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Study of nebulization delivery of aerosolized fluorescent microspheres to the avian respiratory tract

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SUMMARY

This study investigated the delivery of an aerosol of monodisperse microspheres to the respiratory tract of birds following aerosol exposure. Adult domestic pigeons (*Columbia livia domestica*; n=5 birds per timed treatment) were exposed to an aerosol of fluorescent 1.0 μm diameter carboxylate microspheres for 0.5, 1, 2 or 4 hr. During the aerosolization period, the birds were free standing in a plexiglass treatment chamber, and the aerosol was delivered using a commercial nebulizer. Immediately following aerosol exposure the birds were euthanized and the carcasses were intravenously infused with a modified paraformaldehyde/gluteraldehyde fixative. Evaluation of microsphere distribution was performed using a stereoscopic microscope with an epifluorescent module. The results from this study revealed that the amount of aerosolized particles delivered using a commercial nebulizer was proportional to exposure periods. Aerosol exposure periods of 0.5 h or 1 h did not result in a readily observable distribution of 1.0 μm fluorescent microspheres to the cranial thoracic, caudal thoracic, or abdominal air sac membranes. This was partly attributed to the relatively low concentration of the individual monodisperse microspheres in the aerosolized suspension. The 2 and 4 hr exposure periods resulted in readily observable deposition of the 1.0 μm fluorescent microspheres in the cranial thoracic, caudal thoracic, or abdominal air sac membranes with the 4 hr exposure period resulting in the greatest number of particles on the membrane surfaces. For each of the exposure periods there was individual animal variation regarding the distribution and relative number of spheres deposited. This study demonstrates the widespread deposition of particles that had an aerodynamic equivalent diameter of approximately 1 μm and provides a better understanding of particle deposition efficiency within the respiratory system following aerosol exposure in birds.

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Index Terms

nebulization; fluorescent microsphere; inhalation deposition; particle distribution; bird

INTRODUCTION

The avian respiratory system is the most efficient in the animal kingdom for oxygen exchange (15). However, there are three fundamental differences between the respiratory systems of mammals and that of birds that seem to predispose birds to inhaled pathogens (8). First, the conducting airway system of birds through which particles are cleared is composed of only three generations of branching airways. The major sites where pathogens might deposit (i.e., the air sac membranes) are consequently separated by substantial distances from the mucociliary escalator. Second, the surface for gas exchange (i.e., the lung parenchyma) is not a bellows system, as it is in mammals. Instead, birds have extensive series of thin-walled sacs (air sacs) that extend throughout the coelomic cavity and even into their bones. As a consequence, almost every component of the avian body has some communication or contact with the respiratory tract and, in some cases, is separated from sites of pathogen deposition by only a very thin layer of cells. The third unique factor is the unidirectional flow of air through the avian respiratory system in contrast to bi-directional airflow in the mammalian lung. The effect that inhaled particles and pathogens have on the airways of birds depends on numerous factors, such as the concentration, particle size, airflow velocity and relative pathogenicity of the inhaled agent.

Compared to mammals, the number of studies evaluating treatment regimes for respiratory disease in birds is minimal. Treatment often includes direct delivery of an agent to the respiratory mucosa (26). This is especially important in birds due to the avascular nature of their air sac membranes. In human medicine, drugs are delivered directly to the lungs either through the use of pressurized metered dose inhalers (pMDIs), direct inhalation using a dry powder inhaler (DPI), or inhalation of aerosols of aqueous solutions or suspensions generated with a nebulizer(26). The use of pMDI and DPI devices require the cooperation of the subject and are inappropriate in animals due to the inability to control their inhalation. Therefore, nebulization is the most common technique for delivery of aerosols in animals(2) In veterinary medicine, aerosol delivery has been optimized for several laboratory animal models (9) and has also been described for various species of companion animals(7, 12, 13). For years, nebulization techniques for aqueous solutions and suspensions have been used in companion avian clinical practice to deliver prophylactic therapeutics and/or medications such as antibiotics (17) and antifungals. However, little information regarding the anatomic distribution of the drug or dose delivered is available(3, 14, 29).

Studies examining particle distribution in the avian respiratory tract have been reported using a variety of methods and particle types to determine *in vivo* particle deposition patterns. Some of the hallmark studies in birds that detail aerosol deposition in the avian respiratory tract utilized radioactively labeled latex particles, radioactively labeled submicrometric (sulfur colloid) particles, iron oxide and fluorescent microspheres (10, 16, 23, 25). Computerized tomography has also been attempted in chickens (28).

An important therapeutic approach for treating respiratory diseases in avian species is inhalation exposure to aerosolized drug solutions or suspensions using nebulizers. However, the aerodynamic size distribution of the aerosol particles or droplets must be in a size range that allows penetration through the head and upper airways to efficiently deposit in the lungs and air sacs. Medical nebulizers designed for transoral inhalation by people primarily provide aerosols with mass median aerodynamic diameters of about 5–10 μm (22), which provides efficient deposition in both the human bronchial airways and gas exchange region of the lung(21). In nasal-breathing small animals this aerosol size distribution tends to deposit particles primarily in the head rather than in the lungs(20). In unrestrained birds in a treatment chamber, aerosols delivered to the enclosure along with an equal volume of dry diluting air will shrink in size distribution because of water evaporation from the droplets. Using a nebulizer yielding an aerosol with mass median aerodynamic diameter of about 2 μm, it is reasonable to anticipate that the aerosol mass median diameter can be reduced to about 1 μm. This study was designed to verify that particles of about 1 μm in aerodynamic equivalent diameter can be readily inhaled and deposited in both the lungs and the air sacs of pigeons. The particles used were monodisperse and labeled with a readily visualized fluorescent dye.

Although the distribution of various particle types have been studied in birds, the effectiveness of nebulization regimes commonly used in avian practice has not been investigated. The objective of this study was to utilize nebulization as commonly practiced in avian clinical settings and assess the effect of inhalation exposure period on the delivery and distribution of the aerosolized fluorescent microspheres to the avian respiratory tract. Birds were allowed to stand and move freely in a treatment chamber, and the aerosol was delivered for four exposure periods (0.5, 1, 2 or 4 hr) using a commercial nebulizer. The exposure periods were selected based on the range of exposure times used in clinical practice. Deposition and distribution of the fluorescent microspheres was evaluated using stereoscopic fluorescent microscopy.

MATERIALS AND METHODS

Preparation of microspheres

Fluoresbrite™ carboxylate microspheres (1.0 μm diameter, physical density 1.05 g cm^{-3}) were obtained from Polysciences (Warrington, PA) in a 2.5% solids mass aqueous suspension with 4.55×10^{10} microspheres per milliliter of water. Microspheres were impregnated with yellow-green fluorescent dye with a maximal excitation peak of 458 nm and a maximal emission peak of 540 nm.

To reduce the loss of microspheres from the respiratory tract during tissue fixation and processing, microspheres were thinly coated with albumin, which becomes cross linked with other proteins of the respiratory tract by gluteraldehyde and paraformaldehyde fixation(11). A 1 ml aqueous suspension of microspheres was vortexed with 19 ml of distilled water containing 0.125% albumin for one minute. The suspension was allowed to settle and then vortexed a second time before centrifugation at 8,000 “g” for 10 minutes. The solution was decanted, and the microspheres were re-suspended in distilled water containing 0.001% Zwittergent 3-08 surfactant solution (Calbiochem, San Diego, CA) to help the microspheres

repel each other. The microsphere suspension was vortexed, centrifuged, and decanted to further remove residual albumin. The microspheres were resuspended a third time in distilled water with 0.001% Zwittergent 3-08 and sonicated to help break apart aggregates. Although this produced a suspension of mostly single microspheres, a few aggregates remained. Additional washing and sonication between centrifugations helped to further reduce microsphere aggregates. Prior to aerosolization, all microsphere suspensions were evaluated by microscopy to ensure that aggregation was minimal; the goal was to maximize the number of aerosolized water droplets containing only one microsphere.

The final dilution of the microsphere suspension for aerosolization was calculated according to Raabe (19) based on the characteristics of the Acorn II nebulizer (Marquest Medical Products, Inc., Englewood, CO) operated with compressed air at 30 psig(18). The formula used for finding the optimal concentration is

$$y = \frac{F(\mathrm{VMD})^3 e^{4.5\ln^2\sigma_g}\left[1 - 0.5e^{\ln^2\sigma_g}\right]}{(1-R)\,D^3}$$

where:

e = the base of the natural system of logarithms 2.71828…

ln = the natural logarithm

y = the suspension dilution ratio, new volume/old volume = 52

F = the fraction by volume of particles in the original stock solution = 0.0238

VMD = volume median diameter or droplet mass median diameter = 5.7 μm

R = the singlet ratio = 0.9

D = the physical diameter of monodisperse spheres = 1 μm

σ_g = the geometric standard deviation of the aerosolized droplets = 1.7

In order to generate a monodisperse aerosol, only one particle can be in a droplet. To yield 90% single particles after nebulization, the original concentrated suspension was diluted by a ratio of 1:52. Given this dilution, most of the water droplets generated through the aerosolization process did not contain microspheres. The diluted suspension was placed in the reservoir of an Acorn II nebulizer. The volumes added to the reservoir varied from 6–10 ml depending on the exposure times. The nebulizer was turned off while the reservoir was being refilled.

Animal exposures

Adult domestic pigeons (*Columbia livia domestica;* n=20) deemed healthy based on physical examination were used in the study. All experimental procedures were approved by the University of California, Davis, Animal Care and Use Committee. Non-anesthetized animals (n=5 birds per exposure period) were placed free-standing in a plexiglass treatment chamber. In order to mimic a typical clinical setting, the birds were not acclimatized to the treatment chamber prior to the study. The animals' exposure periods were 0.5, 1, 2 or 4 hr.

Air at 30 psig was supplied to the Acorn II nebulizer to produce a flow rate of 5 l/min. The aerosol stream was diluted by adding dry filtered air at a rate of 6 l/min using a commercially available in-line mixer (In-Tox Products, Albuquerque, NM). The aerosol stream continued through a krypton-85 discharging device (24) that was heated to maintain the existing stream at 45°C. In this device, water was completely evaporated from the nebulized microspheres and the electrostatic charge of the aerosol was reduced to the Boltzmann equilibrium. The calculated concentration of the resultant aerosol of monodisperse spheres was 1.4×10^7 particles per liter of air.

Assuming a typical inhalation minute volume of about 130 ml for each pigeon (4) and 20% particle respiratory tract deposition in small animals (20), the expected inhalation deposition in the pigeon respiratory system can be estimated to be about 360,000 microspheres per minute of exposure. For a 1 hr exposure, the calculated average deposition per square millimeter of respiratory tract would be only about 20 microspheres assuming the total respiratory tract surface is about one square meter.

Preservation of the avian respiratory tract

Immediately following aerosol exposure, the birds were anesthetized with ketamine hydrochloride (Vedco, Inc., St. Joseph, MO; 50 mg/kg IM) and xylazine (Vedco, Inc.; 2 mg/kg IM), and the basilic veins of the birds were catheterized bilaterally with 26 gauge catheters. To delay blood clotting, the animals received 2,000 IU of heparin intravenously. The birds were humanely euthanized with an intravenous overdose of pentobarbital. To achieve vascular clearing, immediately following euthanasia, approximately 200–300 ml of 0.9% sodium chloride with 10,000 I.U. of heparin per liter was intravenously infused using a peristaltic perfusion pump (Cole-Parmer, Vernon Hills, IL) until the fluid exiting the opposing catheterized basilic vein was clear. The respiratory tracts of the birds were then fixed *in situ* by intravascular perfusion with 200–300 ml modified paraformaldehyde/ gluteraldehyde fixative (2% paraformaldehyde, 2% gluteraldehyde in phosphate buffered saline, 340 mOsm, pH=7.2) using the peristaltic pump. Immediately following perfusion, the catheters were occluded with an infusion plug. Subsequently, the birds were stored at 4° C for at least 48 hours. This extended storage period optimized the effusion of fixative from coelomic organs to thoracic and abdominal air sac membranes, thereby increasing their stability and integrity. There was minimal effusion of the fixative into the clavicular or the cervical air sacs, therefore, these air sacs were not sampled. After the carcasses were preserved, the coelomic cavity was emptied of the major organs (excluding the respiratory components).

Microscopic Analysis

In situ examination of the respiratory tract (trachea, tracheal bifurcation, cranial and caudal thoracic and abdominal air sacs) for the presence of microspheres was performed using a Leica MZ12 stereoscopic microscope (Leica, Heerbrugg, Switzerland) with an epifluorescent module. Each anatomic region of interest was identified using 6.3× magnification with a further enhanced magnification factor of 30 to 50× to count particles and assess particle distribution. Deposition was graded with the following scores: −−−−: 0 particles; +: 1–5 particles; ++: estimated 5–20 particles; +++: estimated 21–100 particles;

and ++++: estimated >100 particles. In order to clearly examine the inner surfaces of the air sacs, the mesothelium was incised. After microscopic evaluation of the air sac membrane surfaces, gross dissections were performed so that the ostia associated with the air sac membrane could be examined. After the air sac membranes were examined, gross dissection of the trachea and pulmonary parenchyma was performed. The trachea and primary and secondary bronchi were cut in half along their long axes using a razor blade. Dissections of the major airways followed air paths out to the level of the ostia. These airway passages were examined with the same stereoscopic microscope and magnification as previously above.

RESULTS

During the aerosol exposure period, all of the birds remained standing and did not exhibit any ill effects. After euthanasia and gross dissection, the appearance of the respiratory tract tissue *in situ* appeared normal for all birds when examined using the bright-field mode of the stereoscopic microscope. Relative deposited quantities of fluorescent microspheres and their distribution varied with the duration of aerosol exposure and between individual birds (Figures 1a and 1b and Table 1). For the 0.5 hr exposure period, the microspheres were found in very low numbers (<5) in various regions of the branching airways for 3/5 birds. Exposure for 1 hr resulted in observed distribution of the microspheres to the trachea, bronchi (primary and secondary), pulmonary parenchyma and the ostea of the cranial thoracic, caudal thoracic, and abdominal air sacs for 4 of the 5 birds exposed. Compared to the 0.5 hr exposure period the numbers of microspheres were subjectively found to be slightly greater for the 1.0 hr exposure period. For birds that were exposed for 2.0 hr, the microspheres were heavily concentrated at the primary and secondary bronchi and the thoracic and abdominal air sacs. After 2 hours, microspheres were more widely distributed to the thoracic and abdominal air sacs, and after 4 hours of exposure, larger numbers of microspheres were found throughout all respiratory tissues examined in 4 of 5 birds.

DISCUSSION

Aerosolized delivery of vaccines and antimicrobial treatments are frequently used in poultry and pet bird (5). However, an understanding of the distribution of aerosolized treatments to the avian respiratory tract is limited. We previously demonstrated that microspheres ranging in size from 1–3 μm were distributed throughout the respiratory system of anesthetized pigeons when an aerosol dose was delivered for 30 minutes using intermittent positive pressure ventilation (25). Since breathing patterns differ in unanesthetized animals, the goal of this study was to examine the distribution of microspheres in the respiratory tracts of pigeons that could move freely in a treatment chamber, which mimics therapy used in clinical avian medicine. Therefore, this study provided novel information that is more clinically relevant in demonstrating airborne particle delivery to the avian respiratory tract. We found duration of exposure treatment is a primary factor in both the distribution and the dose delivered to highly defined regions of the avian respiratory system. We also found with increasing time of exposure to aerosolized particles, the degree of particle deposition into the avian respiratory system with its unidirectional air flow is enhanced, until equilibrium is attained with approximately uniform particle deposition/translocation to each of the air sacs

of the respiratory system. The results from this study showed that the regional extent that the 1.0 μm fluorescent microspheres were distributed into the avian respiratory tract depended on the exposure time; exposure times of 0.5 to 1. 0 hr resulted in limited numbers of microspheres being found in the trachea, primary and secondary bronchi and the pulmonary parenchyma. Exposure times of 2.0 and 4.0 hr resulted in larger numbers of microspheres being found at the ostia on the air sac membranes. In general, it was not surprising that the degree of particle accumulation in the lungs was greater than the air sac membranes for all of the treatment periods, given the aerodynamics of a aerosolized particle. The main mechanism for a 1 μm inhaled particle that is about unit density to deposit in the airways and lungs is gravitational settling, and upon contact with a surface the particles are irreversibly adhered due to aqueous surface tension. Therefore, high deposition in the lungs would be expected since the distance to the nearest surface is small and the relative surface area compared to the air sac cavities is exponentially larger. In contrast, aerosolized particles passing through the air sacs would have more of a tendency to remain airborne and not deposit on membrane surfaces.

Drug delivery to the lower respiratory tract is critical to treating many respiratory infections in birds(8, 27). Several previous studies have reported limited success with aerosolized treatments given to birds for relatively short time periods. Beernaert *et al*. (1) found that exposing pigeons to aerosolized voriconazole for 15 minutes resulted in only low levels of the drug in lung and plasma and suggested this could be due to insufficient penetration of the respiratory system or low absorption of the drug from the respiratory system into the bloodstream. Corbanie *et al*. (6)found that distribution of microspheres to the lower respiratory tract (air sacs) of young chickens depended on microsphere size, but after 15 minutes of intermittent exposure, the percentage of microspheres reaching the air sacs was relatively low in all cases. However, with longer exposures some success has been reported with aerosol drug delivery. Brown and Butcher (3) successfully treated experimentally induced *Mycoplasma gallisepticum* infections in budgerigars with eight hours of exposure by nebulization.

The results of this study were consistent with the predicted low number of single particles (about 360,000) depositing in the whole airway surface, including lungs and air sacs. The findings from this study demonstrate that the Acorn II nebulizer and treatment chamber were effective in delivering 1 μm microspheres to all areas of the respiratory tract based on the limited 10% deposition that is typical for small animal inhalation deposition (20). Nebulization without evaporation of excess water typically yields droplet size distributions in the 5 to 10 μm median aerodynamic diameter range that has been shown to be ineffective in reaching the lungs in small nose-breathing animals (20). Considerable improvement in treatment efficacy would be expected if nebulized drugs utilized nebulizers having mass median aerodynamic diameters less than 3 μm and if the aerosol were allowed to mix with dry air prior to inhalation to achieve aerosol particles 1 μm or smaller in aerodynamic equivalent diameter. In the future, nebulizers developed for continuous nebulization therapy for humans should be evaluated for treating avian patients given that the mass mean aerodynamic diameter of the aerosol droplets was reported to be about 2.0 μm (21).

In conclusion, this study demonstrated that the number and extent of aerosolized particles delivered to the avian respiratory tract was dependent on the length of exposure periods. This study provides a laboratory method that can be used in future studies for evaluating different nebulizers and treatment regimes for aerosol delivery to birds. If the medical management of respiratory disease in birds could be maximized, relative treatment success might improve and the relative costs might even decrease. This could have important ramifications for avian medicine from an individual bird patient standpoint to a flock perspective.

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Abbreviations

pMDIs	pressurized metered dose inhalers
DPI	dry powder inhaler

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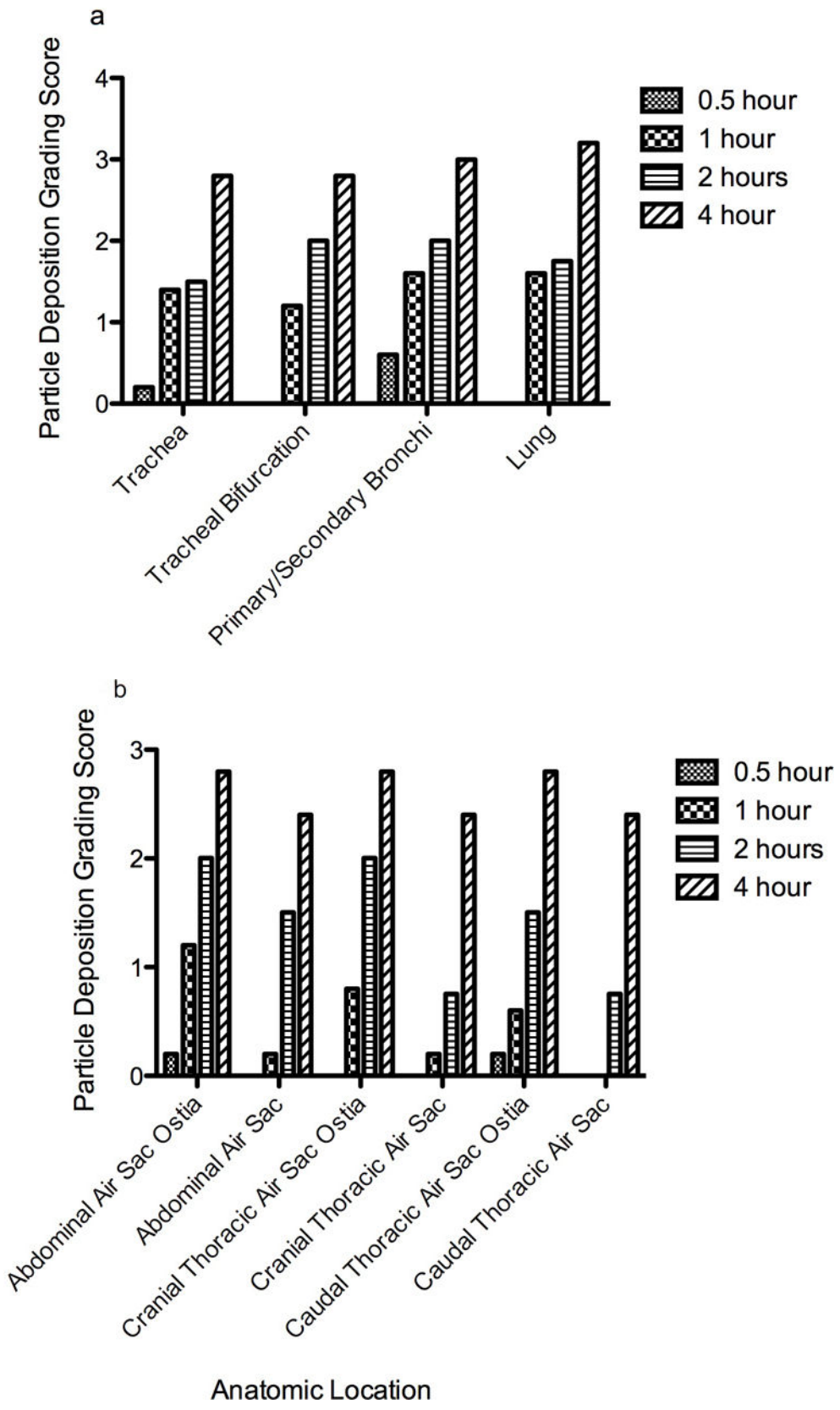

Figure 1.

Bar chart demonstrating the relative number of fluorescent 1.0 μm carboxylate microspheres detected in the trachea and pulmonary parenchyma (a) or air sacs (b) of pigeons' respiratory tracts after aerosol exposure for 0.5, 1, 2, or 4 hr.

Table 1

Characterization (distribution and relative concentration) of particles deposited in the avian respiratory tract during timed exposures.

	Bird	Trachea	Tracheal Bifurcation	Primary/Secondary Bronchi	Lung	Abdominal Air Sac Ostia	Abdominal Air Sac	Cranial Thoracic Air Sac Ostia	Cranial Thoracic Air Sac	Caudal Thoracic Air Sac Ostia	Caudal Thoracic Air Sac
0.5 hour	1	−−−−	−−−−	+	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
	2	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
	3	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
	4	−−−−	−−−−	+	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
	5	+	−−−−	+	−−−−	+	−−−−	−−−−	−−−−	+	−−−−
1 hour	6	++	++	+++	+++	++	−−−−	+	+	−−−−	−−−−
	7	++	++	++	++	++	+	+	−−−−	+	−−−−
	8	+	+	++	++	+	−−−−	+	−−−−	+	−−−−
	9	+	+	+	+	+	−−−−	+	−−−−	+	−−−−
	10	+	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
2 hours	11	+	+++	+	+	++	−−−−	++	−−−−	++	−−−−
	12	+	+	++	+	+	+	+	−−−−	+	−−−−
	13	+	+	++	++	++	++	++	+	++	+
	14	+++	+++	+++	+++	+++	+++	+++	++	++	++
4 hours	15	++++	++++	++++	++++	++++	+++	++++	+++	++++	+++
	16	++++	++++	++++	++++	++++	+++	++++	+++	++++	+++
	17	+	+	+	++	−−−−	−−−−	−−−−	−−−−	−−−−	−−−−
	18	+	+	++	++	++	++	++	++	++	++
	19	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++

Note: −−−−: 0 particles; +: 1–5 particles; ++: estimated 6–20 particles; +++: estimated 21–100 particles; ++++: estimated >100 particles