

Evaluation of respiratory route as a viable portal of entry for *Salmonella* in poultry

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Abstract: With increasing reports of *Salmonella* infection, we are forced to question whether the fecal–oral route is the major route of infection and consider the possibility that airborne *Salmonella* infections might have a major unappreciated role. Today’s large-scale poultry production, with densely stocked and enclosed production buildings, is often accompanied by very high concentrations of airborne microorganisms. Considering that the upper and lower respiratory lymphoid tissue requires up to 6 weeks to be fully developed, these immune structures seem to have a very minor role in preventing pathogen infection. In addition, the avian respiratory system in commercial poultry has anatomic and physiologic properties that present no challenge to the highly adapted *Salmonella*. The present review evaluates the hypothesis that transmission by the fecal–respiratory route may theoretically be a viable portal of entry for *Salmonella* in poultry. First, we update the current knowledge on generation of *Salmonella* bioaerosols, and the transport and fate of *Salmonella* at various stages of commercial poultry production. Further, emphasis is placed on survivability of *Salmonella* in these bioaerosols, as a means to assess the transport and subsequent risk of exposure and infection of poultry. Additionally, the main anatomic structures, physiologic functions, and immunologic defense in the avian respiratory system are discussed to understand the potential entry points inherent in each component that could potentially lead to infection and subsequent systemic infection of poultry by *Salmonella*. In this context, we also evaluate the role of the mucosal immune system as essentially one large interconnected network that shares information distally, since understanding of this sort of communication between mucosal sites is fundamental to establish the next phase of disease characterization, and perhaps immunization and vaccine development. Further characterization of the respiratory tract with regard to transmission of *Salmonella* under field conditions may be of critical importance in developing interventional strategies to reduce transmission of this important zoonotic pathogen in poultry.

Keywords: *Salmonella*, respiratory route, portal of entry, systemic dissemination

Introduction

There is good experimental and epidemiologic evidence that primary infection by *Salmonella* is through the fecal–oral route, along with an established requisite infectious dose. Previous published data have suggested that direct ingestion and contact are the most common routes of transmission for many zoonotic enteropathogens; however, inhalation of infectious particles should not be neglected.^{1,2} Airborne transmission of microbes is not restricted to agents causing respiratory disease, because enteric and other pathogens can also be transmitted by this route. The potential airborne transmission of zoonotic enteric pathogens, such as *Salmonella*, has not been well studied, even after repeated efforts, with reports suggesting that airborne transmission of *Salmonella*

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is possible.^{3–5} Much of the uncertainty regarding airborne transmission and infection by *Salmonella* and other enteric pathogens has been associated with the relationship between bioaerosol particle size and number as well as the dose of pathogen it is carrying.^{6–8} Also, it is an established impression that bioaerosol generation and airborne transmission of a pathogen might be possible, but that infection eventually has to be fecal–oral, because settling of the microbe on feed and other surfaces will lead to infection through ingestion.⁵

The present review discusses the possibilities of airborne *Salmonella* infection pertaining to poultry, followed by discussion involving established poultry practices potentially leading to generation of *Salmonella* bioaerosols at every stage of commercial poultry production. Possible mechanisms of survival and transmission of *Salmonella* in bioaerosols gaining entry to the respiratory system in poultry are then discussed. Further, the role of avian respiratory anatomy, physiology, and immunology in airborne infection of *Salmonella*, making the respiratory route a viable portal of entry for *Salmonella*, is also discussed. A comprehensive understanding of the role of airborne transmission and subsequent infection of *Salmonella* in poultry may allow for more effective prophylactic strategies and interventions for this important economic issue in the poultry industry.

Seriousness of the problem

From the evolutionary point of view, bacteria of the genus *Salmonella* are some of the most remarkable and successful microorganisms, which are able to infect practically every living cell in the animal and vegetable kingdoms, and have extraordinary mechanisms to evade the immune system of plants, invertebrates, and vertebrates. Poultry is a major reservoir for food borne *Salmonella enterica* subspecies *enterica* serovars, with *S. Typhimurium*, *S. Enteritidis*, *S. Heidelberg*, *S. Kentucky*, and *S. Senftenberg* being the most prevalent serovars in US poultry.^{9,10} Nevertheless, these serovars are also linked to leading causes of food borne illness worldwide.¹¹ In addition to the different disease outcomes, there is also a significant range of host specificity. In chickens, infection with host-specific *Salmonella* serovars *S. Gallinarum* and *S. Pullorum* cause septicemia, fowl typhoid, and Pullorum disease, respectively,¹² whereas infections with non-host-specific serovars generally produce no clinical symptoms. In the last decade, significant progress has been made with regard to knowledge about *Salmonella* invasion and pathogenesis in mammalian hosts; however, information regarding *Salmonella* invasion and colonization mechanisms and interactions with host cells in poultry is limited and

poorly defined.¹³ For most bacterial pathogens, virulence is a multifactorial process requiring two general classes of determinants. The first encompasses genes that participate in the physiological processes necessary for survival in host and non-host environments, and these genes are generally found in both pathogenic and nonpathogenic organisms. *Salmonella* infection has a complex pathogenesis. The pathogen is usually taken up by contaminated food. A variety of fimbrial adhesins are involved in initiation of contact with host cells. The first hallmark of bacterial–host cell interaction is invasion of nonphagocytic cells, eg, the epithelial cells of the intestinal mucosa. *S. enterica* is also a facultative intracellular pathogen that survives phagocytosis and is able to proliferate in infected host cells within a specific compartment, ie, the *Salmonella*-containing vacuole.^{14–16}

Pathogenicity islands are genetic elements on the chromosomes of a large number of pathogens and are considered to be a “quantum leap” in the evolution of bacteria.¹⁷ However, the paradigm of pathogenicity islands is broken in some cases. *S. Senftenberg*, a serovar that is more resistant to environmental stresses, is frequently isolated from hatching houses and raw feed materials, and is adapted to colonize and persist in poultry houses for up to 2 years.¹⁸ The prevailing theory of *Salmonella* enteropathogenesis is that bacterial invasion of the intestinal epithelium is essential for virulence, and this requires the virulence-associated genomic region, *Salmonella* pathogenicity island 1 (SPI-1).^{17,19} However, *S. enterica* serovar *Senftenberg* strains isolated from food borne outbreaks have been reported to lack the invasion-associated locus SPI-1, indicating that SPI-1 is not essential for intestinal inflammatory disease.²⁰ Yet, despite its lack of invasiveness, *S. Senftenberg* has emerged as a human pathogen, with the ability to acquire important pathogenic loci from other bacteria, such as the *Yersinia* high pathogenicity island, which encodes a yersiniabactin-mediated iron acquisition system present in highly pathogenic strains of *Yersinia* and several members of the *Enterobacteriaceae* family.²¹ Several studies have illustrated the presence of the high pathogenicity island in *S. Senftenberg* and the importance of poultry and pigs as a reservoir and vehicle for dissemination of zoonotic infection.^{22–25} Experimental and epidemiologic evidence suggests that primary *Salmonella* infection is by the fecal–oral route for poultry. Nevertheless, airborne transmission of *Salmonella* and similar enteric zoonotic pathogens has been historically neglected. Studies involving *Salmonella* bioaerosol production in combination with suggestions of vulnerable avian respiratory architecture indicate the respiratory route as a possible portal of entry for *Salmonella* in poultry.

Poultry practices and bioaerosol generation

Control of *Salmonella* infection is vital at the apex of commercial poultry production, given that this organism can be transmitted vertically from hen to egg.²⁶ Further, it is crucial to keep breeder production flocks free from *Salmonella* because a colonized flock will spread the bacteria to a large number of commercial flocks. Unlike production flocks, which are relatively short-lived, with a total lifespan of approximately 42 days, elite flocks, including breeders, may have a lifespan in excess of one year, so careful management is needed to ensure ongoing freedom from colonization by *Salmonella* and other pathogens.²⁷

In this regard, hazard analysis and critical control point-based intervention strategies have been developed and are used extensively.²⁸ While many of these strategies have proven effective in laboratory or field trials, implementation in commercial operations has proven inconsistent in some cases, especially towards the lower end of the production pyramid. There have been consistent reports of *Salmonella* infection, with most hurdles beginning at the commercial hatcheries, followed by expansion of incidence to processing plants.^{29–31} With increasing reports of *Salmonella* infection, we are forced to question our present intervention strategies, which are established based on fecal–oral transmission as the major route of infection, and consider the possibility that airborne *Salmonella* might have a major unappreciated role.

The hatchery

Ideally, fertile eggs should be enteropathogen-free; however, this is not the commercial reality in many hatcheries. Dust and bioaerosols generated from contaminated eggs within a hatchery incubator, a critical control point, can spread enteropathogens to other areas of the hatchery depending on airflow.³² The presence of *Salmonella* in air samples from hatcheries is irrefutable and has been proven by various sampling and identification methods.^{32,33} Further, it is a general notion that dust carried by airflow settles elsewhere in the hatchery on uninfected eggs and hatched egg shells, and is then assumed to follow a typical fecal–oral route of transmission, with hatchlings pecking these contaminated egg shells. However, the possibility of entry and transmission of *Salmonella* through the respiratory route has historically received little attention.^{4,32,34,35}

In modern poultry production, birds are held in the hatcheries for about 12–24 hours. Hatched birds experience prolonged high-density confinement in the hatchery, where they are exposed to circulating air that can carry *Salmonella* and

other pathogens. Further, transportation of hatchlings from hatcheries to placement facilities adds additional confinement time, during which birds do not have access to feed and water.³⁶ This early feed deprivation has been associated with delayed gut maturation^{37–39} and reduced immunity,^{40,41} increased susceptibility to pathogens, and a reduction in overall performance.^{42–44} Under such stressed conditions, the anatomic, physiologic, and immunologic state of hatchlings increases their vulnerability to infection. Considering these factors, it is reasonable to consider all possible routes of enteropathogen infection, especially the respiratory route, as a potential portal of entry for enteropathogens and *Salmonella* in context. Overall, we hypothesize that the hatchery environment is one of the most critical aspects of commercial poultry production with respect to airborne transmission of *Salmonella*, and hence various intervention strategies have to be developed at this stage to ensure prevention.

Litter

Prolonged use of litter with multiple sequential flocks of birds can harbor pathogens,⁴⁵ and dust originating from litter due to air circulation and movement of birds may lead to generation of bioaerosols from the production system and infect chickens via the respiratory route. Today's large-scale poultry production, with densely stocked and enclosed production buildings, is often accompanied by very high concentrations of airborne microorganisms.^{1,46} In modern broiler houses with tunnel ventilation, large volumes of air are moved through the house to provide an optimal temperature and superior ventilation for growth of broilers. These large volumes of moving air may potentially contain a range of bacteria sourced from the internal environment of the house, including pathogens such as *Salmonella*. Hence, there is a possibility that circulation of air within the poultry housing environment provides opportunities for transfer of these pathogens throughout the production house and to the surrounding air environment.⁴⁷ Several studies have reported the presence of high levels of airborne *Escherichia coli*, *Salmonella*, and *Campylobacter* inside poultry houses.^{29,31,48–53}

Transportation and processing

The goal of on-farm pathogen reduction strategies is to deliver poultry to the processing plant with undetectable levels of *Salmonella*. However, fasting, catching, and transportation of poultry from primary production to processing can be significant in the dissemination of enteric pathogens, via contamination and subsequent cross-contamination because of stress and use of dirty crates, trucks, and catching/pickup crews.⁵⁴

Controlling *Salmonella* contamination of poultry prior to slaughter may include limiting the exposure to bacteria in the final few hours before slaughter during on-farm loading, transportation, and holding at the processing plant. Understanding the kinetics of transmission of *Salmonella* to poultry from the environment during the last few hours before slaughter may identify a critical control point to reduce the number of contaminated birds entering the processing plants. If rapid airborne transmission by inhalation occurs immediately prior to slaughter, controlling dust and reducing transmission at this time point will be important.^{3,55} The first point of increased exposure prior to slaughter may occur when birds are taken off feed for 3–8 hours before loading. This can increase the flock's contact with *Salmonella* through increased ingestion of litter within the barn when in search of feed,^{56,57} which may eventually lead to fecal–oral transmission of infection. Withdrawal of feed is also associated with stress, somewhat similar to that of the hatchery conditions mentioned above. Second, disturbance of litter increases the amount of dust in the air, which may increase the risk of infection due to inhalation of *Salmonella*-contaminated particles or bioaerosols.^{3,56} Third, a large amount of dust is generated during the load-out process, and facilitates potential inhalation of *Salmonella* bioaerosols. Large fans blow air on turkeys to keep them cool during the summer months. From there, infection may spread through dust blown by fans to uninfected birds. These events occur in the last few hours before the birds are killed, and may increase exposure to *Salmonella*-laden dust.^{3,55} Pathogenic contamination of the respiratory tract in birds entering processing plants may contribute to carcass contamination, and *Salmonella* and *Campylobacter* have been detected in the respiratory tracts of broilers prior to entering the scald tank.^{58–62}

Survival of bacteria in bioaerosol

The aerosolization process starts at three interfaces, ie, litter, dust, and air, during any production cycle.⁵³ However, aerosolization and its transfer is a traumatic process for most microorganisms, and survival can be dependent on the mechanisms of aerosolization, the climate into which these organisms are launched, the distance they are travelling and, of course, the time involved in the whole process.^{62–64} *Salmonella* has been shown to be viable in laboratory-generated bioaerosols for more than 2–4 hours.^{48,62} Continued survival of *Salmonella* in source material, such as dust or litter, and subsequent survivability in aerosols, can also depend on the serovar of *Salmonella*. *S. Senftenberg* could persist for more than 2 years despite cleaning, disinfection, desiccation,

and depopulation, and was subsequently able to infect newly placed *Salmonella*-free layers.⁶⁵ Humidity is known to play a major role in the survivability of *Salmonella*,³³ but is one of the many factors that determine its survivability in bioaerosols.^{1,27,62}

Avian upper respiratory tract and bronchial airway

Among the air-breathing vertebrates, the avian respiratory organ is considered to be the most structurally complex and the most functionally efficient gas exchanger.^{66–73} As a consequence, particles and particle-associated microorganisms are inhaled as unavoidable constituents of air flow.⁷⁴ The architecture of the avian respiratory tract is an important component in terms of susceptibility and resistance to infectious agents. In this section, the main anatomic structure, physiologic functions, and immunologic defense mechanisms of the avian respiratory system are described to provide a general view of the conduit through which a pathogen has to travel.

Upper respiratory tract

The upper respiratory system begins at the nares and consists of passages that lead inhaled air and bioaerosol to the larynx. Physiologically, these oronasal structures function to heat and humidify inspired air, so can be expected to do the same to bioaerosols containing *Salmonella*, and indeed may provide favorable conditions for recovery and growth of *Salmonella* after traumatic transfer in bioaerosols.

Immune structures involved in the respiratory system of chickens and turkeys include Harderian glands, and conjunctival-associated, paranasal, and nasal-associated lymphoid tissue. Though these structures are functionally important components of local immunity, especially in the upper respiratory tract. Local immunity of the respiratory mucosal system is ensured by nonspecific defensive reactions with an arsenal of mechanical and cellular defenses, supplemented when necessary by inflammatory and immune responses.^{75–77}

There is no constitutive lymphoid tissue in the trachea. However, studies investigating infections of the respiratory system have shown reactivity of the tracheal mucosa to infections, despite a lack of essential lymphoid tissue.⁷⁸ The tracheal mucosa is highly responsive to infection and reacts with extensive lymphocyte infiltration in the lamina propria and submucosa, followed by proliferation. However, according to some studies, immune responses within the trachea are age-dependent.⁷⁷ The relationship between development of these immune structures and age is an important issue that

requires further consideration for understanding of immune function related to the respiratory system. Conjunctival-associated lymphoid tissue and nasal-associated lymphoid tissue, which are at the forefront of defense, are not fully functional germinal center and plasmatic cells at 4 weeks of age.^{76,79} Further, B-cell responses were not detected in the trachea until 3 weeks post-infection in layer-type chickens, which is typically more than half the lifespan of the average broiler. Having assessed the high risk of contamination of chickens through the aerosol route in hatcheries, these structures appear to have a minute role in preventing infections acquired through inhalation.⁷⁷

Aerosols in the upper respiratory tract

In conventional poultry production, birds are exposed to high loads of aerosolized particles, and considering the high probability of pathogens in these bioaerosols, the bird's upper respiratory tract would be very vulnerable to infection. The respiratory immune system has developed strategies to remove inhaled particles and to adequately respond to those microorganisms that cross the epithelial barrier in order to maintain integrity and function. Inhaled bioaerosol particles are eliminated from the respiratory system by multiple mechanisms, including aerodynamic filtration, mucociliary clearance, and phagocytosis, which are part of the innate defensive system in mucosal immunology.^{75,80–84} Inhaled foreign particles are initially removed by nasal mucociliary action via the mucociliary escalator mechanisms of the trachea, primary bronchi, and secondary bronchi. The primary bronchi and the roots of the secondary bronchi are lined mostly with columnar epithelial cells, which are longitudinally folded and ciliated. However, much of the remaining epithelium of the secondary bronchi is nonciliated, cuboidal, or squamous in structure; hence, mucociliary movement is restricted only up to the proximal end of the secondary bronchus.^{66,85} Thus, clearance mechanisms in the upper respiratory tract through the proximal end of the secondary bronchi primarily rely on particles becoming trapped in mucus, oral mucociliary transport, and expectoration or swallowing of trapped material. However, a detailed study by Hayter and Besch showed that particle size critically affects the site of deposition within the respiratory tract.⁸⁶ This portion of the respiratory system forms the first line of defense against large inspired particles up to 4–7 μm , but does not entrap many smaller particles. Cilia are smaller and fewer in the epithelium of the distal portion of secondary bronchi which raises uncertainty about the effectiveness of this site

in preventing microorganisms in the air stream from entering the lower portions of the respiratory system.⁸⁶

Dose approximation for respiratory route

Considerable evidence regarding generation and survivability of *Salmonella* within bioaerosols raises the question of exposure to infectious doses via respiratory inoculation. Assessment of exposure shows that the concentration of airborne microorganisms in the static air of animal farming environments can reach values of up to 10^{10} cells per cubic meter of air,⁸⁷ and particulate matter having a size of 3 μm can reach below the primary bronchi and may be involved in infection. Aerosols of about this size should be able to carry *Salmonella*, considering its typical diameter of around 0.7–1.5 μm and length of 2–4 μm , and several studies have shown the impact of bioaerosols with regard to *Salmonella* infection in poultry.^{34,46,47,62,88,89}

The impact of poultry house pollutants on particulate clearance from the respiratory system of birds remains largely unknown. Physical (dust), chemical (ammonia), and biological (bacteria or virus) conditions that reduce ciliary motility or disrupt the ciliated epithelium have been associated with increased risk of respiratory infection with several enteropathogens in poultry.^{64,85,90–95} There may not be a direct correlation between total respirable particles and their size and that of the *Salmonella* dose, and larger particles assumed to be removed by mucociliary clearance may also contribute to the dose involved through the respiratory route. Thus, particles that are not cleared by mucociliary mechanisms are exposed to the cellular and immunologic mechanisms of the bronchial airway.

Immune system of the bronchial airway

Most avian species, including chickens, lack draining lymph nodes but have a system of bronchial-associated lymphoid tissue (BALT) structures referred to as lymphoid nodules.⁷⁹ In chickens, these structures are confined to the openings of most caudal secondary bronchi and also the mediadorsal, lateroventral, and laterodorsal secondary bronchi, while a wider distribution pattern including regions of longitudinal foldings of the mucosa has been observed in turkeys.^{79,96} Inspired air containing foreign materials bypasses the medioventral secondary bronchi opening, facilitating deposition of heavier foreign materials in the epithelium. It is not until after passing the filtering mechanism of the lungs and air sacs that air passes through the medioventral openings without BALT.⁹⁷

Development of BALT depends on age and environmental stimulation. In day-old chickens and turkeys, no or very few infiltrating lymphocytes are seen in the primary bronchi region.^{98,99} Lymphoid nodules develop at these locations within the next 3–4 weeks, and cells producing immunoglobulin (Ig)M, IgG, or IgA can be detected; CD4+ and CD8+ T-cells are found at the same time localized in distinctive patterns.^{77,79,80} Germinal centers can first be detected in 2–4-week-old birds. The numbers of cells producing IgG, IgA, or IgM continue to increase during the following weeks; however, it is now evident that BALT requires 6 weeks to develop fully in poultry, which approaches the age of the broilers at the end of a typical production cycle.^{76,78,80}

Mature BALT structures consist of lymphocyte aggregates that are covered by a distinct layer of epithelial cells known as follicle-associated epithelium, which harbors considerable numbers of lymphocytes. In 6–8 week-old birds, almost every bronchial opening was surrounded by a raised ring of lymphoid tissue and germinal centers are found in most lymphoid nodules, and occasionally plasma cells are seen under the follicle-associated epithelium.^{76,77}

Epithelial cells in chicken BALT have been shown to lack microfold cells. However, recent research⁹⁶ has described four epithelial cell types in the follicle-associated epithelium, depending on age, including cells which have irregular microvilli on their luminal surface and close contact with lymphocytes, and therefore are suggested to display some features of microfold cells. However, uptake of particles by these cells has not been observed, and characteristic organelles such as apical vesicles are absent. It has been suggested that these cells may not be involved in antigen uptake and processing, but participate in tissue repair.⁸⁰ In a study that successfully reproduced fowl typhoid via intratracheal *S. Gallinarum* challenge, Basnet et al suggested that *S. Gallinarum* is likely to penetrate both microfold cells and epithelial cells on gastrointestinal lymphoid tissue and epithelial cells on BALT to infect lymphoid cells.¹⁰⁰

The large numbers of heterophils found in the BALT of both chickens and turkeys may be functionally significant. Phagocytes, particularly heterophils, are an important element of avian respiratory defense systems because birds lack a large pulmonary macrophage population. Studies have suggested that macrophages and granulocytes are capable of transporting materials captured in the bronchial lumen to sites where specific immune responses may occur.⁷⁹ Hence, to aid this process, at least two means of handling foreign particles in avian BALT are recognized, the first of which is passage between disrupted epithelial cells directly

to phagocytic cells. On the other hand, follicle-associated epithelium cells are believed to mediate antigen contact with lymphocytes through endocytosis.^{76,96,101}

The appearance of a disrupted epithelial barrier over lymphoid nodules, supported by described endocytosis taking place in epithelial cells, might suggest that antigens would be readily available to lymphocytes and phagocytes in BALT. This demonstration of highly developed and constitutively present BALT structures in chickens and turkeys has encouraged some investigators to suggest that these mucosal-associated lymphoid structures may functionally compensate for the lack of lymph nodes in birds.^{79,83,85,97} However, it remains to be shown if these structures specifically respond to antigenic stimulation by generation of memory B and T lymphocytes. The relationship between development of these immune structures and age is an important issue that needs attention. Having assessed the high risk of contamination of birds through the aerosol route in hatcheries, these immune structures seem to have a very minor role to play in preventing infections. Particulate matter and pathogens that cannot be efficiently cleared by the upper respiratory tract and bronchial airway may enter the parabronchial region or ventilating air sacs.

Parabronchial region and avian air sacs

Avian lungs have a highly intricate anastomotic joining of branches of tubular structures so as to make or become a continuous structure known as the parabronchial system. Hence, the functional unit of the avian lung is the parabronchus, in which terminal air spaces called air capillaries are heavily intertwined with blood capillaries for oxygen exchange.⁷⁰ Air capillaries and blood capillaries form approximately 90% of the exchange tissue.^{70,77,78} The air capillaries are generally strong due to a meshwork created between these and the blood capillaries, and mechanical compression of the lungs does not cause them to collapse. The lungs are ventilated unidirectionally and continuously in a caudocranial direction by the synchronized action of air sacs and parabronchial structures.

Air capillaries in poultry are much smaller in diameter (3–20 µm) than the alveoli of mammals, and are estimated to be a fraction of the size of mammalian alveoli.¹⁰² This results in an increase in the surface area over which gas exchange occurs and aids in increasing the diffusing capacity of the lungs. It is important to note at this point that any particles or pathogens in aerosols passing beyond the secondary bronchi or the mucociliary escalator can potentially end up within

the parabronchial region. Further, the parabronchi are lined by nonciliated cuboidal and squamous epithelium, so do not possess a means of moving inhaled foreign particles in the cranial/oral direction.¹⁰²

Blood–gas barrier

The blood–gas barrier comprises endothelial cells that border blood capillaries, an intermediate extracellular matrix space called the interstitium, a single layer of air capillary epithelium. On average, the tissue thickness of the avian blood–gas barrier is known to be thinner than that in mammals, and various data show that this holds true even for weak flyers, such as domestic chickens.^{66,103–108}

Structural failure in the chicken lung

During a respiratory cycle, avian lungs change in volume by a mere 1.4%.¹⁰⁹ Based upon research on the microanatomy of avian lungs, it has been proposed that support of blood capillaries by surrounding air capillaries contributes to the strength of the capillary walls and thus allows a very thin and stable blood–gas barrier.^{67–69} However, these properties appear to have conflicting roles, because thinness is essential for efficient flux of oxygen by passive diffusion, and strength is crucial for maintaining structural integrity.⁷² Compared with mammals, arterial blood pressures in birds is much higher. Broilers and turkeys frequently experience problems with hypertension that lead to high myocardial stress, resulting in complications such as aortic aneurysm and ascites.^{67,68,109,110} Mechanical failure of the blood–gas barrier due to its thinness, is an important but overlooked factor.^{107,108} In addition, increases in pulmonary capillary blood pressure are probably the foremost direct challenge to the mechanical integrity of the blood capillary wall, especially the blood–gas barrier, and indirectly the epithelial–epithelial cell connections. Due to increases in pulmonary capillary blood pressure, avian lungs are more susceptible to mechanical pulmonary injuries and hence pathogenic diseases. Given these vulnerable conditions, the avian respiratory system would be expected to have a strong and robust immune system. However, the immune system in the avian lung is limited, especially during confinement of poultry under commercial conditions. Now we explore the reasons for and consequences of this limited immune system with respect to airborne *Salmonella* infection in poultry.

Respiratory cellular defenses

Phagocytes constitute the first line of defense against infectious agents in the respiratory system. Microscopic techniques

have shown that macrophages are absent on the surface of air capillaries but present on the epithelial lining of the atria.^{70,111} Interestingly, macrophages were also seen in connective tissue below this epithelium on the floor of the atria^{112,113} and in the interatrial septa,⁸⁰ indicating that phagocytic cells are strategically located at the start of the gas exchange area to clear the air of inhaled particles before it reaches the thin and vulnerable air capillaries.⁸⁵ However, there have been reports of extremely low numbers of macrophages residing in the normal, steady-state respiratory tract of chickens, indicating that poultry lack resident phagocytes in the respiratory system to defend against invading infectious agents.^{82,114–116} Considering the importance of macrophage mediation of heterophil chemotaxis and activation, it is theorized that defense of the respiratory tract in poultry may be more susceptible to respiratory infections.⁷⁵ Alveolar macrophages can easily be harvested by pulmonary lavage in mammals, but this technique is not successful in birds.^{111,115–122}

Avian air sacs

Avian air sacs are thin and transparent membranous ventilating structures connected to the primary and secondary bronchi, and comprise most of the volume of the respiratory system.^{77,85,123,124} The two types of connections are direct when air sacs are coupled to the lungs by the primary bronchus and secondary bronchi, while indirect ones are formed entirely by the parabronchi. The caudal thoracic and abdominal air sacs, often collectively referred to as the caudal air sacs, arise from a second and third set of secondary (lateroventral and mediodorsal) bronchi and from continuation of the intrapulmonary primary bronchus.

The predominant sites of respiratory infection are the caudal air sacs, while cranial air sacs are the most devoid of infection, compared with other parts of the respiratory system. Part of the reason for this may be that the air sacs extend well beyond the limits of the coelomic cavity, with many bones being extensively pneumatized.^{125,126} A key explanation regarding the susceptibility of caudal air sacs to infection lies in the gas flow pathway and the mechanisms present in the parabronchi for particle removal. Due to the arrangement of the parabronchi, avian lungs have a flow-through system of breathing, unlike the tidally ventilated mammalian respiratory system. Fresh inhaled air is mixed with residual stale air in the respiratory airways of the mammalian system, while inspired air completely bypasses the cranially lying openings of the medioventral secondary bronchi in avian systems, a process known as inspiratory aerodynamic valving.^{66,73,80} During the inspiratory phase, as

well as in the expiratory phase, air flows into the mediodorsal and lateroventral secondary bronchi, and this flow pattern results in continuous ventilation of the parabronchial lung in a caudocranial direction.^{67,69–71,73,80} Due to this unidirectional flow, inhaled aerosol particles that could not be eliminated by several independent mechanisms, including aerodynamic filtration, mucociliary clearance, and phagocytosis in the upper respiratory tract end up in the caudal air sacs.⁸⁶ These air sacs, because of their fragility, are very difficult to work with, so little is known about immunity within their structures. Granulocytes make up the majority of immune cells that can be detected in the air sacs, followed by macrophages, while lymphocytes are relatively rare.¹²⁴ As in the lung parenchyma, mononuclear phagocytes have been detected in the connective tissue of the air sacs.¹¹² Overall, the air sacs are deemed very vulnerable because of their location, their thin walls, and their low numbers of resident immune cells. All things considered so far, none of the anatomic, physiologic, or immunologic properties of the avian respiratory system give the impression of offering a major challenge to highly adapted *Salmonella* or any other pathogen invading the respiratory system. Taking into account the predisposing conditions in poultry production leading to bioaerosols of *Salmonella* and the inherent incongruity of the avian immune system, defenses may become overwhelmed and the avian respiratory system is likely to be susceptible to *Salmonella* infection via the respiratory route.

Proposed *Salmonella* infection process via the avian respiratory route

The perceived susceptibility of poultry to respiratory disease is proposed to stem from various human interventions, including extreme genetic manipulation and intensive management for maximum productivity.^{78,85} Since the domestication of the wild

jungle fowl (*Gallus gallus*) in Southeast Asia, the domestic fowl has been selected for efficient weight gain and egg production through intense genetic breeding. Better husbandry and management may be partly responsible for the present performance of broilers, which reach a body mass of 2,500 g in less than 40 days when compared with the late 1940s when broilers took approximately 90 days to reach a slaughter body mass of 1,800 g.¹²⁷ Most of this progress is said to begin with an increased growth rate during the first 2 weeks post hatch. During such “directed, rather enforced, evolutionary selection” for maximal productivity, structural-functional disequilibrium is bound to occur and has been evident.^{70,75}

The remarkable breeding progress in domestic birds is now sometimes associated with a mortality of up to 10% per flock because of various metabolic diseases. Depending on the etiological agent and specific defects in host defenses, a respiratory infection can be overwhelming and have severe consequences. In recent studies published by our laboratory, we have proposed that *Salmonella*, an established enteropathogen, can infect poultry via the respiratory route.¹²⁸ We evaluated this hypothesis using intratracheal administration of *S. Enteritidis* followed by enumeration of recovery of colony forming units (cfu) from the ceca-cecal tonsils and from the liver and spleen. *S. Enteritidis* challenged intratracheal colonized caeca to a similar or greater extent than oral administration at identical challenge levels. Chickens cultured for cfu enumeration from intratracheal-challenged chicks at same dose as orally challenged, resulted in an increase of 1.5 log higher *S. Enteritidis* from ceca-cecal tonsils, and a much lower intratracheal dose of *S. Enteritidis* could colonize the caeca to the same extent as a higher oral challenge (Table 1). This trend of increased cecal colonization due to intratracheal challenge was observed in week-old birds, which are widely considered to be more difficult to infect via the oral route. Incidence data for the liver and spleen showed 33%

Table 1 Evaluation of intratracheal infection of chickens with *Salmonella* Enteritidis^{*,**}

Group	Dose cfu of SE/chick	Route of challenge	Log ₁₀ cfu of SE/g of ceca content	Ceca-cecal tonsil	Liver and spleen	Trachea
1	1.5×10 ⁴	OR	1.89±0.38 ^c	5/12 (41.66%) ^b	0/12 (0%) ^d	1/12 (8.33%) ^d
2		IT	1.13±0.40 ^c	8/12 (66.66%) ^b	6/12 (50%) ^{b,c}	8/12 (66.66%) ^{b,c}
3	2×10 ⁶	OR	1.86±0.40 ^c	8/12 (66.66%) ^b	2/12 (16.66%) ^{c,d}	3/12 (25%) ^d
4		IT	3.20±0.17 ^b	12/12 (100%) ^a	10/12 (83.33%) ^{a,b}	11/12 (91.66%) ^{a,b}
5	1×10 ⁸	OR	5.99±0.51 ^a	11/11 (100%) ^a	1/11 (9.09%) ^d	5/11 (45.45%) ^{c,d}
6		IT	5.11±0.47 ^a	12/12 (100%) ^a	11/12 (91.66%) ^a	12/12 (100%) ^a

Notes: *Chicks were challenged on day 7 with SE intratracheally or orally at concentrations of 1.5×10⁴, 2×10⁶, or 1×10⁸ cfu per chick, and 24 hours post challenge, the ceca-cecal tonsils were cultured to enumerate Log₁₀ SE/g of ceca content; the data are expressed as the mean ± standard error of mean. Ceca-cecal tonsils, trachea, liver, and spleen enrichment data are expressed as positive/total chickens for each tissue sampled (%). **Adapted from Kallapura G, Morgan MJ, Pumphord NR, et al. Evaluation of the respiratory route as a viable portal of entry for *Salmonella* in poultry via intratracheal challenge of *Salmonella* Enteritidis and *Salmonella* Typhimurium. *Poult Sci.* 2014;93:340–346.¹²⁸ <http://creativecommons.org/licenses/by/3.0/legalcode>; ^{a-d}The significant difference between groups is represented by different superscripts (P<0.05), n =12/group. **Abbreviations:** cfu, colony-forming units; SE, *Salmonella* Enteritidis; OR, orally; IT, intratracheally.

of liver and spleen samples to be positive for *S. Enteritidis* administered intratracheally (10^6 cfu/chick), as compared with 0% when administered orally (Table 1). These data suggest that the respiratory tract may be a largely overlooked portal of entry for *Salmonella* infections in chickens.

Current knowledge of *Salmonella* bioaerosol generation, its fate, transport, and survivability, as a means to assess the transport of bioaerosols and subsequent risk of exposure and infection of poultry was discussed previously.^{3,54,56} Further, analyses of poultry anatomy, physiology, and immunology have enabled an understanding of the inherent possibilities of *Salmonella* infection via the respiratory route. At this point, a meaningful understanding of how, where, and when the respiratory defenses are breached and the process of *Salmonella* infection and subsequent dissemination is essential, and is addressed in the following section.

Salmonella survival in the upper respiratory tract

Salmonella transiting through the respiratory tract does not face any of the hurdles faced when transiting through the gastrointestinal tract. Earlier we discussed the inefficacy of mucociliary clearance in the upper respiratory tract and the airway bronchial system, at least under poultry-rearing conditions, the consequences of which are multiple. *Salmonella* in bioaerosols can theoretically reach the parabronchial and caudal air sac regions, which are potentially the most vulnerable areas for infection. Due to the undeveloped immune lymphoid tissue in neonatal commercial poultry, and the impaired mucociliary clearance, the dose and age at which several *Salmonella* species infect birds has been found to be much lower than that required for oral infection.^{76,77,100,128}

Bacterial adhesion and invasion

Adhesion of *Salmonella* to host epithelial cells in the gastrointestinal tract is the crucial step in infection and enables bacteria to colonize the host intestine, and this step is the result of coordination, in time and space, of expression of many virulence genes. As an enteric pathogen, the most formidable barrier that *Salmonella* has to overcome is the epithelial lining of the intestine. Several epithelial cell types are shown to be invaded by *Salmonella*, and Peyer's patches represent the main portal of entry in early *Salmonella* infection.¹²⁹ The process of invasion and subsequent infection is widely accepted to be pathogen-driven, and host cells have a minor or no role to play, whereas in the respiratory system, age-related developmental differences in the nasal-associated lymphoid tissue and BALT structures may have functional

consequences with respect to the pathogenesis of *Salmonella*. The disrupted epithelial barrier and previously described endocytosis could be a relatively easy portal of entry for *Salmonella*.⁸⁰ Thinning of the epithelium, reduced numbers of ciliated cells, and the absence of mucus-producing goblet cells facilitating a relatively open and localized interaction for *Salmonella*, which is known to infect its host through similar structures. In the parabronchial region, the scarcity of respiratory immune cells is clearly a potential advantage to *Salmonella* and any pathogen invading the respiratory system. Infiltration and subsequent phagocytosis of pathogens, to an extent greater than seen in mammals, might essentially be of advantage to *Salmonella*, which already has an established intracellular survival mechanism.^{14–16} It is interesting to note that a rapid change in virulence gene expression occurs during transition from the intestinal lumen into tissue that promotes systemic dissemination of *Salmonella* under enteric conditions.¹³⁰ These rapid changes are considered to be due to various stimuli in the intestinal tract, such as bile and quorum sensing molecules. Because of these findings, one might suggest that transit of *Salmonella* through the respiratory airway, which essentially lacks these signals, might not result in expression of virulence genes, and any infection may be caused in spite of the host-driven mechanisms described above. Nevertheless, these suggestions require methodical evaluation and experimental evidence.

We recently conducted a series of field trials in North and South America to evaluate the association between cecal and tracheal recovery of *Salmonella* in chickens and turkeys from commercial flocks. Environmental humidity and temperature were measured to evaluate their effects on the frequency of positive isolations from the ceca and trachea.¹³¹ In all trials, *Salmonella* was recovered from tracheal samples more often than from cecal samples (Table 2). Environmental conditions were not associated with the incidence of recovery of *Salmonella*. These observations suggest that tracheal contamination can be a good indicator of *Salmonella* infection under commercial poultry conditions, and that tracheal samples can be used as a complementary tissue to the ceca and liver/spleen to increase recovery of *Salmonella* in infected poultry.

Intracellular lifestyle and infection

Salmonella must overcome the defense mechanisms of the enteric immune system through development of an intracellular lifestyle,¹³² which may also be true for infection through the respiratory route. Macrophages were seen in connective tissue below the parabronchial epithelium on the floor of the atria^{111–113} and in the interatrial septa,⁸⁰ indicating that

Table 2 Evaluation of recovery of *Salmonella* in trachea, ceca, and liver/spleen in commercial chickens and turkeys*

Field trial and location	Bird type	Average temperature (°C)		Average % humidity	Salmonella recovery/total (%)		
		Minimum	Maximum		Trachea	Ceca	Liver and spleen
Trial 1 Bucaramanga, Colombia	Broiler	18.9	25.3	82	96/1,061 (9.0%)	ND	ND
Trial 2 Buenos Aires, Argentina	Broiler	21	28	63	8/20 (40%)	0/20 (0%)	2/20 (10%)
Trial 3 Guanajuato, Mexico	Broiler	18	28	65	28/100 (28%)	10/100 (10%)	ND
Trial 4 Arkansas, USA	Turkey	-2.9	4.9	67	34/100 (34%)	17/100 (17%)	ND
Trial 5 Arkansas, USA	Broiler	-2.7	7.7	69	3/150 (2%)	27/150 (18%)	11/150 (7.3%)

Note: *Adapted from Kallapura G, Botero A, Layton S, et al. Evaluation of recovery of *Salmonella* from trachea and ceca in commercial poultry. *J Appl Poult Res.* 2014;23:132-136.¹³¹

Abbreviation: ND, not determined.

phagocytic cells are strategically located at the start of the gas exchange area to clear the air of inhaled particles.⁸⁵ Further, a study by Stearns et al showed that aerosolized inert iron particles with a diameter of 0.18 μm were phagocytosed in the gas exchange area and endocytosed by epithelial cells lining the atria and the proximal parts of the infundibula, resulting in transport to tissue macrophages underneath the atrial epithelium.¹¹⁴ This suggests that, if *Salmonella* invasion occurs, these pathogens surely must adapt to an intracellular lifestyle, although this has not been described specifically.

Taking into consideration that *Salmonella* would not colonize respiratory tissue, it is necessary to recognize its subsequent fate, which unfortunately is unknown and needs elucidation. Under the conditions of the gastrointestinal tract, invasion by *Salmonella* causes a heavy influx of heterophils, a stereotypical yet effective response. Ironically, growth of *Salmonella* is aided by the inflammatory responses aimed at controlling luminal bacteria. Most of the intestinal microbiota is comprised of strictly anaerobic bacteria belonging to the Bacteroidetes and Clostridiales classes that rely on fermentation of amino acids and complex polysaccharides. One fermentation end product generated by the microbiota is hydrogen sulfide (H_2S), a cytotoxic compound that is converted to thiosulfate ($\text{S}_2\text{O}_3^{2-}$) by the colonic epithelium. During inflammation, neutrophils that transmigrate into the intestinal lumen release reactive oxygen species in an attempt to kill bacteria. A byproduct of releasing reactive oxygen species is the oxidation of thiosulfate to tetrathionate ($\text{S}_4\text{O}_6^{2-}$). In contrast with fermenting microbiota, *S. Typhimurium* can use tetrathionate as a terminal electron acceptor to support its growth by anaerobic respiration, which is more efficient for energy production than fermentation. Also, anaerobic tetrathionate respiration facilitates growth on poorly fermentable carbon sources. All these examples imply that the respiratory environment is totally different from that of the gastrointestinal tract, and hence intrabronchial or intraparenchymal persistence is highly unlikely.¹³³

Another mode via which *Salmonella* could potentially infect and spread systemically is by entering into blood directly and causing bacteremia. This mode is quite conceivable, considering the disrupted epithelial barrier of developing BALT structures, described endocytosis by epithelial cells. Conversely, continued survival of these bacteria entering the blood is thought to be unlikely. Recently, we evaluated and compared the infectivity of *S. Senftenberg* following oral gavage, intratracheal, or intravenous challenge in 7-day-old chicks with *S. Senftenberg*. This study was quite interesting in that it yielded new information regarding the ability of *Salmonella* to infect and colonize chickens. The ability of *S. Senftenberg*, a relatively noninvasive serotype, to rapidly colonize the ceca after intratracheal exposure was unexpected. It was remarkable to observe in those studies that intratracheal delivery of *S. Senftenberg* was the only route able to colonize the ceca of the chickens when compared with oral gavage or intravenous challenge in a dose-response fashion, suggesting that *S. Senftenberg* entering the blood is likely to be cleared and will not be able to colonize the ceca to the same extent as that seen with intratracheal challenge.¹³⁴ Reports suggest that the blood-borne bacterial load is rapidly reduced from the circulation by trapping of bacteria in tissue macrophages, and the rate of reduction is significantly enhanced in the presence of opsonic factors in the blood.¹³⁵ Further, passively immunized turkey poults, when challenged with *E. coli*, showed a rapid reduction in bacterial load by approximately $5 \log_{10}$ within 10 minutes post-intravenous challenge, in contrast with nonimmunized poults.¹³⁶ Erythrocytes collected from lavaged chicken lungs readily phagocytosed 1.5 μm diameter polystyrene particles, and 22% of chicken erythrocyte cells engulfed 1–6 particles after 5 hours of coincubation, compared with rat erythrocytes, which could not do the same.⁷⁵ Although little is known about such interactions, the phagocytic activity of these chicken erythrocytes is attributed to nucleated properties. The role, if any, of these inherent phagocytic properties of erythrocytes in the defense of the pulmonary

system and body in general requires further elucidation. As described above, it is currently thought that avian erythrocytes may clear particles from the lungs and deliver them to organs like the liver and spleen, where they may be destroyed or sequestered. Information regarding mucosal effector sites in avian lungs is also limited. The presence of intraepithelial and interstitial lymphocytes and isolation and functional characterization of CD8+ effector T-cells expressing interferon gamma from lung tissue of influenza virus-infected birds has been described.^{137,138}

Unfortunately, these studies did not specify whether T-cells were obtained from the mucosal inducer or effector sides of lung tissue. Similarly, a recent study in an infectious bronchitis virus model in chickens applied microarray analysis to investigate the host response in the lungs but did not discriminate between BALT structures and the interstitial immune system as the source of RNA.¹³⁹ With the chicken genome sequence now available,¹⁴⁰ future work on host-pathogen interaction will increasingly apply this approach of more targeted gene expression analysis to lung tissue.¹⁴¹ Such work should take the distinct anatomic structures of the lung-associated immune system into account, which may very well show strikingly different phenotypic properties and functional activities.

Systemic dissemination and cecal colonization

Theoretically, *Salmonella* thriving and replicating in respiratory macrophages could be transported from the lungs to secondary lymphoid organs, such as the spleen, and spread systemically to the liver and potentially other organs. Systemic dissemination of *Salmonella*, after departing the respiratory organ, might well follow the same mechanisms observed for the fecal–oral route. Similar to the established process of oral infection, macrophages are major effector cells eliciting innate immunity, and also transport *Salmonella* to host tissues,¹⁴² primarily to organs of the reticuloendothelial system, such as the liver and spleen, along with kidney, blood, and reproductive tract.¹³¹ Macrophages provide a protected site for intracellular bacterial replication and further act as a vehicle of dissemination. Colonization of the gall bladder by *Salmonella* is of special importance, since this organ is recognized to be involved in the chronic carrier state. Eventually, *Salmonella* could end up in the intestinal tract via secretion of bile from the gall bladder, and begin its intestinal life cycle for further fecal dissemination.

Even though this may be the most conceivable mode of systemic spread of *Salmonella*, there could be other channels

through which *Salmonella* could spread systemically. A growing body of evidence shows that the mucosa behaves as a systemwide network, suggesting that it should be seen from a holistic viewpoint as a global organ. Recent research evaluating the existence of communication between various mucosal sites indicates hitherto unappreciated levels of cross-talk between mucosal compartments, which influence mucosal immunity.¹⁴³ Any substance that reduces ciliary motility or disrupts the ciliated epithelium could be expected to adversely affect the resistance of birds to microorganisms that normally enter via the respiratory system. Interestingly, an earlier study by Weinack et al, involving eight separate trials, considered this assumption and evaluated any effects of respiratory tract impairment on the gut microflora and gut pathogens, and reached an astounding conclusion. Chickens exposed to aerosolized *Mycoplasma gallisepticum* and infectious bronchitis virus started shedding higher doses of *S. Typhimurium* and *E. coli* more quickly than via oral challenge.⁹⁰ The authors linked this observation to a decrease in intestinal microflora due to respiratory stress. However, they certainly suggested that the possibility of infection via aerosol inoculation for these organisms should not be ruled out.

Collectively, such studies indicate that the mucosal immune system is essentially one large interconnected network and that the individual components are very efficient at sharing information distally. When we extrapolate this concept in the context of the pathogenesis of *Salmonella* and hypothesize those macrophages, as a critical part of the immune system with regard to the migrating capabilities of *Salmonella*, it is possible that these macrophages can actually carry ingested *Salmonella* from the respiratory system to the cecal tissue. An experimental understanding of this sort of communication between mucosal sites is fundamental to the next phase of characterization of the disease, and perhaps vaccine development. One way in which we might provide evidence for such cross-talk is through use of molecular markers. Alveolar macrophages in mammalian systems are the most abundant antigen-presenting cells in the lungs, and play a critical role in regulating pulmonary immune responses to inhaled pathogens and allergens. Striking phenotypic differences between alveolar and peritoneal macrophages have been described, particularly with regard to CD11c expression.¹⁴⁴ Identification of similar markers in the avian system would prove beneficial and might be employed to demonstrate the migration of respiratory macrophages from airway regions to cecal tissue.

Conclusion

The present review has evaluated the hypothesis that transmission by the fecal–respiratory route may be a viable portal of entry for *Salmonella* in poultry. First, we have updated the current knowledge regarding *Salmonella* bioaerosol generation, and its transport and fate at various stages of commercial poultry production. Emphasis is placed on survivability of *Salmonella* in these bioaerosols as a means for assessing the transport and subsequent risk of exposure and infection of poultry. Additionally, the main anatomic structure, physiologic functioning, and immunologic defense of the avian respiratory system have been described to enable an understanding of the potential entry points inherent in each component that could potentially lead to infection and subsequent systemic infection of poultry by *Salmonella*. In theory, it is conceivable that, under field conditions, airborne *Salmonella* may be able to infect poultry by this route. Further clarification of the involvement of the respiratory tract in transmission of *Salmonella* under field conditions may be of critical importance in the development of interventions to reduce transmission of these pathogens in poultry. Understanding the communication between mucosal sites is fundamental to the next phase of disease characterization, and perhaps vaccine development. Further experiments using aerosol exposure are intriguing, because these could lead to insights important when developing live *Salmonella* vaccines, in addition to control of *Salmonella* on the farm.

Disclosure

The authors report no conflicts of interest in this work.

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