0.7 to 2.1 µm with the system “off “ or “on”.

The predicted reduction difference per particle size between the system “off” and “on” obtained with the ACI collector was calculated for the 3 viruses (Fig 5.5). The results indicated a total reduction from 0.14 logs (for particles between 0.7 and 1.1 µm) to a maximum of 1.90 logs (for particles greater than 9 µm) for IAV, a reduction of 0.18 logs (for particles between 2.1 and 3.3 µm) to a maximum of 1.33 logs for the largest particles, and in the case of PEDV, we observed a reduction of 0.73 logs (for particles between 9.0 and 10.0 µm) to a maximum of 1.43 logs (for particles between 3.3 and 4.7 µm).

Total particle removal efficiency

Based on the optical particle counts obtained during study 2, 82.8, 8.1, 2.3, 1.6, 2.0 and 3.2% of total particles were distributed among the size ranges of 0.3-0.5, 0.5-1.0, 1.0-3.0, 3.5-5.0, 5.0-10 and larger than 10µm respectively. Removal efficiency for total particles ≥1 µm was greater than for those <1 µm and ranged between 76 and 82% and between 52 and 56% for these groups respectively (Table 5.2). On the other hand, the removal efficiency of virus particles from aerosols generated by infectious animals ranged from 88 to 99.9 %

for IAV, negative removal efficiency to 100% for PRRSV, and 58.8 to 96.8% in the case of PEDV. Total particle size distribution measured by the OPC is shown in the Figure 5.6.

Virus viability results

Of all air samples collected using the air cyclonic collector, 90%, 17.6% and 100% tested positive for IAV, PRRSV and PEDV by RT-PCR respectively (Table 5.3). IAV was isolated in six out of 27 (22.2 %) positive air samples. From those, 5 were isolated with the EPI system “off” and 1 with the EPI system “on”. In the case of PRRSV, it was isolated in 9 out 12 (75%) samples of which 6 were isolated from the system “off” and 3 with the system “on”. Results from the bioassay performed in pigs demonstrated the presence of infectious PEDV in samples treated with the EPI system “on” and “off”. All 3 inoculated pigs experienced moderate to severe diarrhea, with fecal number of RNA copies/mL ranging from 3.96×10^{10} to 7.57×10^{10}. Pigs had histopathological lesions of moderate to marked atrophic enteritis, while the pig from the negative control group showed no clinical signs, had normal intestinal histomorphology, and tested negative by RT-PCR.

Discussion

Developing biocontainment strategies for production animals is a priority for food animal industries. A particular challenge is the prevention of spread of

emergent pathogens of economic (i.e. PRRSV, PEDV) and zoonotic importance (i.e. IAV, *S. aureus)* than may be transmitted among farms via aerosols. Our results indicate an overall reduction on the quantity of airborne pathogens in air treated with the EPI system. However distance to the source of ions, particle size and type of infectious agent affected the removal efficiency of the system. Notably, the EPI system produced a reduction in the number of viable airborne viruses within the airspace of infected pigs.

In translating this approach to industry, it is important to understand if the physical orientation of the system will influence pathogen reduction. We found that removal efficiency was influenced by pathogen type and location of the EPI line. Distance to the ion source greatly influenced the efficiency of the system resulting in higher ion concentrations as distance to the EPI line decreased. Greater removal efficiency was observed for influenza and PRRS viruses than for *S. aureus*. Similar results have been found for *Bacillus subtilis* vegetative cells, *Pseudomonas fluorescens, Candida albicans*, toxins and allergens mechanically generated (MacFarlane, et al., 1999; Yao, et al., 2009; Xie, et al., 2011). This is the first report showing that proximity to the source of ions also influences reduction of airborne viruses.

Removal efficiency of viruses by the EPI system was shown for mechanically generated aerosols, and for aerosols generated by experimentally infected animals. In the case of IAV our results demonstrated a difference in concentration between 0.35 and 1.9 logs after the EPI treatment on particles

ranging between 2.1 and > 9 µm containing viable virus. This log difference can be translated to a removal efficiency of 2.3% and 98% respectively. Considering that virus particles are positively associated to larger size ranges, and that previous studies did not report their results based on particle size, this total removal efficiency could be considered higher than that of the use of feed additives, changes in ventilation rates and air distribution, and other electrostatic precipitators (Takai, et al., 1996; MacFarlane, et al., 1999; Yao, et al., 2009; Xie, et al., 2011) but similar to a total of 85% obtained when a combined method of spraying an oil mixture controlled by an animal activity sensor was used (Takai and Pedersen, 2000). Removal efficiencies by particle size were higher for aerosols generated by animals than mechanically, particularly for IAV. It is unclear why the differences existed but stability and nature of aerosols generated by animals could have played a role. Non biological particles such as NaCl carry fewer electric charges compared to biological particles (Mainelis, et al., 2001) so results obtained from mechanically generated aerosols could underestimate the system efficiency due to their dilution on PBS media. Location of source of aerosols with regards to the air sampler's position within the room (2.8m height in study 1 vs. 30 cm from the animals in study 2) could have also influenced the results.

Our methods also allowed us to quantify the removal efficiency of total airborne particles and compare those to the viral removal efficiency of particles of different sizes. We observed that removal efficiency increased with particle size

as measured by the OPC and these results were very consistent during the entire study. Notably, similar results were observed with the linear regression modeling analysis for IAV and PRRSV. For both viruses, total viral reduction was highest for particles ranging between 3.3 and those > 9µm diameter from aerosols generated by infected animals. Interestingly, total reduction of PEDV particles was the highest when associated to particles between 3.3 and 4.7 µm. For the nature and scope of this study, it remains unknown if removal mechanisms of particles such as gravity, impaction or interception played a significant role in the total removal efficiency of virus. More research is needed to corroborate these results and evaluate whether other mechanisms besides ionization may have played a role in the removal efficiency parameters.

Virus viability results indicated a trend towards reduced viability of airborne pathogens when the EPI system was turned on. In the case of PEDV, we did not rely on cell culture methods because PEDV is difficult to grow *in vitro*, but assessed viability with a bioassay study. Although with the bioassay we were only able to assess presence or absence of virus, it provided an estimate of PEDV viability. However, we do not know whether the EPI system decreased the overall amount of PEDV in the air. Nevertheless, samples collected with the EPI system “on” were still infectious.

Our study also suggests potential for reducing exposure of zoonotic pathogens to swine workers and veterinarians. Removal efficiency of S. aureus (in study 1) and IAV (in study 2) was significant for all particle sizes measured

(0.4 to >9 µm, with the exception of those ranging between 0.7-1.1 µm) at 3 m distance to the source of ions and those greater than 3.3 µm in aerosols generated by infected animals. These results indicate, for the first time, the size of particles with which these zoonotic pathogens associate once aerosolized and the size ranges at which the system was more effective at removing them from the air. Decreasing the quantity of these pathogens in the occupational environment could reduce pathogen transmission between animals and people although further research is needed to determine whether this reduction is sufficient to halt transmission of zoonotic agents from animals to people.

Throughout the study we found instances where the removal efficiency was negative (meaning there were more particles in the air after the EPI system was on). These results were unexpected and they may be due to the sensitivity of the PCR technique where small variations in RNA copies can result in significant differences affecting the removal efficiency ratio. Alternatively, there could have been changes in the experimental conditions during the time the EPI system was “on” or “off” that could have affected the results although this is unlikely since negative removal efficiencies were not observed when removal efficiency was calculated for total airborne particle concentrations.

Relative humidity has also been reported to impact the efficiency ionization (MacFarlane, et al., 1999), however under the conditions of our study our results did not show a statistically significant effect of the EPI in IAV removal efficiency at the two RH tested although there was a trend towards higher

efficiency at higher humidity. The relationship between negative ion concentration (an indicator of particle removal efficiency) and air humidity levels is complicated and involves hydration and chemical reactions of negative air ions with water in air (Wu, et al., 2006). Further studies are needed to fully establish the impact of RH on the EPI system efficiency taking into account interactions with other environmental factors.

In conclusion, ionization of airborne particles reduces the quantity of airborne pathogens. Decreasing the infectious pathogen load should mitigate the risk of occupational exposure and should help contain pathogen spread from farms and improve overall regional biosecurity in pigs.

Table 5.1 Summary of study parameters.

Study parameters	Study 1	Study 2
Aerosol source	Mechanically generated (6-jet Collison nebulizer)	Naturally generated (infection in pigs)
Aerosol generation height	2.8 m*	0.3 m
Pathogens tested	IAV, PRRSV and *S. aureus*	IAV, PRRSV and PEDV
EPI line height	3 m, 2 m, 1 m	1.3 m
Air collector locations	0.2 m	1.2 m
Optical particle counter location	0.2 m	1.2 m
Ionizer performance analyzer location	0 m	0 m
Replicates	3	2 (17 days) or 1 (6 days)
Total tests run	27	40

*All height distances measured above floor

Table 5.2. Removal efficiency by particle size of the EPI system for total particles and viral particles of influenza A virus (IAV), porcine reproductive and respiratory virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) from aerosols generated by experimentally infected animals.

Total particles (by OPC)		Viral particles (by RT-PCR analysis)			
Particle size range (µm)	Average removal efficiency (%)	Particle size range (µm)	Average removal efficiency (%)		
			IAV	PRRSV	PEDV
0.3-0.5	51.6	0.4-0.7	77.1	98.4	89.9
0.5-1.0	56.1	0.7-1.1	52	-*	95.5
1.0-3.0	76.2	1.1-2.1	77.7	-	58.8
3.0-5.0	82.2	2.1-3.3	77.5	97.8	89.8
5.0-10.0	79.8	3.3-4.7	98.2	99.7	96.4
>10	76.2	4.7-5.8	96.5	98.8	89.7
		5.8-9.0	97	98.2	85.1
		>9.0	98.2	99.9	84.3

*Removal efficiency could not be calculated because both values obtained with the system “off” and “on” were the PCR limit of detection

Table 5.3 Number of positive results of influenza A virus (IAV), porcine reproductive and respiratory virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) by RT-PCR and virus isolation (VI) or bioassay in air samples from acutely infected animals.

Virus	Diagnostic technique	EPI system		Total samples
		"off"	"on"	
IAV	[1]PCR	14/15 (93.3%)	14/15 (93.3%)	27/30 (90.0%)
	[2]VI	5/14 (35.7%)	5/14 (35.7%)	6/27 (22.2%)
PRRSV	PCR	8/34 (23.5%)	8/34 (23.5%)	12/6 (17.6%)
	VI	6/8 (75.0%)	6/8 (75.0%)	9/12 (75.0%)
PEDV	PCR	6/6 (100%)	6/6 (100%)	12/12 (100%)
	[3]Bioassay	2/2 (100%)	2/2 (100%)	3/3 (100%)

[1]RT-PCR results presented as number of positives of total samples tested (%)

[2]VI results presented as positive samples of total RT-PCR positive samples tested (%)

[3]Bioassay results are presented as positive pigs of the total inoculated (%)

Figure 5.1 Diagram of the research unit utilized in study 1, depicting the release of mechanically generated aerosols, the location of the EPI line, the ion release, and the placement of the Andersen cascade impactor and liquid cyclonic air collectors during the collection of air samples.

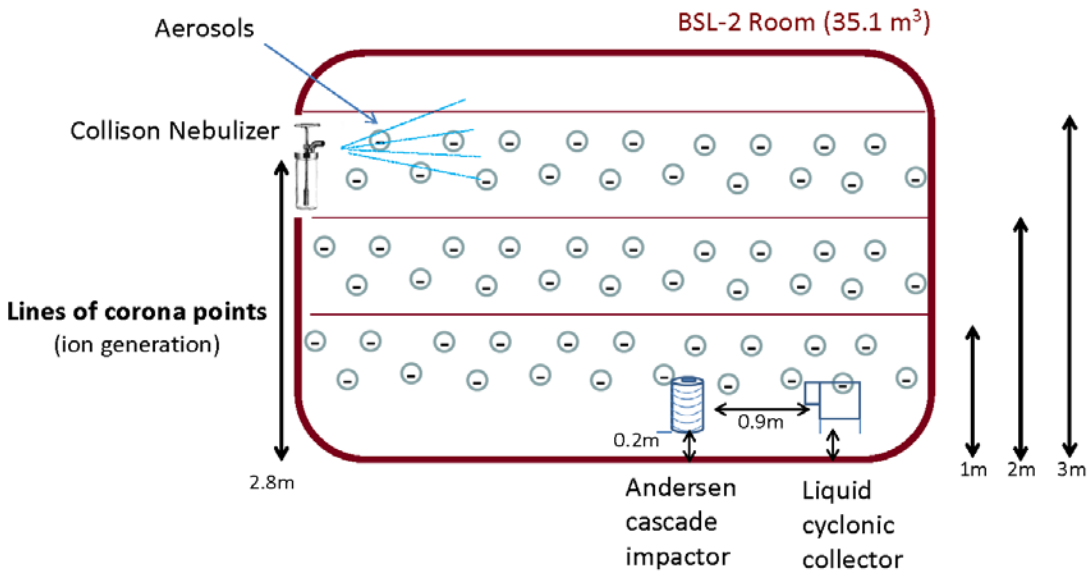


Figure 5.2 Diagram of the research unit utilized in study 2, depicting the animal space, the location of the EPI line, the ion release, and the placement of the Andersen cascade impactor and liquid cyclonic air collectors during the collection of air samples.

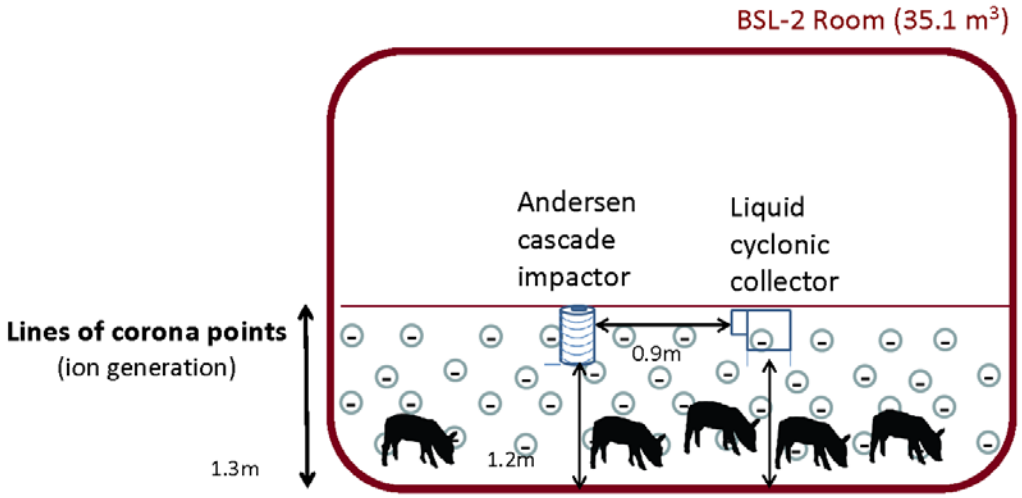
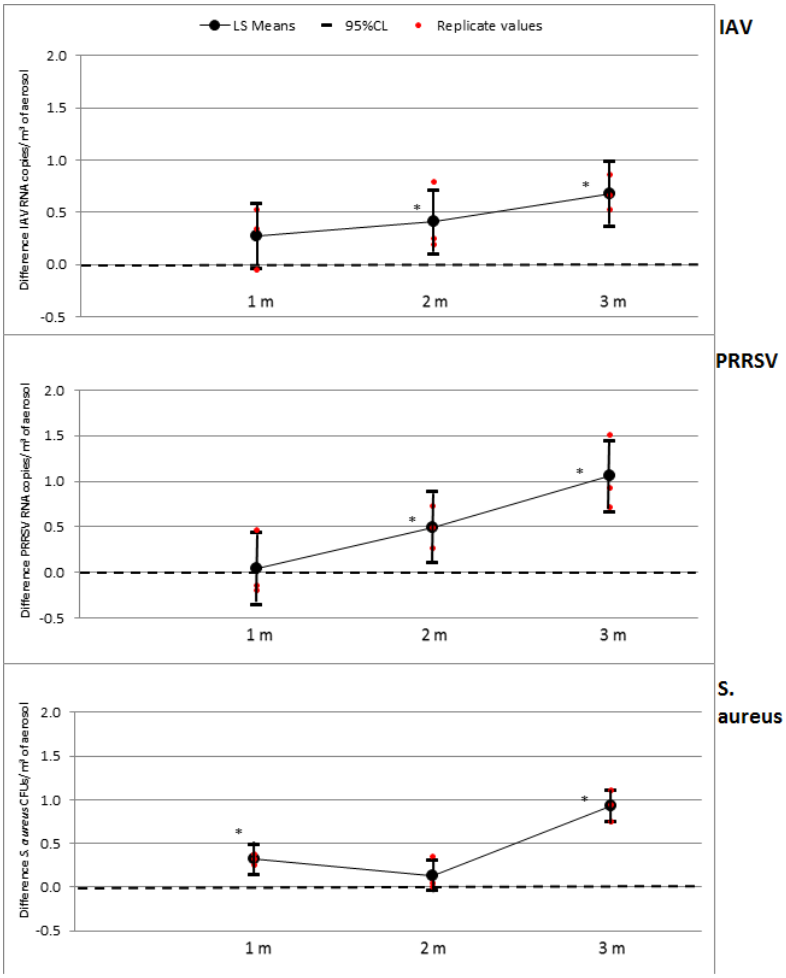
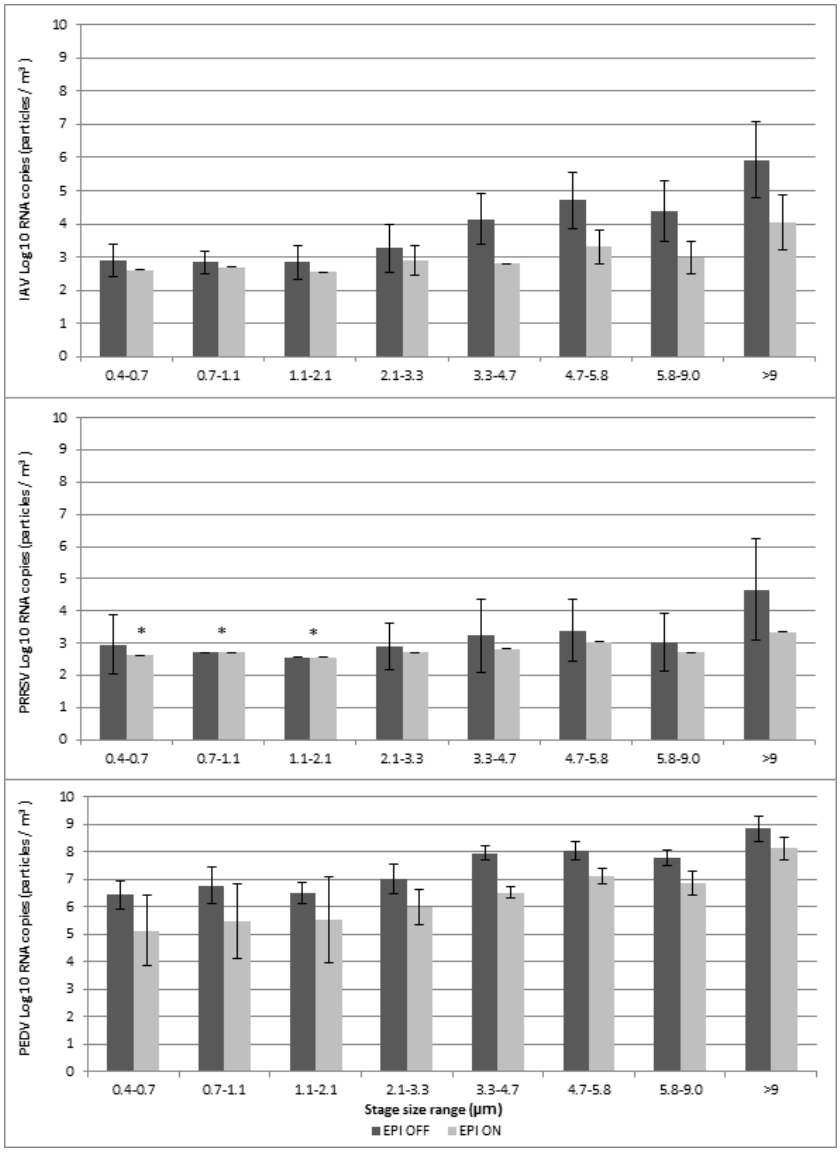


Figure 5.3 Linear regression modelling for total reduction of influenza A virus (IAV), porcine reproductive and respiratory virus (PRRSV) and *Staphylococcus aureus* with the EPI system "on" at various distances from the ground. Least square means of concentration difference of RNA copies/m^3 or CFUs/ m^3 of aerosol by distance of the EPI line to the ground with the Andersen Cascade Impactor sampler after adding all pathogen particles of all stages. Dashed line indicates the null value.



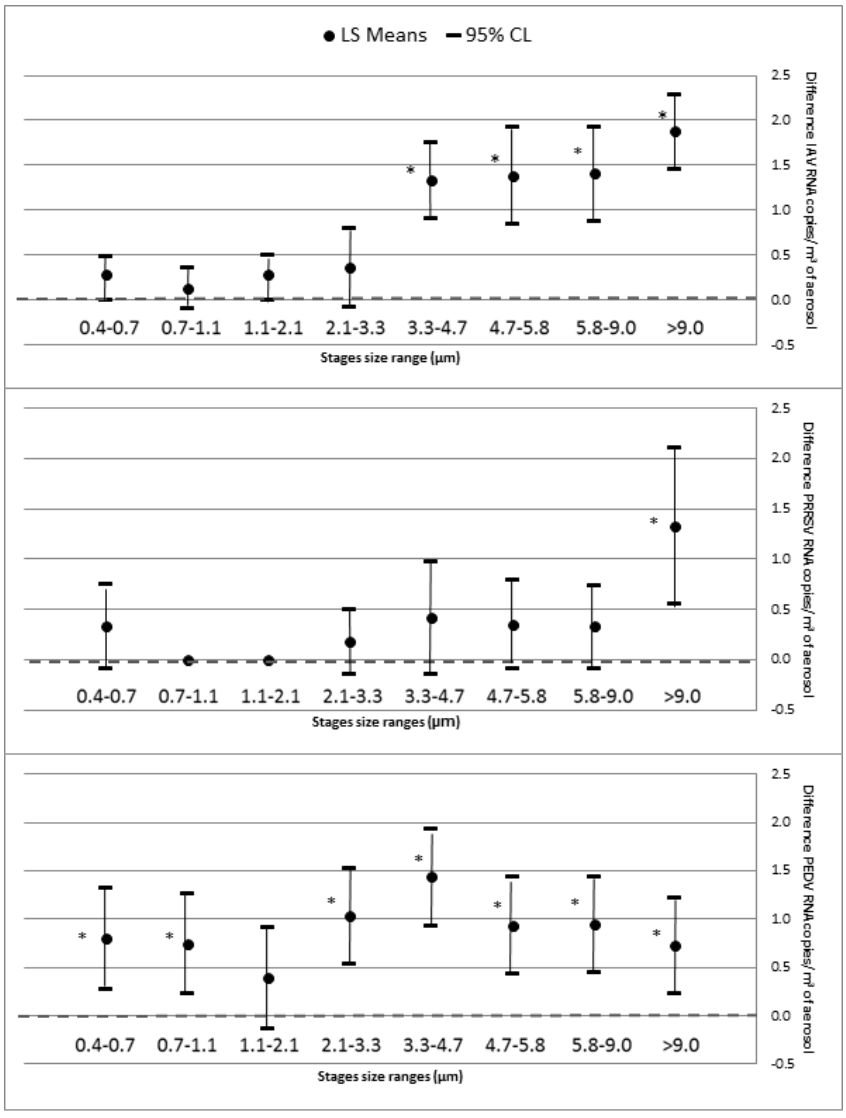
***** 95% CI does not include the null value, p value < 0.05

Figure 5.4 Concentration of influenza A virus (IAV), porcine reproductive and respiratory virus (PRRSV) and porcine epidemic diarrhea virus (PEDV) in the air (RNA copies/ m^3) after animal challenge with the EPI system “off” and “on” by particle size.



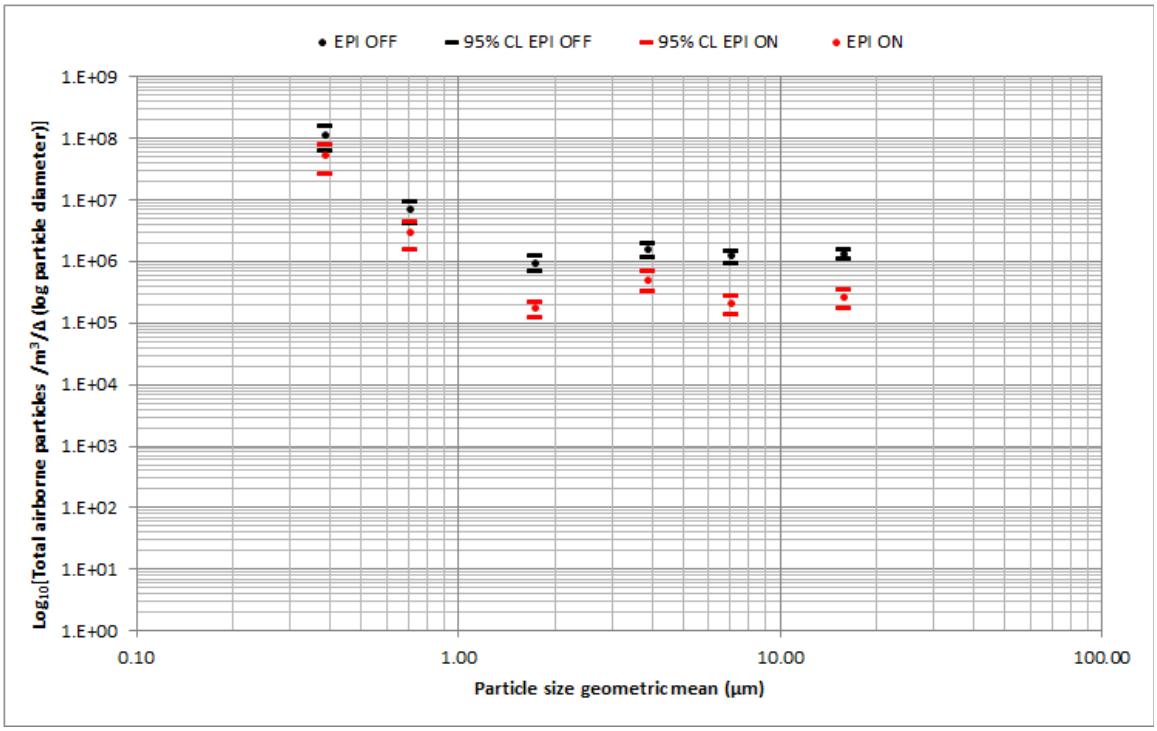
* Polymerase chain reaction (PCR) limit of detection.

Figure 5.5 Linear regression modelling analysis. Total reduction of influenza A virus (IAV), porcine reproductive and respiratory virus (PRRSV), and porcine epidemic diarrhea virus (PEDV) from experimentally infected animals measured with the ACI sampler with the EPI system “on” by particle size. Dashed line indicates the null value.



***** 95% CI does not include the null value, p value < 0.05

Figure 5.6 Distribution of total airborne particles (geometric mean of number of particles/m^3 and 95% confident interval) measured using an optical particle counter with the EPI system “off” and the system “on”.



Chapter 6: General discussion and conclusions

Improving the understanding of infectious bioaerosols generated by animals is crucial to prevent airborne transmission of diseases within and between farms, and between animals and people. The main objective of this thesis was to characterize bioaerosols generated by infectious animals in regards to concentration, particle size distribution and infectivity of viruses affecting pigs and to evaluate control strategies to reduce the risk of airborne pathogen spread. Studies were conducted in both experimental and field settings to characterize bioaerosols under controlled and natural conditions and to assess the potential role of bioaerosols in disseminating specific viruses during outbreaks. Viruses responsible for devastating outbreaks in swine, as well as in poultry, were shown to become airborne and remain infectious. Specifically for porcine reproductive and respiratory syndrome virus (PRRSV) and influenza A virus (IAV), our work supported previous findings that showed that these viruses can be airborne. In addition, we reported for the first time that, despite being an enteric virus, porcine epidemic disease virus (PEDV) could also be found in air samples inside and outside premises. We also confirmed that highly pathogenic avian influenza (HPAI) was present in air samples collected inside and outside premises during active outbreaks. For all these viruses, it was observed that viral RNA occurred at variable concentrations in association with particles ranging widely in size. However, the infectivity of airborne viruses appeared to be particle size dependent.

Methods used to quantify organisms in air may greatly influence observed results. We validated methods to determine the recovery efficiency of a filter-based air sampler used to study airborne pathogen transmission between farms. Furthermore, using size selective air sampling methods, we tested the efficacy of a commercial air sanitation technology, the EPI (electrostatic particle ionization) system and demonstrated that it reduced the airborne concentration of viruses under experimental conditions.

The introduction and first chapter of this thesis provided background information on disease transmission emphasizing all aspects related to airborne transmission, and reviewed important airborne viruses of swine, and control methods directed at minimizing the risk of airborne spread in food animal production. The review showed a significant gap with regards to particle size associated with viruses generated by infected animals including measurements under field conditions. Particle size determines distance across which pathogens can be transported, as well as the site of deposition in the host and the survivability of the pathogen, and it is also used to classify the mode of transmission of pathogens as droplet (short distance transport, larger particles) or airborne (long distance transport, smaller particles) after being aerosolized. As a result, particle size is central to the epidemiology of airborne diseases and a focus of this thesis. Our review also highlighted the limited methods available to sample viruses associated to ranging particle sizes in particular for diluted aerosols where virus concentrations are lower.

In chapter 2, the concentration and size distribution of inhalable particles that transport IAV, PRRSV, and PEDV generated by acutely infected pigs was characterized, and virus viability assessed for each particle size range. IAV, PRRSV, and PEDV were shown to be aerosolized and viral RNA was detected in all size particles measured, with the exception of particles between 0.7 and 2.1µm in the case of PRRSV. This demonstrates that these airborne viruses generated naturally by animals may be transmitted across both short and long distances. Notably, PEDV was detected in all particle size ranges and in higher quantities than IAV or PRRSV, which may be attributable to the large volumes of diarrheic material with high concentrations of PEDV that is excreted by acutely infected animals.

Viability assays for PRRSV and IAV demonstrated the presence of infective viruses in particles larger than 2.1 microns, but not in smaller particle size ranges. However, particle size is positively associated with virus concentration, and infectivity in cell culture is virus concentration dependent. Therefore, further studies are needed to assess virus viability using equal concentrations of viruses across specific size ranges of particles.

As a limitation of this chapter it is important to highlight that this study was only replicated once. However, the study was conducted throughout a 24 day period to cover the peak of shedding of the viruses tested. Therefore the estimates obtained were robust. It is acknowledged however, that other

environmental testing conditions and in particular different challenge strains and immune status of the animals could result in different estimates.

Because the sensitivity of existing air sampling methods may be insufficient for detecting low virus concentrations, we compared the performance of the conventional Anderson Cascade impactor (ACI) and the Tisch cascade impactor (TCI) a filter based high volume impactor. Overall, the higher airflow and collection principles of the high volume TCI sampler did not improve the efficiency of detection of the airborne viruses compared to the conventional ACI sampler. This is likely due to the lower recovery efficiency of the viruses from the TCI filter substrates since, under the conditions of our study, the recovery efficiency was only 26% or lower, and decreased with decreasing virus concentration. Differences between spiked and aerosolized methods to load the filters as well as the combination of the fiber glass substrate and high air flow responsible for a possible structural damage and desiccation of the virus particles should be considered when analyzing efficiency differences observed between collectors. Our observations underline the difficulty of estimating the viral concentration in bioaerosols based on filter-based air sampling techniques, due to collection and processing inefficiencies of the methods.

As a limitation of this chapter it is important to highlight that the filter substrate extraction efficiency was measured as traditionally done in other studies by loading the filtrate substrates using spiked virus directly into the filter. Previously it has been shown that the loading method of the filter impacts the

recovery efficiency and that spiked tests tend to overestimate recovery efficiencies compared to loading the filter using an aerosol. In our study, when the recovery efficiency of the collectors was compared, the filters were loaded with aerosols. Therefore it is likely that our TCI results had underestimated the capabilities of the sampler when compared with the ACI. Furthermore, recovery efficiency was only assessed using one type of filter substrates. In retrospect, we should have included more than one type of filter substrate or have used the TCI sampler impact platforms similarly to the aluminum impact plates used for the ACI.

In chapter 4, the TCI and ACI were used to estimate concentrations of airborne viruses both within and outside swine (PRRS, PED) or poultry facilities (HPAI) in the face of acute disease outbreaks. This enabled comparison of the performance of both air samplers under field conditions where the distributions and concentrations of viruses in large volumes of air were expected to differ from those created under experimental conditions. All 3 viruses were detected in the air samples collected inside and, in the case of HPAI and PED viruses, they were also detected outside of the tested facilities. In contrast to previous studies, PRRSV was not detected immediately outside swine facilities supporting the hypothesis that long distance airborne transmission of PRRSV may be less likely to occur than previously reported. Differences between testing methods, climatic conditions, virulence of PRRSV strains and infectious status of the animals at

time of sampling should be considered when interpreting the results between studies.

PEDV and HPAIV were detected both inside and outside affected swine or poultry farms, suggesting a higher likelihood of dissemination of these viruses between farms. Our results support the hypothesis that both direct airborne transport and deposition of virus particles onto surfaces surrounding infected premises could provide pathways for the dissemination of viruses between premises. Furthermore, despite of the limited enteric clinical signs in the pigs of the 2 PEDV positive farms investigated, PEDV was again found in larger quantities in air samples compared to HPAIV and PRRSV, concordant with the experimental observations reported in chapter 2. These results highlight the need to enforce biosecurity measures even when clinical signs are not obvious. Overall, when sampling diluted aerosols under field conditions no significant differences in collection efficiency were detected between air collectors. The sole exception was for samples collected for PRRSV inside swine facilities, in which viral RNA was detected with the ACI sampler but not the TCI sampler. Low concentrations of airborne PRRSV combined with low recovery efficiencies from the TCI substrates may be factors that could account for this difference between collectors. During the field study, we acknowledge the testing of a limited number of farms with differences among them and their infectious status. Therefore, adding additional farms with better characterized times of infection and environmental conditions should help generate more robust estimates for risk of

viral transmission. Nevertheless, to our knowledge this is the first study that characterized the particle size of specific viruses in aerosols from swine and poultry barns which is very much needed information to design transmission models and interventions to prevent the viral spread. Clearly, further studies should be conducted to address these limitations and better understand the role of infectious aerosols in the dissemination of specific viruses during outbreaks under field conditions.

Lastly, chapter 5 investigated the efficiency of an air sanitation technology, the electrostatic ionization technology or EPI. Measurements of the quantity and viability of IAV, PRRSV, PEDV and *S. aureus* were obtained using experimentally generated aerosols and in aerosols generated by infected animals. The ionization of airborne particles reduced the quantity of airborne pathogens in the order of magnitude of 0.5 and 1.9 logs. However no significant differences were observed in regards to infectivity. Higher removal efficiencies of viruses were observed with aerosols generated from infected animals compared to those experimentally generated. The nature of the particles in aerosols (non-biological particles, such as NaCl, compared to biological particles) and location of source of aerosols with regards to the air sampler's position within the room are factors that could have influenced the observed data. Overall, the study showed potential for the EPI system to reduce airborne pathogens inside contaminated facilities. However, further studies are needed to compare the results obtained in our studies with those generated under field conditions where

particle concentrations, relative humidity and temperature conditions are different. Determining whether this reduction is sufficient to halt zoonotic transmissions from animals to people or to prevent the spread of pathogens between farms are also a crucial questions that remain unanswered. Nevertheless, decreasing the quantity of these pathogens in the air of occupational environments has the potential to reduce pathogen transmission between animals and people.

The work conducted in this thesis generated new questions that can be addressed in follow up studies. Because evidence is still limited on the collection of viruses outside facilities, alternative systems are needed for virus sampling using high-volume size differentiating air samplers such us liquid concentrators or other sampling methods that can capture viable viral particles in diluted air without using an extraction process. More precise information of particle size distribution and concentration of infectious aerosols at different distances from the point of generation would help to develop better models for predicting spread among farms. Such models should also consider the acute and chronic nature of herd infection, and the stages (early, middle, late) of outbreaks in epidemics. There are currently no estimates comparing risks of acutely versus endemically infected herds.

A key observation derived from our studies is also the deposition of airborne pathogens on surfaces outside infected premises. Therefore, the airborne route may play a role not only in the direct transport of particles between

farms, but also as a source of contamination of the surrounding environment. Future studies should evaluate the role of airborne particles contaminating surfaces close to the facilities and its potential for virus spread between facilities. This information would be helpful to better identify biosecurity strategies to biocontain an infection within a herd. Currently, there are very limited methodologies directed at doing so in food animal production systems.

Lastly, the methodology developed during this thesis provides a valid platform to test the capabilities of other sanitation technologies, such as photocatalytic technologies with higher biocide efficiencies, ultraviolet light and others. Further studies are needed to evaluate these technologies in food animal production systems.

Altogether, the work presented in this thesis provided science-based information on the particle size distribution and infectivity of airborne viruses generated by animals with the goal to prevent airborne transmission of diseases within and between farms and from animals to people. The information provided, analyzed and discussed here has direct implications for the animal food industry and public health, and can assist producers and veterinarians to evaluate strategies to control and prevent airborne transmission within and between infected animal populations.

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