Structural and functional effects of Bordetella avium infection in the turkey respiratory tract

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STRUCTURAL AND FUNCTIONAL EFFECTS OF BORDETELLA AVIUM INFECTION IN THE TURKEY RESPIRATORY TRACT

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Structural and functional effects of *Bordetella avium* infection in the turkey respiratory tract

by

William George Van Alstine

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY Major: Veterinary Pathology

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GENERAL INTRODUCTION

The poultry industry in the United States accounts for approximately 11 billion dollars worth of poultry products each year and the turkey industry contributes 20 percent of the total product value.\(^1\) Large flocks, multiple ages of turkeys on a farm, intensified rearing practices, and concentrated production in limited geographic areas have contributed to increased incidence of multiple respiratory infections. The total loss from disease and management related problems costs the poultry industry approximately 2 billion dollars each year.\(^1\) Respiratory infections account for 50 percent of the losses due to mortality and morbidity.\(^1\)

Bacterial pathogens, such as *Bordetella avium* and *Escherichia coli*, are important causes of turkey respiratory disease. Turkey coryza, caused by *B avium*, is a highly contagious upper respiratory disease of turkeys and chickens. It has been reported in most major turkey producing regions of the United States.\(^2\) In the 1983 Iowa turkey health survey, turkey coryza and colibacillosis-coryza syndrome were estimated to be the most important causes of monetary loss from disease in Iowa market turkeys (W. J. Owings, Department of Poultry Science, Iowa State University, Ames, IA, 1983). Turkey coryza was classified as 1 of the top 10 priorities for turkey respiratory disease research in 1986.\(^1\)

Turkey coryza is characterized clinically by excessive
oculonasal discharge, sneezing, tracheal collapse, and decreased rate of weight gain.\textsuperscript{3-5} Although \textit{B. avium} is noninvasive, it persistently colonizes the ciliated epithelium of the nasal cavity and trachea leading to inflammation of the upper airways, loss of ciliated epithelial cells, and accumulation of nasal and tracheal mucus.\textsuperscript{3,6} Morphological and functional alterations caused by \textit{B. avium} infection in the turkey lung have not been described.

Mechanisms responsible for damage to the ciliated epithelium are not known, but a bacterial toxin has been incriminated in several studies.\textsuperscript{7,8} Heat-labile toxin, prepared from sonicated live \textit{B. avium}, is lethal for turkeys when injected parenterally, but the contribution of this toxin to the clinical signs and tracheal lesions of turkey coryza is unknown.\textsuperscript{9}

Turkey coryza can be a clinically mild disease with low mortality, but infected turkeys may be predisposed to other respiratory pathogens.\textsuperscript{4} Excessive mortality has been reported when \textit{B. avium}-infected turkeys are exposed to other respiratory pathogens.\textsuperscript{10-12} \textit{Escherichia coli}, the cause of avian colibacillosis, is the most common secondary bacterial pathogen isolated from \textit{B. avium}-infected turkeys.\textsuperscript{2} Vaccination of \textit{B. avium}-infected turkeys with live Newcastle disease virus vaccine can result in high death losses due to colibacillosis.\textsuperscript{12} Mechanisms by which \textit{B. avium} predisposes
turkeys to colibacillosis and other respiratory diseases are not known.

The objectives of this study were to i) evaluate the effects of *B. avium* infection on the pulmonary clearance of *E. coli* in turkeys, ii) determine if *B. avium* influences the adherence and colonization of *E. coli* in the turkey trachea, iii) describe the pulmonary lesions and changes in bronchus-associated lymphoid tissue in turkeys infected with *B. avium*, and iv) characterize the morphological and metabolic alterations caused by *B. avium* heat-labile toxin on turkey tracheal organ cultures.

This dissertation is presented in the alternate format and consists of four manuscripts submitted to refereed scientific journals. The format used is that of the American Journal of Veterinary Research. The review of the literature precedes the first manuscript. The first, second, and third manuscripts have been submitted for publication to the American Journal of Veterinary Research. The fourth manuscript has been accepted for publication in Avian Diseases. A general summary and discussion follow the last manuscript. A list of references appears at the end of each manuscript. Literature cited in the general introduction, literature review, and general summary and discussion appears at the end of the dissertation.
The Ph.D. candidate, William George Van Alstine, was the principal investigator for each study.
Turkey coryza - Turkey coryza, caused by *Bordetella avium*, is a highly contagious upper respiratory disease of turkeys characterized clinically by excessive oculonasal discharge, sneezing, coughing, dyspnea, tracheal collapse and decreased rate of weight gain.\(^3,4,11,13-16\)

Morbidity within *B avium*-infected flocks often reaches 100\%, but mortality usually remains low unless turkeys are concurrently infected with a second respiratory pathogen.\(^4,17\)

Chickens and quail can be infected with *B avium*, but clinical signs and lesions are far less severe than in turkeys.\(^18-23\)

Initial searches for the cause of this disease centered on isolating respiratory viruses. Respiratory adenoviruses were associated with the disease, but turkey coryza could not be consistently reproduced with these viruses.\(^24,25\)

In 1967, Filion et al. described 2 outbreaks of respiratory disease in Canadian turkeys with clinical signs similar to turkey coryza.\(^26\) An organism similar to *Bordetella bronchiseptica* was isolated from the affected turkeys and the disease was experimentally reproduced in inoculated and contact turkeys.\(^26\)

In 1978, Hinz et al. reported outbreaks of upper respiratory disease in West Germany and isolated a *Bordetella bronchiseptica*-like bacillus from the respiratory tracts of affected turkeys.\(^17\) This bacillus was used to reproduce the disease in turkeys, and the disease was termed
turkey bordetellosis. In 1979, Simmons et al. isolated a gram-negative bacillus from naturally infected turkeys in North Carolina and concluded this organism was the primary etiologic agent of turkey coryza. Based on physical and biochemical properties, Simmons et al. identified this organism as Alcaligenes faecalis and termed the disease alcaligenes rhinotracheitis. Since 1979, a gram-negative, motile, aerobic, nonfermentative bacillus has been isolated from affected turkeys throughout the United States. This organism is now classified as Bordetella avium based on morphological, physiological, biochemical, and serological studies, and analysis of bacterial DNA and RNA composition. The disease is called turkey coryza.

Gross lesions found in turkeys infected with B avium typically include excessive foamy ocular and nasal exudate, excessive thick tracheal mucus, tracheal collapse, mild submandibular swelling, and staining of periocular, cervical and wing feathers by brownish exudate.

Like other Bordetella species, B avium has specific tissue tropism for ciliated respiratory epithelium. Colonization of the nasal cavity and trachea by B avium causes mild catarrhal rhinitis and fibrinopurulent tracheitis within the first 10 days of infection. From 14 to 21 days postinfection (PI), tracheal lesions include multifocal loss of ciliated cells, epithelial hyperplasia, depletion of
mucus, and diffuse infiltration of lymphocytes and macrophages into the mucosa.\textsuperscript{3,6,16} From 21 to 40 days PI, the tracheal mucosa is composed of basophilic, low columnar to cuboidal, nonciliated cells; squamous metaplasia occurs sporadically.\textsuperscript{3,6} Mucus glands become cystic and lined by cuboidal epithelium.\textsuperscript{3} Tracheal rings may flatten dorsoventrally and become severely distorted.\textsuperscript{3,6} By 53 days PI, tracheas return to normal except for residual distortion of tracheal rings and shortened luminal epithelium.\textsuperscript{3,6} \textbf{Bordetella avium} can be isolated from infected tracheas for at least 53 days PI.\textsuperscript{3} Histological evaluations of lung of \textit{B} avium-infected turkeys have been included in several early studies of turkey coryza, but pulmonary lesions attributed to \textit{B} avium infection were either not found or not reported.\textsuperscript{4,31-33}

Attempts at treatment and prevention of turkey coryza have met with only limited success in commercial turkey flocks.\textsuperscript{13,34,35} \textbf{Bordetella avium} can persist at least 6 months in litter, and can survive wide ranges of temperature (10 C - 40 C) and humidity (32% - 78%).\textsuperscript{13,32,34} Because \textit{B} avium is resistant to many environmental influences, antibiotic therapy and vaccination have been the focus of control measures. Antibiotics, administered orally, parenterally, or as an aerosol, cause only temporary remission of clinical disease.\textsuperscript{16,35,36} Vaccination of turkey
breeder hens with *B. avium* bacterins has increased survival rates, improved growth rates, and delayed the onset and severity of clinical disease in progeny of vaccinated hens. Vaccination of young turkeys with a temperature-sensitive mutant, attenuated live cultures, or formalin-inactivated cultures of *B. avium* produce incomplete or short-lived protection. These procedures are usually not sufficient to eliminate the disease from a flock.

Isolates of *B. avium* have been divided into 2 groups based on colony morphology, biochemical profiles, and hemagglutination reactions. Type I isolates, described by Jackwood et al., are similar to group I isolates described by Rimler and Simmons. Type I (group I) isolates are pathogenic for turkeys. These isolates agglutinate erythrocytes of turkeys, chickens, sheep, and guinea pigs, and form round, raised, colorless colonies on bovine blood agar and MacConkey agar. Type II isolates are nonpathogenic for turkeys and do not agglutinate erythrocytes. Colonies of type II isolates are larger than those of type I isolates. Type II isolates form round, raised, opaque, mucoid colonies on bovine blood agar and MacConkey agar. Rimler and Simmons reported that group II isolates have colony morphology similar to type II isolates and do not agglutinate erythrocytes; however, some group II isolates are pathogenic for turkeys. Type I and type II isolates can also be
distinguished by comparing the cellular fatty-acid content of the bacteria.\textsuperscript{30}

Potential virulence factors of \textit{B. avium} include: hemagglutinins\textsuperscript{44}, plasmids for antibiotic resistance\textsuperscript{45,46}, lipopolysaccharide\textsuperscript{7}, histamine-sensitizing factors\textsuperscript{47}, heat-labile dermonecrotic toxin\textsuperscript{9}, heat-stable toxin\textsuperscript{48}, and surface pili.\textsuperscript{49} However, the contribution of these factors in the pathogenesis of turkey coryza is unknown. \textit{Bordetella avium} may bind via a surface ligand to a monosaccharide receptor on tracheal epithelial cells or to N-acetylmuramylpentapeptide and D-galactose moieties of tracheal mucus, but the mechanism by which \textit{B. avium} adheres to tracheal epithelium is incompletely understood.\textsuperscript{7,50}

Adherence of \textit{B. avium} causes ciliostasis and loss of ciliated cells in turkey tracheal organ cultures and degeneration and loss of ciliated cells in vivo.\textsuperscript{7,8,51} Mechanisms responsible for the local effects of \textit{B. avium} infection on the trachea are unknown. \textit{Bordetella avium} is noninvasive yet marked alteration of the tracheal mucosa results from bacterial colonization. Such local injury is suggestive evidence for bacterial toxins as mediators of local tracheal lesions\textsuperscript{7}, and adherence of the bacteria to tracheal epithelium appears to be a prerequisite for local cytopathic effects.\textsuperscript{7} One study, utilizing turkey tracheal organ cultures, suggested that acute lethal injury to
ciliated cells appeared to be induced by *B. avium* itself or toxic bacterial metabolites rather than the host's tissue response to the colonizing bacteria.\(^8\)

Several physiological changes have been found in turkeys infected with *B. avium*. McCorkle et al. reported that tissue levels of norepinephrine, dopamine, and serotonin were lower in *B. avium*-infected turkeys compared to *B. avium*-free controls after administration of alpha methylparatyrosine. Based on these findings, they suggested that postganglionic sympathetic neurons in poults may be involved in the pathogenesis of turkey coryza.\(^5\) In another study, McCorkle et al. found increased levels of serum corticosterone in *B. avium*-infected turkeys compared to normal turkeys.\(^5\) Simmons et al. described a histamine-sensitizing factor similar to that reported for *Bordetella pertussis* and suggested this factor may be responsible in part for the excessive lacrimation and mucus production observed in *B. avium*-infected turkeys.\(^4\) Edens et al. found depressed colonic and foot pad temperatures in *B. avium*-infected turkeys and suggested that endotoxin was responsible for this change.\(^5\) In each instance, the authors suggested these effects may be due to the action of a toxin. However, no toxin which induces these effects has been isolated, and the relationship of heat-labile toxin or heat-stable toxin to the clinical disease or to these physiological effects is
unknown.\textsuperscript{9,48} Whether these physiological changes are specific for \textit{B. avium} infection or represent a nonspecific response to any bacterial infection of the respiratory tract is unknown.

Based on field observations, \textit{B. avium}-infected turkeys may have impaired resistance to other infectious diseases.\textsuperscript{2} Simmons et al. reported that \textit{B. avium} infection caused impaired function of the cellular immune system as evidenced by depressed \textit{in vitro} lymphocyte stimulation by concanavalin A and disrupted thymic architecture.\textsuperscript{55} However, later studies using delayed hypersensitivity\textsuperscript{56}, graft versus host reaction\textsuperscript{57}, leucocyte migration\textsuperscript{58}, and anaphylactic response\textsuperscript{59} in \textit{B. avium}-infected turkeys demonstrated normal or enhanced cellular immune responses. No definitive impairment of the immune system has been demonstrated in \textit{B. avium}-infected turkeys.\textsuperscript{2}

Uncomplicated infections of \textit{B. avium} cause a mild disease which, in most cases, results in low mortality.\textsuperscript{4,13} However, serious losses may result when \textit{B. avium}-infected turkeys are simultaneously exposed to other respiratory pathogens such as adenovirus\textsuperscript{10}, Yucaipa virus\textsuperscript{11,60}, Newcastle disease virus\textsuperscript{61}, CELO virus\textsuperscript{10}, \textit{Mycoplasma gallisepticum}\textsuperscript{10}, and \textit{Mycoplasma meleagridis}.\textsuperscript{10} \textit{Escherichia coli} is the most common secondary bacterial invader, but other gram-negative bacteria such as \textit{Pasteurella multocida} can concurrently infect \textit{B. avium}—
infected turkeys.\textsuperscript{2} Vaccination of \textit{B avium}-infected flocks with live Newcastle disease virus can result in high death losses due to colibacillosis.\textsuperscript{12} The mechanisms by which \textit{B avium} predisposes turkeys to other infectious agents have not been characterized.

\textbf{Avian colibacillosis -} Colibacillosis, caused by \textit{Escherichia coli}, is a serious and potentially fatal disease of chickens and turkeys and is responsible for major economic losses in the poultry industry.\textsuperscript{62} Infection with \textit{E coli} can produce a variety of lesions in poultry including enteritis\textsuperscript{63}, salpingitis\textsuperscript{64}, airsacculitis\textsuperscript{65}, colisepticemia\textsuperscript{66}, osteomyelitis and synovitis\textsuperscript{67}, coligranuloma\textsuperscript{68}, and cloacal bursitis.\textsuperscript{69} Three forms of respiratory disease have been described in naturally-occurring colibacillosis.\textsuperscript{70} First, colisepticemia occurs in young birds and causes typical lesions of acute gram-negative bacterial septicemia affecting the respiratory tract, heart, liver, spleen, and lymphoid tissues. The second and most common form of respiratory disease is subacute fibrinopurulent polyserositis and airsacculitis in birds surviving acute septicemia. A third and sporadic form of \textit{E coli} infection is chronic granulomatous pneumonitis, hepatitis, and enteritis in flocks some time after acute septicemia or subacute polyserositis.\textsuperscript{70}

Attenuation of normal pulmonary defense mechanisms may
be important in the pathogenesis of avian colibacillosis. Epizootics of *E coli* infection are commonly associated with viral or mycoplasmal respiratory disease, vaccination, and environmental stress.\(^{64,71-73}\) *Bordetella avium* infection is considered to be one of the most important predisposing factors involved in outbreaks of colibacillosis following Newcastle disease virus vaccination in turkeys.\(^{74}\)

Colibacillosis is believed to occur in turkeys by inhalation of poultry house dust contaminated with feces. Infection may extend from the lower respiratory tract into the bloodstream.\(^{70,75}\) Pathogenic serotypes of *E coli* are commonly found in poultry house dust and litter.\(^{76,77}\) *Escherichia coli* septicemia has been reproduced experimentally by exposing turkeys to virulent serotypes of *E coli* via air sac, intratracheal, aerosol, and oral routes.\(^{64,69,78,79}\) The mechanism whereby *E coli* enters the bloodstream from the respiratory tract remains a mystery; however, *E coli* bacteremia is necessary for most, if not all, manifestations of colibacillosis in turkeys.\(^{80}\)

Strains of *E coli* vary in their capacity to produce disease.\(^{81}\) This variation may be due in part to differences in their capacity to adhere to the respiratory mucosa. Virulent strains of *E coli* are more capable of colonizing chicken tracheal epithelium compared with avirulent strains.\(^{82}\) This colonization is considered an important
initial step in the pathogenesis of colibacillosis in the chicken.\textsuperscript{82} Type 1 (F1) fimbriae can promote adherence of \textit{E coli} to urinary tract and oral epithelium in mice and humans by binding to mannose residues on mucosal epithelial cell membranes.\textsuperscript{83–85} However, the involvement of F1 fimbriae in the pathogenesis of disease in mammals and poultry is controversial.\textsuperscript{86,87} Naveh et al. reported that \textit{E coli} expressing type 1 fimbriae adhere to chicken tracheal epithelial cells; however, they did not prove that type 1 fimbriae were responsible for the adherence.\textsuperscript{88} Higher morbidity and mortality were reported in chickens inoculated with \textit{E coli} expressing type 1 fimbriae compared with chickens inoculated with nonfimbriated \textit{E coli}.\textsuperscript{88} Gyimah and Panigrahy reported partial protection in chickens challenged with pathogenic \textit{E coli} after vaccination with a vaccine containing type 1 fimbriae prepared from \textit{E coli} homologous to the challenge strain.\textsuperscript{89} In this study, neither \textit{E coli} adherence nor clearance in the respiratory tract were evaluated.

Most strains of \textit{E coli} which are virulent for turkeys produce type 1 fimbriae when cultured in vitro.\textsuperscript{81,90} In turkeys, type 1 fimbriae do not play a significant role in adherence of pathogenic \textit{E coli} to intestinal epithelium, but may promote bacterial trapping or adherence to intestinal mucus.\textsuperscript{91} Antibodies to type 1 fimbriae do not enhance clearance of fimbriated \textit{E coli} from the bloodstream.\textsuperscript{92} The
role of type 1 fimbriae in the adherence of *E. coli* to the tracheal mucosa of turkeys is unknown.

Other mechanisms of virulence for *E. coli* have been proposed. Virulent *E. coli* resist the bactericidal effects of serum and resist phagocytosis by producing exopolysaccharide capsular material and external carbohydrate moieties of lipopolysaccharide. The roles of plasmid-mediated bacterial products such as toxins, adhesins, and siderophores as virulence factors have been reviewed. 62, 69, 80

**Anatomy of the avian respiratory tract** - The respiratory system in turkeys begins at the nostrils and consists of the nasal cavities, oropharynx, trachea, bronchi, lungs, and air sacs. The trachea is composed of complete interlocking cartilage rings and is covered by pseudostratified ciliated columnar epithelium with numerous shallow mucous glands. 93-95 The distal end of the trachea connects to the syrinx just inside the thoracic inlet. Two extrapulmonary primary bronchi arise at the syrinx. These are relatively short and enter the lungs on the ventromedial aspect. Structurally, the extrapulmonary bronchi resemble the trachea. 93, 94

Instead of dicotymously branching airways terminating in alveoli as in mammalian lung, the avian lung is composed of a series of interconnecting bronchi. 94 The tubular system of the lung consists of 3 bronchial subdivisions: the intrapulmonary primary bronchus, various secondary bronchi,
and many parabronchi (tertiary bronchi). The cartilage rings of the extrapulmonary primary bronchi are replaced by a layer of smooth muscle in the intrapulmonary primary bronchi. Within the lung, the mucosa of the primary bronchi is composed of low columnar, ciliated, pseudostratified epithelium with goblet cells and occasional mucous glands. Primary bronchi pass through the lung with a slight S-bend while giving rise to several groups of secondary bronchi before entering the abdominal air sacs. Each secondary bronchus gives rise to many parabronchi. Parabronchi, which communicate with several secondary bronchi, contain numerous invaginations or atria that lead to smaller round openings termed infundibula. Extending from the infundibula are the air capillaries, anastomosing cylindrical tubes which function in gaseous exchange. Air sacs are thin walled extensions of the respiratory tract connecting to the lung by the primary and secondary bronchi and the parabronchi.

In the chicken, lymphoid nodules project into the airways at the bifurcations of the primary and secondary bronchi. The epithelium over this bronchus-associated lymphoid tissue (BALT) is flattened, lacking in cilia and goblet cells, and is similar to lymphoepithelium of mammalian BALT. Microfold (M) cells within the lymphoepithelium are believed to selectively transfer soluble and particulate antigens from the airway lumen into the internal lymphoid
Below the epithelium, BALT consists of a well organized reticulin network filled with lymphocytes and macrophages. In the chicken, lymphocytes in BALT commonly express immunoglobulin A on the cell surface, but immunoglobulins are only rarely found within lymphocytes. Although antigen does not appear to be necessary for the development of BALT, antigen is necessary for expanding the size and number of lymphoid aggregates. Unlike mammalian BALT, germinal centers are commonly found in BALT of chickens. Similar BALT has not been described in the turkey lung.
PAPER I. EFFECTS OF BORDETELLA AVIUM INFECTION ON THE PULMONARY CLEARANCE OF ESCHERICHIA COLI IN TURKEYS
Effects of *Bordetella avium* infection on the pulmonary clearance of *Escherichia coli* in turkeys

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SUMMARY

One-day-old turkeys were inoculated intranasally with *Bordetella avium*. Noninoculated hatchmates were housed separately. At 2 and 4 weeks of age, *B avium*-infected (BA+) and *B avium*-free (BA-) turkeys were exposed to an aerosol of virulent *Escherichia coli*. The remaining turkeys were used as controls not exposed to *E coli*. Turkeys were necropsied on postaerosolization days 0 (immediately after aerosolization), 1, 3, 5, and 7. Lung and tracheal samples were collected from each turkey for bacterial quantitation and histopathologic examination. A 1-ml blood sample was collected for detection of bacteremia. Numbers of *E coli* in lung from both 2 and 4-week-old turkeys were not significantly different between BA+ and BA- groups using pooled data over time, however numbers of *E coli* isolated from trachea were significantly greater in BA+ turkeys compared with BA- turkeys at most sample times. Pulmonary abscesses and *E coli* bacteremia were much more frequent in 2-week-old turkeys than in 4-week-old turkeys, but no differences were found between BA+ and BA- turkeys. Air sacculitis was more common and more severe in BA+ turkeys at both ages. Hyperplastic bronchus-associated lymphoid tissue was more common in BA+ turkeys than in BA- turkeys and appeared to be the first site of heterophil infiltration following *E coli* aerosolization.
INTRODUCTION

Turkey coryza, alcaligenes rhinotracheitis, and avian bordetellosis are terms used to describe a specific upper respiratory disease of young turkeys and chickens caused by *Bordetella avium* (*Alcaligenes faecalis*). This highly contagious disease is characterized by bacterial colonization of the nasal and tracheal mucosae, rhinitis, conjunctivitis with frothy exudate, tracheitis, tracheal collapse, and decreased weight gain. Although turkey coryza is usually associated with low mortality, affected turkeys may be predisposed to *E. coli* infection resulting in increased mortality and condemnations.

The high incidence of *E. coli* infection in *B. avium*-infected turkeys and a report of simultaneous seroconversion to both Newcastle disease virus (NDV) and *B. avium* suggest a potential interaction between these respiratory pathogens. In flocks where *B. avium* is endemic, increased mortality due to *E. coli* is seen following Newcastle disease virus vaccination. Other pathogens which have been isolated from *B. avium*-infected flocks include adenovirus, CELO virus, and *Mycoplasma meleagridis*.

The purpose of this study was to determine the effects of *B. avium* infection (turkey coryza) on pulmonary and tracheal clearance of pathogenic *E. coli*. 
MATERIALS AND METHODS

Experimental design - From a group (n=72) of 1-day-old turkeys (Nicholas strain) of both sexes, 1 group of 36 turkeys was inoculated intranasally with B avium; the remaining 36 turkeys were raised as B avium-free controls. At 2 weeks of age, 15 B avium-infected (BA+) turkeys and 15 B avium-free (BA-) turkeys were exposed to an aerosol of pathogenic E coli. Three BA+ turkeys and 3 BA- turkeys were randomly selected for necropsy from each treatment group 1 day prior to aerosolization and at postaerosolization day (PAD) 0, 1, 3, 5, and 7. Three turkeys from each group were necropsied and cultured prior to aerosolization to confirm that BA- turkeys were free of B avium and that turkeys were free of E coli. Turkeys necropsied at PAD 0 were killed and sampled within 30 to 60 minutes after aerosolization. A blood sample was collected for detection of bacteremia, and gross pathologic changes were recorded. Lung and trachea were collected from each turkey for bacterial quantitation and histopathologic examination. The above procedures were repeated using 18 BA+ and 18 BA- turkeys when the remaining 36 turkeys were 4 weeks old.

Bacteria and culture conditions - Turkeys were inoculated with B avium strain 838 as previously described. Briefly, 0.05 ml of inoculum was placed on one nostril for inhalation into the oronasal cavity. The inoculum contained
1.2 \times 10^7 \text{ colony-forming units (CFU) of } B \text{ avium per ml in phosphate buffered saline solution (PBSS).}

The \textit{E coli} strain, ECl (078:K80:H9:F1), is pathogenic for turkeys and has been used in a previous experiment. A stock culture maintained at -60°C was thawed and used to inoculate 3 tissue culture flasks containing 60 ml of brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI), and a sample of the stock culture was streaked onto solid medium to verify a pure culture. The flasks were incubated aerobically at 37°C with agitation for 24 hours. Cultures were centrifuged at 3000 x g and the bacterial pellet was resuspended in PBSS to the original volume. The bacterial suspension was maintained at 5°C and used for aerosolization within 1 hour of preparation. For aerosolization of 2-week-old and 4-week-old turkeys, the \textit{E coli} suspension contained 2.6 \times 10^9 \text{ CFU/ml}.

\textbf{Aerosolization of \textit{E coli} - BA+ and BA- turkeys were commingled in an aerosol chamber (0.35 m$^3$) with air exchange of 4 liters per minute. The \textit{E coli} suspension was administered through an ultrasonic nebulizer (Model 65, DeVilbis Co., Somerset, PA) with power adjustment and air flow at maximum settings. The nebulizer delivered 125 ml of \textit{E coli} suspension over a period of 30 minutes. At the end of aerosol exposure, the chamber was exposed to ultraviolet light for 10 minutes before turkeys were removed.}
Necropsy procedure - At each necropsy time, 3 BA+ and 3 BA- turkeys were selected at random. A blood sample (1 ml) was aseptically collected from the wing vein, and the turkeys were anesthetized with pentobarbital sodium (5-10 mg) given intravenously. The femoral vessels were severed and the turkeys were maintained with the cranial end elevated to facilitate exsanguination. Turkeys were immersed in a disinfectant solution for 1 minute before exposure of the body cavities. Gross lesions in the thoracic and abdominal viscera were noted. A 1 cm section of the midcervical trachea was removed aseptically, opened longitudinally, and placed in 9 ml cold (5 C) PBSS for bacterial quantitation. The left lung was removed aseptically and placed in 45 ml cold PBSS for homogenization and bacterial quantitation. The lower trachea, right lung and air sacs were perfused with 10% neutral buffered formalin and fixed in situ for 30 minutes before being removed from the turkey.

Bacterial quantitation - Blood samples were placed in 3 ml of BHI broth and incubated at 37 C for 24 hours. Tubes with turbidity were subcultured onto solid media for bacterial identification. Tracheal sections, each in 9 ml PBSS, were mixed on a vortex-mixer (Vortex-Genie, Fisher Scientific, Pittsburgh, PA) at maximum speed for 1 minute. A 1-ml sample of PBSS from each tracheal segment was serially diluted 10-fold 3 times, and 50 ul of each dilution were
placed as a band on MacConkey medium. Lungs were homogenized for 1 minute with a motorized tissue grinder (Model 23, The Virtis Co., Gardiner, NY) operated at 70% power. Lung homogenates were serially diluted 10-fold 3 times and 50 ul of each dilution was placed on MacConkey medium. Additionally, 0.5 ml of the lowest dilution of lung and tracheal tissues was inoculated onto MacConkey medium. All plates were incubated 36 hours at 37 C and colonies were counted for determining CFU/cm of trachea and CFU/lung. Each colony morphotype was tested by slide agglutination with antisera specific for B avium or E coli ECl.

**Microtiter agglutination test** - Serum was harvested from 6 of 36 2-week-old and 6 of 36 4-week-old turkeys prior to E coli aerosolization. Sera were tested for antibody to E coli by microtiter agglutination as previously described, except that live E coli ECl cells in 2% NaCl were used as the antigen. The sera were also tested for antibody to avian mycoplasmas (bacterial agglutination) and NDV (microtiter hemagglutination-inhibition test) (Dr. M. S. Hofstad, VMRI, Iowa State University, Ames, IA).

**Histopathology** - Lung and tracheal tissues were fixed 48 hours in 10% neutral-buffered formalin, paraffin embedded, sectioned at 3 um and stained with hematoxylin and eosin by routine methods. For each turkey, 2 sections of trachea and 2 sections of lung were examined.
Statistical analysis - Numerical data means from the 30 turkeys exposed to *E coli* in each experiment were evaluated for significant differences by the F test (Statistical Analysis System, ANOVA procedure).
RESULTS

Bacterial numbers in tissue - Numbers of E. coli in lungs from 2-week-old and 4-week-old turkeys were not significantly different (P > 0.3) when BA+ and BA- groups were compared using data pooled over time (Figs 1 & 2). An initial decrease in E. coli numbers was followed by stable or increased numbers at PAD 3 in 2-week-old turkeys and PAD 5 in 4-week-old turkeys.

The number of E. coli isolated from trachea was significantly greater (P < 0.05) in BA+ turkeys compared with BA- turkeys at most sample times. In 2-week-old turkeys, numbers of E. coli in trachea generally increased with time. At PAD 3 to 7, E. coli numbers were significantly greater (P < 0.05) in BA+ turkeys compared with BA- turkeys (Fig 3). In 4-week-old turkeys, numbers of E. coli were significantly greater (P < 0.05) in tracheas of BA+ turkeys compared with BA- turkeys at most sample times (Fig 4). Escherichia coli was not detected in tracheas of BA- turkeys after PAD 5. Numbers of E. coli were generally greater in the tracheas of 4-week-old BA+ turkeys compared with 2-week-old BA+ turkeys.

Detection of bacteremia after E. coli aerosolization was much more frequent in 2-week-old turkeys than in 4-week-old turkeys (Table 1). All positive blood cultures produced a single colony morphotype on MacConkey medium and colonies from each culture agglutinated with antisera specific for
**E. coli ECl.**

*Bordetella avium* was isolated from all turkeys inoculated with *B. avium*. Numbers of *B. avium* ranged from $10^5$ - $10^7$ CFU/cm trachea. Colonies isolated from each turkey agglutinated with antiserum specific for *B. avium*.

Lungs of both 2 and 4-week-old BA+ turkeys contained $10^3$ - $10^6$ CFU of *B. avium* per lung. At PAD 7, *B. avium* was isolated ($10^5$ CFU/cm) from trachea, but not from lung, in 2, 2-week-old turkeys that had not been inoculated with *B. avium*. *Bordetella avium* was not isolated from 4-week-old BA- control turkeys.

**Lesions** - Gross lesions were restricted to the respiratory tract and were similar in 2 and 4-week-old turkeys. All BA+ turkeys had mucopurulent oculonasal exudate and increased tracheal mucus. Mild to severe dorso-ventral flattening of the tracheal mucus was present in 15 of 30 BA+ turkeys. BA- turkeys had no gross lesions of rhinitis or tracheitis.

Histological changes in the tracheas of BA+ turkeys were similar to those previously described and included mild suppurative tracheitis with loss of ciliated epithelial cells, depletion of mucus, bacterial colonization, and diffuse infiltration of mononuclear cells in the lamina propria. Nonciliated epithelial cells were hyperplastic in the tracheas of 4-week-old turkeys. Tracheas of BA- turkeys
had no lesions \textit{B. avium} infection.

Tracheal lesions following \textit{E. coli} aerosolization were similar in all BA+ turkeys and consisted of low numbers of heterophils in the lamina propria at PAD 1 through PAD 7. Tracheas of BA- turkeys had a mild infiltration of heterophils in the lamina propria at PAD 1, but not at later times.

The predominant lung lesion in \textit{B. avium}-infected turkeys was massive peribronchial lymphocytic hyperplasia (Fig 5) around the intrapulmonary primary bronchus. This lesion persisted throughout the experiment in 27 of 30 BA+ turkeys; only 9 of 30 BA- turkeys had similar lesions (Table 1).

Lung lesions following \textit{E. coli} aerosolization were present at all PAD sample times. At PAD 0, locally-extensive heterophil infiltrates, within and around peribronchial lymphoid nodules (Figs 6, 7), were accompanied by mild diffuse heterophil infiltration throughout the lung. By PAD 1 most of the inflammatory response centered around primary bronchi. The peribronchial connective tissue contained serofibrinous exudate and massive infiltrates of heterophils with fewer macrophages. Bacteria were rarely found within the lesions. Similar, but less severe changes were present in adjacent secondary bronchi. Microabscesses, more common in 2-week-old turkeys than in 4-week-old turkeys, were found around primary bronchi or within secondary bronchi (Table 1).
From PAD 3 to 7, the acute suppurative inflammatory response gradually subsided and macrophages and lymphocytes became the predominant inflammatory cells.

Forty-nine of the 60 turkeys exposed to the *E coli* aerosol developed airsacculitis (Table 2). Mild lesions, common in BA- turkeys, involved only the thoracic and/or abdominal air sacs. Mildly affected air sacs were slightly cloudy and contained occasional 1-3 mm white to yellow nodules composed of aggregations of macrophages and lymphocytes. Epithelial surfaces were separated by serofibrinous fluid and small numbers of heterophils and macrophages diffusely infiltrated the interstitium.

Severe air sac lesions were more common in BA+ than in BA- turkeys and more common in 2-week-old than in 4-week-old turkeys (Table 2). Severe air sac lesions usually involved all air sacs and occasionally the pericardium. The air sacs were opaque to white and contained multiple 5-7 mm raised yellow caseous nodules. Microscopically these nodules were pyogranulomas and lymphoid nodules in the connective tissue of the air sacs. Although bacteria were present in some pyogranulomas, no bacteria were isolated from these lesions.

**Serology** - All sera were negative for antibody to *E coli* ECl, *Mycoplasma meleagris*, *M. gallisepticum*, *M. synoviae*, and Newcastle disease virus.
TABLE 1 - Incidence of pulmonary lesions and *E coli* bacteremia

<table>
<thead>
<tr>
<th>Turkeys</th>
<th>Peribronchial lymphoid hyperplasia</th>
<th>Pulmonary abscesses</th>
<th><em>E coli</em> bacteremia</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-week-old BA+</td>
<td>12/15</td>
<td>9/15</td>
<td>9/15</td>
</tr>
<tr>
<td>2-week-old BA-</td>
<td>5/15</td>
<td>8/15</td>
<td>8/15</td>
</tr>
<tr>
<td>4-week-old BA+</td>
<td>15/15</td>
<td>5/15</td>
<td>2/15</td>
</tr>
<tr>
<td>4-week-old BA-</td>
<td>4/15</td>
<td>4/15</td>
<td>1/15</td>
</tr>
</tbody>
</table>

BA+ = *B avium*-infected; BA- = *B avium*-free

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TABLE 2 - Incidence of air sac lesions

<table>
<thead>
<tr>
<th>Turkeys</th>
<th>Mild</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-week-old BA+</td>
<td>5/15</td>
<td>9/15</td>
</tr>
<tr>
<td>2-week-old BA-</td>
<td>9/15</td>
<td>3/15</td>
</tr>
<tr>
<td>4-week-old BA+</td>
<td>10/15</td>
<td>5/15</td>
</tr>
<tr>
<td>4-week-old BA-</td>
<td>8/15</td>
<td>0/15</td>
</tr>
</tbody>
</table>

BA+ = *B avium*-infected; BA- = *B avium*-free
Fig 1 - Recovery of viable *E. coli* from lungs of 2-week-old turkeys. Data expressed as the mean (n=3) + standard error of the mean

Fig 2 - Recovery of viable *E. coli* from lungs of 4-week-old turkeys. Data expressed as the mean (n=3) + standard error of the mean
B. avium free
B. avium infected
Minimum detection limit

Days Postaerosolization

Log₁₀ CFU/Lung

B. avium free
B. avium infected
Minimum detection limit

Days Postaerosolization
Fig 3 - Recovery of viable *E. coli* from tracheas of 2-week-old turkeys. Data expressed as the mean (n=3) + standard error of the mean.

Fig 4 - Recovery of viable *E. coli* from tracheas of 4-week-old turkeys. Data expressed as the mean (n=3) + standard error of the mean. *Escherichia coli* was not isolated from tracheas of *B. avium*-free turkeys at 7 days postaerosolization.
Days Postaerosolization

Log$_{10}$ CFU/cm Trachea

- **B. avium free**
- **B. avium infected**
- **Minimum detection limit**

Days Postaerosolization

Log$_{10}$ CFU/cm Trachea
Fig 5 - Peribronchial lymphocytic hyperplasia in the primary bronchus of *B. avium*-infected turkey. H&E stain; X 70

Fig 6 - Heterophils (inset) infiltrating a peribronchial lymphoid nodule following *E. coli* aerosolization at PAD 0. H&E stain; X 450.
Fig 7 - (Inset of Fig 6) - Heterophils (arrows) in a peribronchial lymphoid nodule following \textit{E coli} aerosolization (PAD 0). H&E stain; X 1100
DISCUSSION

*Bordetella avium* infection had no effect on numbers of *E coli* in lung, but was associated with increased numbers of *E coli* in trachea, particularly in the 4-week-old turkeys. The two principal mechanisms for pulmonary clearance of inhaled bacteria are: mechanical removal by the mucociliary escalator of the upper respiratory tract and removal by pulmonary macrophages. Although the importance of mucociliary clearance is unknown in turkeys, reduced tracheal clearance may exist when ciliated epithelial cells are damaged.\(^{10,11}\) In this study, *B avium* caused severe damage to ciliated epithelial cells of the trachea, yet pulmonary clearance of *E coli* was similar in BA+ and BA- turkeys. Our results suggest that mucociliary clearance is of minor importance in removal of *E coli* from the lungs of turkeys.

Greater *E coli* numbers in the trachea of BA+ turkeys in this study could be due to colonization of the trachea by *E coli* or to increased mucociliary transit time of *E coli* being cleared from the lung. Gram-negative bacteria may opportunistically adhere to tracheal mucosa damaged by infectious or mechanical agents.\(^{12-14}\) *Bordetella*-damaged trachea may be a primary site for *E coli* adherence and colonization.

Large numbers of *E coli* on the *B avium* injured tracheal mucosa may play a key role in the pathogenesis of air sac
lesions. **Bordetella avium** and **E. coli** together produced severe airsacculitis without the presence of other respiratory pathogens. **Escherichia coli** dislodged from the trachea may act as a constant source of bacteria seeding the lower respiratory tract resulting in the more severe airsacculitis commonly found in **B. avium**-infected turkeys. Under field conditions, **E. coli** contaminated poultry house dust may act as the initial aerosol exposure of pathogenic bacteria to the poult.$^{15}$

Pathogenic **E. coli** in the respiratory tract may enter the bloodstream of turkeys when the respiratory lining epithelium is sufficiently damaged.$^{11,16}$ This concept is supported in the present study because 14 of 17, 2-week-old turkeys with **E. coli** bacteremia had gross or histological evidence of pulmonary abscessation beginning PAD 1. The reason for increased resistance to bacteremia in 4-week-old turkeys is unclear. Turkeys may have age-related maturation of their local (mucosal) and systemic immune responses.$^{17}$ Factors involved in bacterial clearance and killing such as macrophage and heterophil function, complement, and immunoglobulins may be less developed in 2-week-old turkeys than in 4-week old turkeys.

The acute inflammatory response to **E. coli** aerosol was focused on the pulmonary bronchi and peribronchial lymphoid tissue. The large peribronchial lymphoid nodules in BA+
turkeys represent a response to chronic antigenic stimulation in the bronchi. The early accumulation of heterophils among peribronchial lymphoid tissue suggests this may be a site of bacterial uptake and antigen processing. The bronchus-associated lymphoid tissue (BALT) may be an initial site of antigen uptake and processing following bacterial challenge by the aerosol route. Low cuboidal nonciliated cells covering the lymphoid nodules may be similar to lymphoepithelial M cells which function in antigen uptake and processing in mammals. Further research will be needed to characterize the nature and importance of BALT in the turkey and its role in respiratory immunity.
REFERENCES


PAPER II. INFLUENCE OF BORDETELLA AVIUM INFECTION ON ADHERENCE AND COLONIZATION OF ESCHERICHIA COLI IN THE TURKEY TRACHEA
Influence of *Bordetella avium* infection on adherence and colonization of *Escherichia coli* in the turkey trachea

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SUMMARY

Four-week-old *Bordetella avium*-infected (BA+) and *B avium*-free (BA-) turkeys were inoculated with *Escherichia coli* expressing F1 fimbriae or with *E coli* not expressing F1 fimbriae. The tracheal adherence of *E coli* at 1 postinoculation hour (1 PIH) and tracheal colonization of *E coli* at 6 PIH were determined using an in vivo bacterial adherence assay. Significantly greater numbers of *E coli* were isolated at both 1 PIH and 6 PIH from the tracheas of BA+ turkeys compared with BA- turkeys. The expression of F1 fimbriae on *E coli* had no significant effect on numbers of *E coli* in the trachea. The greater number of *E coli* in tracheas of BA+ turkeys was probably due to passive retention of bacteria in tracheal exudates. Neither specific adherence nor colonization could be demonstrated for the association of fimbriated or nonfimbriated *E coli* with the *B avium*-damaged trachea.
INTRODUCTION

*Bordetella avium* is the cause of turkey coryza, a highly contagious upper respiratory tract disease of young turkeys. The disease is characterized by ocular nasal discharge, sneezing, tracheal collapse, and decreased rate of weight gain. *Bordetella avium* is a noninvasive, nonfermentative, gram-negative bacterium that persistently colonizes the ciliated epithelium of the respiratory tract leading to inflammation of the upper airways, loss of ciliated epithelial cells, and accumulation of nasal and tracheal mucus. Although turkey coryza can be a clinically mild disease by itself, infected turkeys may be predisposed to other respiratory pathogens. High mortality rates have been reported in *B avium*-infected turkeys exposed to other respiratory pathogens.

Attenuation of the tracheal mucosa by *B avium* infection may enhance adherence and colonization by gram-negative respiratory pathogens such as *E coli*. Bacterial adherence to normal or altered host epithelium is a prerequisite to colonization of mucosal surfaces and subsequent infection of deeper tissues. The normal respiratory tract efficiently removes most bacteria from the airways; however, mechanical, chemical, or viral injury to the airway epithelium may predispose the airways to bacterial infection. In turkeys, mycoplasmal and viral
respiratory infections predispose to colibacillosis.\textsuperscript{15-17} Turkeys experimentally infected with \textit{B avium} develop severe fibrinopurulent airsacculitis after aerosol exposure to \textit{E coli}, whereas similar aerosol exposure of normal turkeys results in only mild, transient airsacculitis.\textsuperscript{10}

In chickens, virulent strains of \textit{E coli} apparently colonize the tracheal epithelium as a preliminary step in the development of colibacillosis.\textsuperscript{18} Type 1 (F1) fimbriae are nonflagellar, filamentous surface appendages produced by \textit{E coli} and other bacteria\textsuperscript{19,20}, which may enhance the attachment of \textit{E coli} to ciliated tracheal epithelium in the chicken.\textsuperscript{21} Although F1 fimbriae may enhance adherence of \textit{E coli} to a variety of cells\textsuperscript{22-26}, the role of F1 fimbriae in the pathogenesis of disease is unclear.\textsuperscript{19,27,28} The role of F1 fimbriae in the association of \textit{E coli} with the \textit{B avium}-damaged trachea is unknown.

The purpose of the present study was to determine if pre-existing \textit{B avium} infection affects the adherence and colonization of fimbriated (F1+) and nonfimbriated (F1-) \textit{E coli} in the turkey trachea.
MATERIALS AND METHODS

Experimental design - Turkeys inoculated at 6 days of age with B. avium were housed separately from an equal number of noninoculated hatchmates. Adherence of E. coli was determined in 5 B. avium-infected (BA+) and 5 B. avium-free (BA-) turkeys using an F1+ E. coli inoculum; another 5 BA+ and 5 BA- turkeys were treated in a like manner using an F1- E. coli inoculum. Adherence of E. coli was determined 1 hour after E. coli inoculation in isolated tracheal segments as described.

To evaluate E. coli colonization, BA+ and BA- turkeys were inoculated with either F1+ or F1- E. coli as described above except the number of E. coli associated with the tracheal mucosa was determined 6 hours after inoculation with E. coli.

Eight turkeys (4 BA+ and 4 BA-) were treated intratracheally with phosphate-buffered saline solution (PBS, pH 7.2) to serve as noninoculated controls.

Turkeys - Forty-eight one-day-old turkeys (Nicholas strain) were obtained commercially (Midwest Turkey Hatchery Inc., Dike, IA). They were maintained for 10 days in heated brooders and, for the remainder of the study, in metal cages with slatted floors. Turkey starter and water were provided ad libitum. Serum samples, collected 1 day before E. coli inoculation, were assayed for agglutinating antibody to
somatic and fimbrial antigens of \textit{E coli} EC1 by microtiter agglutination.\textsuperscript{4} Turkeys were used in the study at 4 weeks of age.

**Bacteria and culture conditions** - Turkeys were inoculated with \textit{B avium} strain 838 at 6 days of age as previously described.\textsuperscript{4} Briefly, 0.05 ml of inoculum was placed on one nostril for inhalation into the oronasal cavity. The inoculum contained 2.4 x 10^6 colony-forming units (CFU)/ml in PBSS.

The \textit{E coli} strain, EC1 (078:K80:H9:F1), is pathogenic for turkeys and has been used in previous studies.\textsuperscript{19,30} A stock culture of \textit{E coli} EC1 was stored in glycerine and brain-heart infusion broth (BHI) (Difco Laboratories, Detroit, MI) 1:1 at -60 C. For preparation of \textit{E coli} inocula, bacteria were grown aerobically on a tryptose agar slant for 24 hours at 37 C. From this slant, \textit{E coli} was subcultured (static and aerobically) in BHI broth, 2 times at 24 hour intervals, either at 37 C to enhance the expression of F1 fimbriae or at 18 C to suppress the expression of F1 fimbriae. The bacterial cultures were centrifuged 20 minutes at 3000 X g, and the supernatant was discarded. The bacterial pellet was suspended with PBSS, maintained at 5 C, and used within 1 hour of preparation. A sample of each \textit{E coli} inoculum was examined for expression of fimbriae by negative-stain electron microscopy\textsuperscript{31}, mannose-sensitive
agglutination of guinea pig erythrocytes, and slide agglutination in antiserum to F1 fimbriae. For slide agglutination, 1 drop of each E coli inoculum in BHI broth was placed on a clean glass slide and mixed at room temperature for 2 minutes with antiserum to F1 fimbriae. Visible white clumps of bacteria indicated a positive agglutination. The number of CFU/ml was determined for each inoculum immediately before use by standard plate counting methods.

Bacterial adherence assay - This assay is a modification of a previously described in vivo tracheal adherence assay. Feed and water were withheld from turkeys 1 hour before they were anesthetized with 0.25 ml of a 4:1 mixture of ketamine hydrochloride (100 mg/ml) (Vetalar, Parke-Davis, Morris Plains, NJ) and acepromazine maleate (10 mg/ml) (Acepromazine, Fort Dodge Laboratories, Fort Dodge, IA) administered IV. Turkeys were placed on a rack to support the head and extend the neck. The midcervical area was sprayed with 70% ethanol and a 1 cm section of skin was removed to expose the trachea. Tracheal adventitia was opened, and the trachea was separated from subjacent tissues. A Satinsky cardiovascular forceps (V. Mueller, Chicago, IL) was applied to the trachea to occlude the lumen. A catheter (3.5 Fr. Tom Cat Catheter-open, Monoject, St. Louis, MO) was passed through the glottis to the base of the isolated
cranial tracheal segment, and after the trachea was completely filled with the bacterial inoculum, the inoculum was aspirated from the trachea and the Satinsky forceps was removed. Each turkey was laid on its side to recover from anesthesia. At 1 or 6 postinoculation hours (PIH), turkeys were euthanatized, and a 1-cm section of trachea was obtained from each turkey as previously described. The *E coli* CFU/cm trachea was determined by standard plate counting methods as described.

To compensate for slight differences in the concentration of *E coli* inocula, an adjusted CFU/cm trachea was calculated as follows:

\[
\log_{10} \left( \frac{1 \times 10^9 \text{ CFU/ml}}{\text{CFU/ml inoculum}} \right) \times \text{observed CFU/cm trachea}
\]

The adjusted CFU/cm of trachea was used for statistical analysis.

**Statistics** - Numerical data were analyzed using analysis of variance procedures. Statistical differences between treatment means were determined by F tests and least significant differences (P = 0.05).
RESULTS

**Bacterial numbers in trachea** - The presence of *B. avium* had a pronounced effect on *E. coli* numbers in the trachea. *Bordetella avium*-infected turkeys had significantly greater (P < 0.01) numbers of *E. coli* in tracheal segments compared with BA- turkeys at both 1 and 6 PIH.

Numbers of *E. coli* in tracheal segments were not significantly different (P > 0.5) when data from BA+ turkeys killed at 1 PIH were compared with data from BA+ turkeys killed at 6 PIH (Fig 1). However, *E. coli* numbers in tracheas were significantly less (P < 0.05) in BA- turkeys killed at 6 PIH compared with BA- turkeys killed at 1 PIH (Fig 1).

The presence of F1 fimbriae had no effect on *E. coli* numbers in the trachea. Numbers of F1+ and F1- *E. coli* were not significantly different in the tracheas of either BA+ or BA- turkeys at 1 PIH (P > 0.4) and 6 PIH (P > 0.12) (Table 1).

All BA+ turkeys exhibited clinical signs of severe rhinotracheitis evidenced by frothy nasal exudate, excessive tracheal mucus, mild tracheal flattening, and congestion of the tracheal vasculature. *Bordetella avium* was isolated (10^4-10^7 CFU/cm trachea) from all BA+ turkeys, but was never isolated from BA- turkeys. All BA- turkeys remained clinically normal. *Escherichia coli* was not isolated from BA+ or BA- turkeys inoculated with PBSS.
**Escherichia coli inocula** - The F1+ inocula, but not the F1- inocula, caused agglutination of guinea pig erythrocytes within 30 seconds. Hemagglutination was prevented when the F1+ inoculum was suspended in PBSS containing 0.5% D-mannose. The F1+ inocula, but not the F1- inocula, agglutinated when mixed on a slide with antiserum to F1 fimbriae. In F1+ inocula, but not the F1- inocula, most *E coli* contained surface filaments with size and morphologic features typical of F1 fimbriae.34

**Serology** - All sera were negative for antibody to somatic and fimbrial antigens of *E coli* ECl.
TABLE 1 - Numbers of *E coli* (Log$_{10}$ CFU/cm) recovered from turkey tracheas following *E coli* inoculation

<table>
<thead>
<tr>
<th>BA+ turkeys</th>
<th>BA- turkeys</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIH</td>
<td>F1+</td>
</tr>
<tr>
<td>1</td>
<td>4.34±0.28</td>
</tr>
<tr>
<td>6</td>
<td>4.54±0.37</td>
</tr>
</tbody>
</table>

Data (n=5/group) are expressed as means ± standard error of the mean.

BA+ = *B avium*-infected; BA- = *B avium*-free; PIH = post-inoculation hours; F1+ = fimbriated *E coli*; F1- = non-fimbriated *E coli* inoculum.
Fig 1 - Recovery of viable \textit{E coli} from tracheas of \textit{B avium}-infected and \textit{B avium}-free turkeys. Data expressed as the mean (n=10/group) + standard error of the mean
B. avium free

B. avium infected

Log_{10} CFU/cm Trachea

Hours postinoculation

1

6
DISCUSSION

In turkeys inoculated intratracheally with *E. coli*, greater numbers of *E. coli* remained associated with *B. avium*-damaged tracheas compared with normal tracheas. In mammals, mechanical, chemical, and viral damage to the tracheal epithelium is known to facilitate adherence of gram-negative bacteria.\(^{14,35}\) These opportunistic bacteria, including *E. coli*, can specifically adhere to epithelial cell receptors or to mucus of the tracheal mucosa.\(^{36-38}\) The excessive, tenacious mucus, which is consistently present in *B. avium*-damaged tracheas\(^4,6\), may be the site for increased *E. coli* adherence. However, altered receptor profiles in the *B. avium*-damaged tracheal epithelium could potentially promote *E. coli* adherence.

In the present study, it was not possible to determine whether increased association of *E. coli* with the *B. avium*-damaged trachea was due to specific adherence to epithelium or mucus, or whether the bacteria were just passively retained in tracheal exudates. Severe damage to ciliated epithelium by *B. avium* probably inhibits clearance of the tracheal exudates and any bacteria associated with it.\(^ {39}\) Perhaps rinsing the trachea to remove excessive mucus and exudate would be useful in determining the site of *E. coli* association.

Bacterial colonization following adherence is an
important feature in the pathogenesis of bacterial infections of mucosal surfaces. Colonization represents a balance between clearance and killing of bacteria and multiplication of adherent bacteria. *Bordetella avium* has a specific tropism for tracheal epithelium, and colonization of the tracheal mucosa can be demonstrated within 6 PIH. In the present study, we attempted to demonstrate *E coli* colonization based on an increase in *E coli* numbers between 1 PIH and 6 PIH. Six hours incubation was considered sufficient time for *E coli* to colonize the tracheal mucosa based on colonization studies using *B avium*. In BA- turkeys, a 10-fold decrease in *E coli* numbers between 1 and 6 PIH indicated significant bacterial clearance from the trachea. In contrast, numbers of *E coli* in the BA+ tracheas were unchanged between 1 and 6 PIH; therefore, *E coli* colonization of the BA+ trachea could not be clearly demonstrated. The present study provided no evidence of a specific *E coli* adherence mechanism for the normal or *B avium*-damaged tracheal mucosa as suggested for the chicken trachea.

The presence of F1 fimbriae did not affect the numbers of *E coli* in tracheas of BA+ or BA- turkeys. Although F1 fimbriae promote the colonization of several strains of *E coli* to urinary tract and oral epithelium of humans and mice, there is no evidence to suggest F1 fimbriae
mediate adherence of \textit{E coli} in the turkey. F1 fimbriae are not important for adherence of pathogenic \textit{E coli} to turkey intestinal epithelium, and antibodies to F1 fimbriae do not enhance clearance of F1\textsuperscript{+} \textit{E coli} from the bloodstream of turkeys\textsuperscript{19,27}. In chickens, F1 fimbriae are thought to facilitate the adherence of \textit{E coli} to tracheal epithelial cells\textsuperscript{21}, and vaccines containing F1 fimbriae of \textit{E coli} provide partial protection in chickens experimentally challenged with virulent \textit{E coli}\textsuperscript{41}. A role for \textit{E coli} F1 fimbriae in the pathogenesis of colisepticemia of turkeys has yet to be established.
REFERENCES


PAPER III. HISTOPATHOLOGY OF LUNG AND BRONCHUS-ASSOCIATED LYMPHOID TISSUE IN YOUNG TURKEYS INFECTED WITH BORDETELLA AVIUM
Histopathology of lung and bronchus-associated lymphoid tissue in young turkeys infected with *Bordetella avium*

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Supported by the US Department of Agriculture, Special Research Grants Program, Grant No. 82-CRSR-2-1082.
SUMMARY

One-day-old turkeys were inoculated intranasally with *Bordetella avium*. Noninoculated hatchmates were housed separately. At 1, 2, 3, 4, and 5 weeks postinoculation (PI), *B. avium*-infected (BA+) and *B. avium*-free (BA-) turkeys were necropsied; tracheas, intrapulmonary primary bronchi, and lung adjacent to primary bronchi were cultured. Lung was collected for histopathological examination. Lungs, perfused with acetic acid, were collected to determine the size, number, and distribution of lymphoid nodules associated with primary bronchi. *Bordetella avium* was isolated from trachea and primary bronchi of all BA+ turkeys, but was never isolated from lung parenchyma. Acute purulent bronchitis was associated with colonization of the primary bronchi by *B. avium* from 1 to 3 weeks PI. Macrophages and lymphocytes persisted in the peribronchial connective tissue for 5 weeks PI. Bronchus-associated lymphoid tissue (BALT) consisted of discrete lymphoid nodules protruding into the lumen of primary bronchi. Lymphoid nodules, morphologically similar in BA+ and BA- turkeys, were composed of nonciliated, cuboidal epithelium covering a zone of loosely arranged lymphocytes and macrophages and a deeper, sharply demarcated lymphoid follicle. Compared with BALT of BA- turkeys, lymphoid nodules of BALT in BA+ turkeys were more numerous and widely distributed along primary bronchi. In both BA-
and BA+ turkeys, the mean diameter of lymphoid nodules doubled between 1 and 5 weeks of age.
INTRODUCTION

*Bordetella avium* is the cause of turkey coryza, a highly contagious upper respiratory disease of young turkeys.¹⁻³ The clinical disease is characterized by excessive oculonasal discharge, sneezing, dyspnea, tracheal collapse, and decreased weight gain.¹,² Histological lesions in the nasal cavity and trachea consist of fibrinopurulent rhinitis and tracheitis associated with bacterial colonization of the ciliated epithelium. Loss of ciliated cells, epithelial hyperplasia, depletion of mucus, and diffuse infiltration of mononuclear inflammatory cells into subepithelial tissues are consistent features of *B avium* infection in turkeys.¹,⁴

The significance of *B avium* as a pulmonary pathogen in turkeys is unknown. When lung tissue was cultured in previous studies of turkey coryza, *B avium* was rarely isolated.²,⁵,⁶ In these experiments, lung was apparently cultured without specifically attempting to culture the primary bronchus. However, *B avium* is consistently isolated when lung homogenates from experimentally infected turkeys are cultured.⁷ Therefore, bacterial colonization may be restricted to a small portion of lung such as the ciliated epithelium of large airways.

Histological examination of lungs from *B avium*-infected (BA+) turkeys was included in several early studies of turkey coryza, but lesions were not reported.²,⁶,⁸,⁹ In contrast, a
recent study described massive hyperplasia of the bronchus-associated lymphoid tissue (BALT) in lungs of turkeys infected with *B. avium*. The purposes of the present study were to determine the distribution of *B. avium* in lung and to describe the changes in lung and BALT of turkeys experimentally infected with *B. avium*.
MATERIALS AND METHODS

Experimental design - Turkeys (n=30) were inoculated intranasally with *B. avium* at 1 day of age; a second group of turkeys (n=25) was raised as *B. avium*-free (BA-) controls. At 1, 2, 3, 4, and 5 weeks PI, 6 BA+ and 5 BA- turkeys were randomly selected for necropsy. Using 3 BA+ and 2 BA- turkeys, trachea, intrapulmonary primary bronchus, and lung adjacent to the primary bronchus from each turkey were cultured for *B. avium*, and lung was collected for histopathology. Lungs collected at each necropsy time from a second group of turkeys (3 BA+ and 3 BA-) were fixed in acetic acid and examined using a dissecting microscope (Bausch and Lomb, Rochester, NY) to determine the size, number, and distribution of lymphoid nodules associated with primary bronchi.

Turkeys - One-day-old turkeys (Nicholas strain) were obtained commercially (Cuddy Farms, Ellsworth, IA). A group of 30 turkeys was inoculated with *B. avium* at 1 day of age as previously described. Briefly, 0.05 ml of inoculum was placed on one nostril for inhalation into the oronasal cavity. The inoculum contained 2.6 x 10^8 colony-forming units of *B. avium* per ml phosphate-buffered saline solution. The remaining 25 turkeys were housed separately and served as noninoculated controls. All turkeys were maintained for the first 10 days in heated brooders and, for the remainder of
the experiment, in metal cages with slatted floors. Turkey starter and water were provided ad libitum. Serum from 3 BA+ and 3 BA- turkeys was collected at each necropsy time and tested for antibody to avian mycoplasmas (bacterial agglutination) and Newcastle disease virus (microtiter hemagglutination-inhibition test) (Dr. L. D. Koehnk, Jewell, IA).

**Necropsy procedures** - Turkeys were anesthetized with pentobarbital sodium (5-10 mg) given IV. The femoral vessels were severed, and turkeys were maintained with the craniad part of the body elevated to facilitate exsanguination. The body cavities were opened, and the left lung was removed aseptically. Left primary bronchi were opened longitudinally and cultured at the middle with a disposable swab (Calgiswab, American Can Co., Glenwood, IL). The lung parenchyma 1-cm lateral to the primary bronchus was flamed, opened longitudinally with a sterile knife, and cultured with a swab. Skin over the midcervical trachea was removed. The trachea was opened with a sterile knife and cultured with a swab. Lower trachea and right lung from each turkey were perfused with 10% neutral-buffered formalin and fixed in situ for 30 minutes before being removed from the turkey.

In turkeys used to determine the size, number, and distribution of lymphoid nodules associated with the primary bronchi, birds were anesthetized, exsanguinated, and tracheas
cultured as above. The lower trachea and both lungs were perfused with cold (5 C) 2.5% acetic acid and fixed in situ for 30 minutes before being removed. The primary bronchus of each lung was opened longitudinally, and the lungs were placed in 2.5% acetic acid for 24 hours before examination under a dissecting microscope. Lymphoid nodules were counted, and the diameter of at least 7 nodules in each primary bronchus was measured using a calibrated eyepiece in the dissecting microscope.

**Bacterial isolation** - Swabs from trachea, bronchi, and lung parenchyma were streaked onto blood agar plates. All plates were incubated aerobically at 37 C for 36 hours. Representative small, isolated, translucent colonies were tested by slide agglutination with antiserum specific for *B. avium*.

**Histopathology** - Formalin-perfused lungs were fixed 48 hours in 10% neutral-buffered formalin, paraffin embedded, sectioned at 3 um and stained with hematoxylin and eosin (H&E) by routine methods. Additional sections were stained with Masson's trichrome for collagen and periodic acid Schiff-Alcian blue (PAS-AB) for mucosubstances. From each turkey, a cross section of the entire lung was taken at the junction of the extrapulmonary and intrapulmonary primary bronchi. Also, a longitudinal section of the lung was taken through the intrapulmonary primary bronchus.
RESULTS

Clinical signs, gross lesions, and serology - Clinical signs of turkey coryza were present in all BA+ turkeys by 1 week PI and persisted throughout the study. Signs included sneezing, excessive oculonasal discharge, and staining of the periocular and cervical feathers with brownish exudate. Gross lesions in the nasal cavity and trachea included frothy nasal exudate, excessive thick tracheal mucus, mild to severe dorso-ventral flattening of the trachea, and hyperemia of the tracheal mucosa. Clinical signs and gross lesions of turkey coryza were not present in BA- control turkeys. All sera were negative for antibody to Mycoplasma meleagris, Mycoplasma gallisepticum, and Newcastle disease virus.

Bacterial isolation - Bordetella avium was isolated from trachea and intrapulmonary primary bronchus of all BA+ turkeys at all necropsy times; however, B avium was never isolated from lung parenchyma adjacent to primary bronchi. All positive cultures produced a single colony morphotype on blood agar and colonies from each culture agglutinated with antiserum specific for B avium. Bordetella avium was not isolated from BA- control turkeys.

Histopathologic lesions in lung and BALT - Pulmonary lesions in BA+ turkeys were limited to primary bronchi. Diffuse peribronchial infiltration of heterophils was common in turkeys (5 of 6) at 1 and 2 weeks PI. Less common was
locally extensive infiltrations of heterophils within and around BALT (Fig 1). Primary bronchi contained exudates of mucus and cellular debris. From 3 to 5 weeks PI, the acute inflammatory response subsided, but lymphocytes and macrophages remained scattered throughout the peribronchial connective tissue. Lymphoid nodules were more numerous and often contained more germinal centers in BA+ turkeys compared to BA- turkeys. Occasionally, multinucleated giant cells were present within BALT. At 5 weeks PI, one lymphoid follicle contained a small granuloma.

Ciliated, columnar epithelium of the primary bronchi remained intact and without degenerative changes throughout this study. Similarly, mucosubstances stained with PAS-AB were continually present in goblet cells at all levels of primary bronchi. Increased peribronchial connective tissue was not apparent in sections stained with Masson's trichrome. Rarely, single bacteria were found adhered to the cilia of bronchial epithelium on H & E stained sections of lung. No lesions were present in BA- control turkeys.

Morphology of BALT - Greater numbers of lymphoid nodules were present in BA+ turkeys compared with BA- turkeys (Table 1). Numbers of lymphoid nodules were similar between right and left lungs. Lymphoid nodules ranged from 0.07 mm to 1.33 mm in diameter and were slightly smaller in BA+ turkeys compared to BA- turkeys (Table 2). The average
diameter of lymphoid nodules approximately doubled between 1 and 5 weeks of age in both BA+ and BA- turkeys.

In BA+ and BA- turkeys, BALT appeared as discreet, white, round to oval nodules on a clear to slightly opaque background of bronchial mucosa after fixation with acetic acid (Figs 2 and 3). Lymphoid nodules were concentrated at junctions of primary and secondary bronchi in BA- turkeys, but were more randomly distributed along the primary bronchus in BA+ turkeys. In BA+ and BA- turkeys, each lymphoid nodule contained a lymphoid follicle located immediately below the epithelium of the bronchus (Figs 4 and 5). The ciliated columnar bronchial epithelium changed to nonciliated, low-cuboidal cells over centers of BALT (Fig 6). The low-cuboidal cells lacked mucous granules. Collagen fibers and bundles of smooth muscle, which were regularly present under the surrounding columnar epithelium, were absent under the low-cuboidal epithelium covering lymphoid follicles of BALT.

Lymphoid follicles were well demarcated and loosely encapsulated with connective tissue on the lateral and antiluminal borders. Immediately under the epithelium, lymphoid follicles were composed of a zone of loosely arranged lymphocytes and large cells with foamy cytoplasm, presumably macrophages (Figs 4 and 5). Deeper in the follicle, lymphocytes were more densely arranged in sheets except around blood vessels where they were concentrically
aligned with vessels. Blood vessels were located at the periphery of follicles on lateral and antiluminal borders. Germinal centers, composed of lymphocytes and surrounded by a delicate collagen capsule, were occasionally present in lymphoid follicles. In BA+, but not in BA- turkeys, small to moderate numbers of heterophils were present in lymphoid follicles.
TABLE 1 - Mean number of lymphoid nodules associated with the primary bronchi of *B. avium*-infected (BA+) turkeys and *B. avium*-free (BA-) turkeys

<table>
<thead>
<tr>
<th>Weeks PI</th>
<th>BA+</th>
<th>BA-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right/1 Lung</td>
<td>Left/1 Lung</td>
</tr>
<tr>
<td>1</td>
<td>26/1</td>
<td>25.3/1</td>
</tr>
<tr>
<td>2</td>
<td>68/1</td>
<td>62.6/1</td>
</tr>
<tr>
<td>3</td>
<td>67.6/1</td>
<td>56/1</td>
</tr>
<tr>
<td>4</td>
<td>116.6/1</td>
<td>115/1</td>
</tr>
<tr>
<td>5</td>
<td>153/1</td>
<td>130/1</td>
</tr>
</tbody>
</table>

1n = 3/group.

2PI = postinoculation.

3n = 6/group.
TABLE 2 - Diameter (in mm) of lymphoid nodules associated with primary bronchi of turkeys

<table>
<thead>
<tr>
<th>Weeks PI</th>
<th>BA+ 1</th>
<th>BA- 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>1</td>
<td>0.13-0.47</td>
<td>0.29</td>
</tr>
<tr>
<td>2</td>
<td>0.07-0.73</td>
<td>0.39</td>
</tr>
<tr>
<td>3</td>
<td>0.13-1.07</td>
<td>0.36</td>
</tr>
<tr>
<td>4</td>
<td>0.07-1.07</td>
<td>0.43</td>
</tr>
<tr>
<td>5</td>
<td>0.07-1.20</td>
<td>0.56</td>
</tr>
</tbody>
</table>

1 BA+ = *B* avium-infected.
2 BA- = *B* avium-free.
3 PI = postinoculation.
4 n = 6/group.
Fig 1 - Heterophils (arrows) within and around BALB in *B. avium*-infected turkey at 2 weeks PI.

H&E stain; x 450
Fig 2 - Lymphoid nodules (N) in primary bronchus of *B. avium*-infected turkey at 4 weeks PI. C = cartilage rings. L = lung parenchyma. x 15

Fig 3 - Lymphoid nodules (N) around openings of secondary bronchi (arrows). x 35
Fig 4 - Lymphoid nodule with lymphoid follicle in *B. avium*-infected turkey at 4 weeks PI. Loose zone of macrophages and lymphocytes between black arrowheads. Blood vessels (white arrows). H&E stain; x 180

Fig 5 - Lymphoid nodule with lymphoid follicle in *B. avium*-infected turkey at 4 weeks PI. Loose zone of macrophages and lymphocytes between black arrowheads. H&E stain; x 180
Fig 6 - Transition from ciliated, columnar epithelium (C) to nonciliated, cuboidal epithelium overlying loosely arranged macrophages and lymphocytes. H&E stain; x 1100
DISCUSSION

Bordetella avium has a striking tropism for the ciliated respiratory epithelium extending from the nasal cavity to the lung.\(^1,4,11\) In the lung, ciliated epithelium is limited to the primary bronchi.\(^12\) As in the trachea, B avium can be consistently isolated from the primary bronchi of turkeys with coryza, but is never isolated from the lung parenchyma adjacent to primary bronchi. This limitation of B avium to primary bronchi helps to explain the inconsistent isolation of B avium from lungs of BA+ turkeys in previous studies.\(^2,5,6\)

Inflammation of the bronchial mucosa was interpreted to be a stereotypic response to persistent injury.\(^1\) Pulmonary lesions associated with B avium infection were limited to primary bronchi. Mild diffuse purulent bronchitis subsided and changed to a predominantly mononuclear inflammatory cell response by 3 weeks PI. Similar progression of inflammatory change occurs in the tracheal and nasal mucosae of BA+ turkeys.\(^1,4\) Loss of ciliated epithelial cells, epithelial cell hyperplasia, depletion of mucus, and microcolonization of ciliated epithelial cells are consistent tracheal lesions in BA+ turkeys\(^1\), but these lesions are absent in intrapulmonary bronchi. Differences in lesions between lung and bronchi could be due to greater colonization of the trachea by B avium as compared to the lung.\(^7\)
BALT in turkeys occurs as prominent, well demarcated lymphoid nodules projecting into the lumen of primary bronchi. Similar BALT has been described in chickens. In BA- turkeys, lymphoid nodules were clustered around openings of secondary bronchi which are important sites for inertial impaction of inhaled particles. The more random spacial development of BALT in BA+ turkeys may result from the diffuse and persistent colonization of \textit{B avium} throughout primary bronchi.

Greater numbers of lymphoid nodules were present in BA+ turkeys compared to BA- turkeys at each necropsy time, although BALT was present in all turkeys. Antigen may not be required for the development of BALT, but may be necessary for expansion and proliferation of this tissue. Proliferation of BALT is not specific for \textit{B avium}, but is a nonspecific response to persistent antigenic stimulation in bronchi. In both BA- and BA+ turkeys, the mean diameter of lymphoid nodules doubled in size between 1 and 5 weeks of age. A similar age related development of BALT occurs in other species.

The low cuboidal epithelium covering each lymphoid nodule is morphologically similar to the lymphoepithelium of BALT and Peyer's patches of chickens and mammals. This lymphoepithelium lacks cilia and mucus-producing cells. Microfold (M) cells, present in the lymphoepithelium of many
species, have the capacity to transport soluble and particulate antigens from the bronchial lumen to the interior of BALT.\textsuperscript{15,16} Acute inflammation of BALT in the present study and in turkeys exposed to an \textit{Escherichia coli} aerosol suggests that BALT in turkeys may be a preferential site of antigen uptake and processing.\textsuperscript{7} Further studies will be needed to characterize the role of BALT in mucosal immunity of turkeys.
REFERENCES


PAPER IV. EFFECTS OF BORDETELLA AVIUM TOXIN ON TURKEY TRACHEAL ORGAN CULTURES AS MEASURED WITH A TETRAZOLIUM-REDUCTION ASSAY
Effects of *Bordetella avium* toxin on turkey tracheal organ cultures as measured with a tetrazolium-reduction assay

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Supported by the US Department of Agriculture, Special Research Grants Program, grant No. 82-CRSR-2-1082
SUMMARY

Turkey tracheal organ cultures (TOCs) were exposed to 1 of the following *Bordetella avium* fractions or controls: live *B avium*, formalin killed *B avium*, *B avium* sonicate, heat-inactivated sonicate, culture supernatant, heat-inactivated culture supernatant, phosphate buffered saline solution, and brain heart infusion broth. After the TOCs were incubated for 2 hours with the bacterial fractions, the cellular metabolism of each TOC was evaluated using a tetrazolium chloride reduction assay and cellular morphology was determined by light microscopy. Additionally, bacterial fractions and controls were injected into turkeys to test lethality. Although the bacterial sonicate, containing heat-labile toxin, was lethal for turkeys, neither the sonicate nor any other *B avium* fraction significantly affected the metabolism or morphology of turkey TOCs.
INTRODUCTION

_Bordetella avium_ is the cause of turkey coryza, an upper respiratory disease of turkeys and chickens.\textsuperscript{1–3} The disease is characterized by rhinitis, conjunctivitis, ocuonalosal discharge, tracheal collapse, and decreased weight gain.\textsuperscript{3} Colonization is restricted to ciliated respiratory epithelium of the nasal, tracheal, and bronchial mucosae.\textsuperscript{4} Although _B avium_ is noninvasive, it causes extensive damage to the colonized mucosae.\textsuperscript{4} In 1 study, visual estimates of ciliary activity in turkey tracheal organ cultures (TOCs) were used as a subjective measurement of ciliary activity, and results suggested that intact live _B avium_ is cytotoxic to tracheal epithelium.\textsuperscript{5} Systemic effects reported with _B avium_ infection include histamine sensitization, alterations in tissue monoamines, and alterations in immune functions.\textsuperscript{6–8} Mechanisms responsible for the local and systemic effects of _B avium_ infection have not been clearly elucidated. A toxin prepared from sonicated live _B avium_ is lethal for turkeys when injected parenterally, but the contribution of this toxin to the clinical signs and lesions of turkey coryza is unknown.\textsuperscript{9}

Tetrazolium reduction is a chemical reaction used to quantitate a variety of metabolic activities in viable cells.\textsuperscript{10,11} The reduction of tetrazolium chloride provides an objective biochemical measurement of cellular metabolism.
in organ cultures. Tetrazolium acts as an artificial electron acceptor for pyridine nucleotide-linked enzymes, including several tissue dehydrogenases. Colorless solutions of tetrazolium salts are reduced to insoluble red formazans at the site of enzymatic oxidation in metabolically active cells. Tetrazolium reduction has been used to detect the viability of ferret tracheal explants, dehydrogenase activity in skin grafts, cellular activity in myocardial cells following ischemia, neutrophil function, and bacterial growth and motility. In hamsters, tetrazolium reduction has been used to quantitate effects of *Mycoplasma pneumoniae* on metabolism of tracheal organ cultures.

In the present study, TOCs from young turkeys were exposed to *B. avium* and a variety of potentially toxic *B. avium* culture products. A tetrazolium reduction assay was used as an objective measure of *B. avium*-induced metabolic derangements in turkey TOCs.
MATERIALS AND METHODS

Experimental design - Tracheal organ cultures produced from 5-week-old turkeys were prepared daily and incubated for 2 hours with 1 of 8 B avium fractions or controls. The TOCs were removed from the fractions, rinsed, and incubated with tetrazolium chloride. After 2 hours, the TOCs were removed, rinsed, blotted, and extracted with acetone. The optical density of the formazan-containing acetone and the dry weight of each TOC were determined. The optical density per mg trachea was calculated daily using 5 TOCs for each bacterial fraction and control. This procedure was repeated on 5 consecutive days. On the last day of the experiment, 3 ml of each bacterial fraction and control was injected intravenously into 3, 5-week-old turkeys to determine lethality.

Tracheal organ cultures - One-day-old broad breasted white turkeys (Nicholas-strain) were obtained commercially (Midwest Turkey Hatchery, Inc., Dike, Iowa) and raised to 5 weeks of age. Turkey starter and water were provided ad libitum. For 5 consecutive days of the experiment, 2 turkeys were killed by anesthetic overdose with pentobarbital sodium, and tracheas were aseptically removed. Tracheas were immediately placed in a petri dish containing Tyrode's balanced salt solution (TBSS) (Sigma Chemical Corp, St. Louis, MO) pH 7.4 supplemented with 1.2% sodium succinate.
(Sigma). Fascia and muscles were carefully removed from the external surface of tracheas. The tracheas were cut into 2-mm wide rings and incubated at 37 C. Ciliary activity was confirmed by examining each ring under an inverted phase-contrast microscope. On each of the 5 days, TOCs from 2 turkeys were pooled and used within 1 hour of preparation.

**Bacterial fractions and controls - Bordetella avium** strain P-4148 (obtained from Dr. R. B. Rimler, National Animal Disease Center, Ames, IA) was used, because this strain is capable of producing turkey coryza and a heat-labile lethal toxin.9

Live bacteria - **Bordetella avium** strain P-4148 was grown in brain heart infusion broth (BHI) (Difco Laboratories, Detroit, MI) for 18 hours at 37 C, aerobically, with agitation. The bacterial suspension was centrifuged (3000 X g, 20 minutes) and the supernatant was discarded. The bacteria were resuspended in phosphate-buffered saline solution (PBSS) to a concentration of $10^9$ colony forming units (CFU)/ml. This inoculum was prepared fresh for each day of the experiment.

Killed bacteria - **Bordetella avium** strain P-4148 was cultured and centrifuged as above. The supernatant was decanted and saved. The bacteria were resuspended in PBSS to a final concentration of $10^9$ CFU/ml and formalin was added to a final concentration of 0.02%. The formalinized bacteria
were mixed, incubated at 37 C for 1 hour, refrigerated (5 C) overnight, and then centrifuged (3000 X g, 20 minutes). The supernatant was discarded and the bacteria were resuspended in an equal volume of PBSS. This fraction was maintained at 5 C until needed. Sterility was confirmed by placing a sample of this fraction on blood agar plates.

Sonicate - A sterile filtered sonicate of B avium strain P-4148 was prepared by Dr. R. B. Rimler as previously described. Protein content, 312 ug/ml, was determined by a modified Lowry method. The sonicate was maintained at -60 C until needed.

Heat-inactivated sonicate - This fraction was prepared by heating the sonicate prepared as above to 56 C for 30 minutes.

Culture supernatant - BHI supernatant saved from preparation of the killed bacterial fraction was filtered through a 0.45 um filter followed by a 0.22 um filter (Millipore Corp., Bedford, MA). The filtered supernatant was cultured to confirm sterility. This fraction was maintained at -60 C until needed.

Heat-inactivated culture supernatant - For each day, culture supernatant, prepared as above, was thawed and heated to 56 C for 30 minutes.

Controls - Phosphate-buffered saline solution pH 7.2 and BHI were used.
**Tetrazolium-reduction assay** - On each day of the experiment, 48 TOCs were equally divided among 8, 16 x 100 mm glass test tubes (Vacutainer, Becton-Dickenson Company, Rutherford, NJ) containing 8 ml TBSS supplemented with 1.2% sodium succinate. To each tube, 3 ml of 1 of the 8 fractions were added. Rubber stoppers were replaced on the filled tubes and air bubbles were removed with a needle and syringe. A ninth tube was prepared by placing 6 ethanol fixed (killed) TOCs with 11 ml TBSS supplemented with 1.2% sodium succinate to serve as a negative control. All tubes were placed on a rocking platform (Model 4651, Ames Company, Elkhart, IN) and incubated for 2 hours at 37 C. From each tube, 5 TOCs were removed, rinsed four times in TBSS and placed in another test tube containing 11 ml of a solution of 0.02% 2,3,5 triphenyltetrazolium chloride (Sigma) in TBSS supplemented with 1.2% sodium succinate. All tubes were placed on a rocking platform and incubated for 2 hours at 37 C. After the incubation, TOCs were removed from the tubes, rinsed 4 times in distilled water, and blotted. Formazan was extracted from each TOC with 3 ml acetone for 10 minutes. The optical density of the acetone-formazan suspension was measured spectrophotometrically (Spectronic 20, Bausch and Lomb, Rochester, N.Y.) at 490 nm. Dry weight of each TOC was measured and the optical density per mg was calculated for each TOC. On each experimental day, 5 values were generated
for each of the 8 fractions and the ethanol fixed TOCs.

**Histopathology** - After 2 hours incubation with the fractions in TBSS, 1 TOC from each fraction on days 3, 4, and 5 of the experiment was fixed in 10% neutral buffered formalin. After fixation, the tracheas were routinely processed and embedded in paraffin, sectioned at 3 um, and stained with hematoxylin and eosin.

**Statistical analysis** - Differences in numerical data among treatment groups were analyzed for significance by the F test and least significant differences (Statistical Analysis System, Analysis of Variance) using a randomized block design.
RESULTS

Following incubation with tetrazolium, red pigment was visible on the tracheal mucosa of all TOCs except those fixed in ethanol. No statistically significant differences in tetrazolium reduction were found between the PBSS control and PBSS-containing bacterial fractions including live B avium, killed B avium, sonicate, and heat-inactivated sonicate (P > 0.2) (Table 1). No statistically significant differences were found between the BHI control and BHI-containing fractions including culture supernatant and heat-inactivated culture supernatant (P > 0.2) (Table 1). A significant difference (P < 0.05) was found between the PBSS control and the BHI control. The ethanol-fixed TOCs did not reduce tetrazolium to formazan; the optical density per mg trachea was zero.

Of all B avium fractions, only the sonicate was lethal for turkeys when injected intravenously. All 3 turkeys injected intravenously with the sonicate were depressed within 1 hour and dead within 3 hours following injection. Turkeys injected with all other fractions appeared healthy during 4 days of observation.

Histologically, no differences were seen among TOCs incubated in the different fractions. In all fractions, the epithelium appeared intact. Occasional bacteria lined the cilia in both the live and killed B avium-containing
fractions. Changes in all TOCs attributed to incubation in artificial media were mild diffuse vacuolation of mucosal epithelial cells, clumped chromatin in mitotically active basal cells, and pyknosis of leukocyte nuclei.
TABLE 1. Optical density (at 490 nm) per mg trachea for 
TOCs exposed to *B. avium* fractions and controls

<table>
<thead>
<tr>
<th>Fraction^1</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Mean±SE^2</th>
<th>Lethal^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBSS</td>
<td>37.3*</td>
<td>54.1</td>
<td>42.9</td>
<td>35.7</td>
<td>41.1</td>
<td>42.2±7.2 a</td>
<td>-</td>
</tr>
<tr>
<td>LBA</td>
<td>56.8</td>
<td>58.9</td>
<td>36.9</td>
<td>21.5</td>
<td>16.8</td>
<td>38.2±19.4 a,b</td>
<td>-</td>
</tr>
<tr>
<td>KBA</td>
<td>47.3</td>
<td>45.2</td>
<td>45.3</td>
<td>23.8</td>
<td>41.1</td>
<td>40.5±9.6 a,b</td>
<td>-</td>
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<tr>
<td>SON</td>
<td>43.1</td>
<td>39.6</td>
<td>42.7</td>
<td>26.3</td>
<td>41.1</td>
<td>38.6±7.0 a,b</td>
<td>+</td>
</tr>
<tr>
<td>H-SON</td>
<td>44.7</td>
<td>40.9</td>
<td>39.8</td>
<td>28.5</td>
<td>17.2</td>
<td>34.2±11.3 a,b</td>
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<tr>
<td>SUPN</td>
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<td>33.1±4.2 a,b</td>
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<tr>
<td>H-SUPN</td>
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<td>47.5</td>
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<td>28.5</td>
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<td>39.2±7.1 a,b</td>
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<tr>
<td>BHI</td>
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<td>34.3</td>
<td>32.4</td>
<td>28.8</td>
<td>20.2</td>
<td>30.3±6.2 b</td>
<td>-</td>
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</table>

^1 PBSS = phosphate-buffered saline solution; LBA = live 
*B. avium*; KBA = formalin-killed *B. avium*; SON = sonicate; H-SON = 
heat-inactivated sonicate; SUPN = culture supernatant; H-
SUPN = heat-inactivated supernatant; BHI = brain heart 
infusion broth.

^2 Mean ± standard error of the mean for each fraction. 
Means with same small letters do not differ significantly 
(P < 0.05). Optical density was multiplied by 10^3.

^3 Lethality following injection of each fraction into 3 
turkeys.

^4 Each number represents a mean of five TOCs.
DISCUSSION

The tetrazolium reduction assay failed to detect metabolic differences among TOCs exposed to the various bacterial fractions and their comparable controls. Several authors have suggested that toxins of B. avium may be responsible for the local and systemic effects of B. avium infection.\textsuperscript{5,19,20} Cytotoxicity, measured by visual estimates of ciliostasis, "blebbing" of the cell surface, and sloughing of ciliated epithelium in TOCs, was seen 10 minutes after addition of live B. avium to 12 hour or older TOCs.\textsuperscript{5} The mild cytoplasmic vacuolization seen histologically in our 3 hour TOCs suggests that cellular metabolism and morphology may be altered in artificial medium.

The differences between PBSS control and BHI control TOCs were expected. BHI has a detrimental effect on the tracheal mucosa and on B. avium adherence to tracheal mucosa (unpublished data).

Tracheal organ cultures taken directly from the lethal sonicate had no detectable morphological or biochemical evidence of cytotoxicity to suggest a direct effect of this toxin on tracheal epithelium. Similar to Bordetella affecting mammals, B. avium contains endotoxin and both heat-labile and heat-stable toxins.\textsuperscript{7,9,19} Endotoxin may have been present in the toxic sonicate used in this study. The role these toxins have in the production of tracheal cytopathology
is unknown.

Tracheal organ cultures are isolated from the immunological and hormonal influences of the living animal. An interaction between bacterial toxins and immunological mechanisms in the living animal may be necessary for cytotoxicity.¹⁹,²¹ Because these interactions would not be possible in the in vitro system used in this study, an in vivo model may be useful for studying these mechanisms.
REFERENCES


GENERAL SUMMARY AND DISCUSSION

This series of experiments was designed to evaluate the structural and functional effects of *B. avium* infection in the turkey respiratory tract. Study 1 was designed to determine whether *B. avium* infection predisposes turkeys to *E. coli* airsacculitis and to evaluate the effect of *B. avium* infection on pulmonary clearance of *E. coli*. Study 2 was designed to determine if *B. avium* infection affects tracheal adherence and colonization of *E. coli*. Study 3 was designed to determine the distribution of *B. avium* in lung and to describe changes in lung and bronchus-associated lymphoid tissue of turkeys experimentally infected with *B. avium*. Study 4 was designed to determine if *B. avium* heat-labile toxin would induce metabolic or morphological alterations in turkey tracheal organ cultures.

In these studies, turkeys experimentally infected with *B. avium* developed severe fibrinopurulent airsacculitis after aerosol exposure to *E. coli*, whereas similar aerosol exposure of normal turkeys resulted in only mild, transient airsacculitis. Association of *E. coli* with the trachea may be an important step in the pathogenesis of airsacculitis in turkeys. Numbers of *E. coli* in tracheas of *B. avium*-infected turkeys were significantly greater than numbers of *E. coli* in tracheas of *B. avium*-free turkeys following aerosol exposure to *E. coli*. Possibly, *E. coli* dislodged from the trachea act...
as a constant source of bacteria seeding the lower respiratory tract resulting in more severe airsacculitis in *B. avium*-infected turkeys. Increased numbers of *E. coli* in tracheas of *B. avium*-infected turkeys were probably due to passive retention in tracheal exudate rather than specific adherence or colonization of *E. coli* to tracheal epithelium.

No significant differences in pulmonary clearance of aerosolized *E. coli* were found between *B. avium*-infected and *B. avium*-free turkeys. Although the importance of mucociliary clearance of inhaled bacteria is unknown in turkeys, these results suggest that mucociliary clearance may be of minor importance in removal of *E. coli* from the lungs of turkeys. In turkeys exposed to aerosolized *E. coli*, pulmonary abcesses and *E. coli* bacteremia were much more common in 2-week-old turkeys compared to 4-week-old turkeys, but no differences were found between *B. avium*-infected and *B. avium*-free turkeys. Further studies will be needed to understand the development of this age-related resistance in turkeys.

*Bordetella avium* was found to colonize primary bronchi and persist in the lung of turkeys for at least 5 weeks postinoculation (PI). Pulmonary lesions in *B. avium*-infected turkeys were limited to the primary bronchi and consisted of acute purulent bronchitis changing to a mononuclear response by 3 weeks PI. A similar progression of inflammatory lesions has been observed in tracheas of *B. avium*-infected turkeys.
However, severe tracheal lesions such as loss of ciliated epithelial cells, epithelial cell hyperplasia, depletion of mucus, and microcolonization of ciliated epithelial cells did not occur in the lung. Differences in lesions between primary bronchi and trachea are probably due to smaller numbers of colonizing *B. avium* in bronchi compared to colonization of trachea.

Compared to *B. avium*-free turkeys, *B. avium*-infected turkeys had greater numbers of lymphoid nodules in primary bronchi. This increase in the number of lymphoid nodules represents a nonspecific proliferation of bronchus-associated lymphoid tissue (BALT) in response to persistent *B. avium* colonization. Average diameter of lymphoid nodules approximately doubled in both *B. avium*-infected and *B. avium*-free turkeys between 1 and 5 weeks of age. A similar age-dependent increase of BALT occurs in other species.96

Bronchus-associated lymphoid tissue in *B. avium*-infected and *B. avium*-free turkeys appeared as prominent, sharply demarcated lymphoid nodules projecting into the lumen of primary bronchi. Lymphoid nodules, histologically similar in *B. avium*-infected and *B. avium*-free turkeys, were composed of nonciliated, cuboidal epithelium overlying a single lymphoid follicle. Adjacent to the epithelium, the lymphoid follicle was composed of a zone of loosely arranged lymphocytes and macrophages. Deeper in the follicle, lymphocytes were more
densely arranged in sheets or concentrically around blood vessels. Some lymphoid follicles contained germinal centers. In turkeys, BALT may be an important site of antigen uptake and processing as evidenced by inflammatory responses centering on lymphoid nodules in turkeys exposed to an aerosol of \textit{E coli}. Further studies will be needed to characterize the role of BALT in mucosal immunity of turkeys.

Sonicates of \textit{B avium} strain P-4148 contain a heat-labile toxin which is lethal for turkeys.\textsuperscript{9} Neither \textit{B avium} sonicate containing heat-labile toxin, nor any other \textit{B avium} bacterial fraction significantly altered the metabolism or morphology of turkey tracheal organ cultures. Therefore, heat-labile toxin is probably not directly responsible for local cytopathology in tracheal mucosa of \textit{B avium}-infected turkeys.

From these studies, we conclude that \textit{B avium} can colonize the primary bronchi of turkeys. This colonization results in inflammation focused on the primary bronchi and in proliferation of bronchus-associated lymphoid tissue. Secondly, \textit{E coli} aerosolization in \textit{B avium}-infected turkeys can result in severe fibrinopurulent airsacculitis. Although \textit{B avium} infection had no effect on clearance of \textit{E coli} from lung, turkeys retained a significant \textit{E coli} population in their tracheas. \textit{Escherichia coli} associated with the tracheal mucosa may serve as a continual source of infection to the airsacs. Thirdly, neither specific adherence nor
colonization could be demonstrated for the association of *E coli* with the normal or *B avium*-damaged trachea. Finally, heat-labile toxin of *B avium* does not alter cellular metabolism or epithelial morphology in turkey tracheal organ cultures.

Although these studies have contributed to our understanding of the structural and functional effects of *B avium* infection in the turkey respiratory tract, important questions remain. Why is *E coli* airsacculitis more severe in *B avium*-infected turkeys? Is mucociliary clearance from the lower respiratory tract impaired by *B avium* infection? What role does mucosal immunity have in resistance to *B avium* infection and is mucosal immunity age dependent? What role does BALT have in mucosal immunity? How does colonization of the respiratory tract by *B avium* lead to mucosal injury? And, do the toxins of *B avium* reported in the literature contribute to the pathogenesis of turkey coryza?
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