Characterization of Atypical Hemolytic *Ornithobacterium rhinotracheale* Isolates and Comparison with the Normal Non-Hemolytic Phenotype

Jessica Nicole Walters

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biomedical and Veterinary Sciences

F. William Pierson
Nammalwar Sriranganathan
Robert D. Evans
Audrey P. McElroy
Tanya LeRoith

October 24th, 2014
Blacksburg, Virginia

Keywords: *Ornithobacterium rhinotracheale*, ELISA, hemolysis, plasmid, turkeys

Characterization of Atypical Hemolytic *Ornithobacterium rhinotracheale* Isolates and Comparison with the Normal Non-Hemolytic Phenotype

Jessica Nicole Walters

ABSTRACT

Ornithobacterium rhinotracheale (ORT) is a Gram-negative bacterium that causes respiratory disease in poultry characterized by rhinitis, tracheitis, and pneumonia with mortality averaging 2-3%. In the Shenandoah Valley of Virginia, the seroprevalence for ORT among turkey flocks as determined by enzyme-linked immunosorbent assay (ELISA) was found to be 70.9% (n=175). Additionally, the seroprevalence for hemorrhagic enteritis virus (vaccine induced), *Bordetella avium*, and paramyxovirus-1 was 100%, 74.8%, and 6.3% respectively. No significant interactions were detected.

The type strain of ORT is characteristically non-hemolytic at least for 96 hours at 37^{o}C on Columbia Blood Agar. In recent years, atypical isolates that rapidly produce hemolysis have been isolated with increasing frequency. A variety of *in vitro* tests were used to determine differences between representative isolates of the hemolytic (H) and non-hemolytic (NH) phenotypes. Findings suggest that the H isolate contains a 4 kb plasmid similar to that found in *Reimerella anatipestifer*. No plasmid was found in the NH isolate. Differences in growth characteristics and resistance to tetracyclines were also noted. No differences in proteins, biochemical characteristics or 16S rRNA sequences were found, the latter serving as confirmation that the isolates were both ORT. Embryo inoculation was used to assess virulence. No significant differences were observed and most embryos survived through to the day of hatch (pip) despite the fact that ORT could be re-isolated.

In turkey poults however, the H phenotype did appear less virulent. A significant depression in weight gain was noted for birds inoculated intratracheally with the NH isolate at 7 days post-inoculation (dpi). NH inoculates also had significantly higher antibody levels on ELISA at 14 and 21 dpi and histopathological lesion scores for lung at 7, 14, and 21 dpi. The NH isolate could be re-isolated from NH-inoculated poults through 21 dpi; whereas the H isolate could only be re-isolated through 14 dpi.

In conclusion, there are numerous differences between the NH and H isolates found in the field with the H isolate appearing less virulent and as such, making it a potential vaccine candidate. The phenotypic difference appears to correlate with this, but may not suffice to explain it.

Acknowledgements

I would like to thank my family, my husband, and my friends, all of who have supported me through the duration of this process and who have dealt with the pain and aggravation associated with it. Thank you especially to my parents and my grandfather for pushing me, for helping me financially, and for never allowing me to give up on my dreams. To Mat, my husband, thank you for always dealing with my stress and for supporting me and pushing me even when I was nowhere close to being easy to deal with. To my friends- I couldn't have done this without your backing and constant friendship. You have all been there to pick me up when I'm feeling down, and to always question my sanity but know when I've reached my breaking point. You all are wonderful.

Thank you to my committee members for their knowledge and support of this dissertation. Dr. Pierson- thank you for helping me learn and for pushing me to branch outside of my comfort zone during my research. Dr. Bob- thank you for being an amazing role model and friend, and for always taking time to help me learn about the poultry industry. Thank you for igniting the spark for me wanting to do a PhD and for wanting to be a poultry veterinarian. Dr. Nathan- thank you for the many, many enlightening conversations and for the many hugs when I've felt like giving up. Dr. McElroy- thank you for initiating my interest in poultry during my undergraduate career, and for introducing me to the great contacts who have helped make my dreams happen. Dr. LeRoith- thank you for being not only a support system, but a friend to talk to and to help me work through the hard times during this dissertation. I don't know how I would've done it without all of you.

I would also like to acknowledge Dr.'s van Empel and Hafez- both of who were great resources for the development of my trials and who never hesitated to assist with my quest for knowledge.

Dedication

This dissertation and research conducted within are dedicated to my grandmothers Naomi "NaNa" Walters, and Beulah "Granny" Osborne. Both of these women were not only supportive in my quest to fulfill my dreams, but were instrumental in teaching me how to become an independent, strong, and intelligent woman. Neither of them could be here to see me complete the final leg of this journey, but I absolutely believe that they have been there to support and guide me through every step of the process. Granny taught me how to kill and process my first chicken, and NaNa never hesitated to take the time so that I could be outside or with animals. Their lessons in life are those that I will never forget, and I will value their support and guidance forever.

TABLE OF CONTENTS

List of Figures:

List of Tables:

List of Abbreviations:

Chapter Two
ORT (*Ornithobacterium rhinotracheale*), ELISA (Enzyme-Linked Immunosorbent Assay)

Chapter Three
ORT (*Ornithobacterium rhinotracheale*), CFU (Colony Forming Units), CBA (Columbia Blood Agar), ddH_20 (double-distilled H_20), EtBr (Ethidium Bromide)

Chapter Four
ELISA=Enzyme-Linked Immunosorbent Assay, HEV= hemorrhagic enteritis virus, ORT=*Ornithobacterium rhinotracheale*, PBS= phosphate buffered saline, dpi=days post-inoculation

Introduction

In Virginia, poultry represents the largest sector of the animal agricultural industry with farm to table revenues for broilers, turkeys, and eggs topping one billion dollars in 2013 (7). In regards to production ranking in Virginia agricultural commodities, broilers are first, turkeys are fourth and layers/eggs are tenth (7). The poultry industry in Virginia is heavily localized in the Shenandoah Valley (Rockingham, Augusta, Shenandoah and Page counties) and consists of approximately 1000 owner managed, contract farms which provide birds to large vertically integrated companies. These companies own the birds, produce the feed and provide the final processed poultry products to retailers. In turkey production as an example, farm owners are paid for each bird they are able to raise to market weight for the companies. Profit for the integrators is based off of the price per pound they receive from retail purchasers. Therefore, disease in flocks not only affects the bottom-line for individual farmers, but overall corporate profitability. Losses may be directly attributed to mortality, decreased feed efficiency (feed conversion ratio), decreased rate of gain and reduced final body weight. Additional expenses include things like vaccination, medication, environmental conditioning, and labor. Losses associated with slowed line speeds and decreased output at the processing plant, where labor and operational costs are the highest, tend to exceed those in all other categories.

Respiratory and gastrointestinal diseases are the two most significant causes of illness in commercial poultry and account for the greatest economic losses. A variety of etiological agents are responsible for illness in birds and have traditionally included species such as Coccidia, *Salmonella, Escherichia coli, Bordetella avium, Pastuerella multocida,* Infectious Bronchitis Virus, Infectious Laryngotracheitis Virus, and others. Each of these disease-causing agents can

contribute to morbidity, mortality, decreased productivity, and associated expenses due to treatment, control and prevention in subsequent flocks.

Ornithobacteriosis caused by the bacterium *Ornithobacterium rhinotracheale*, also known as ORT, is a growing problem, especially in commercial turkeys. Common clinical signs include coughing, nasal discharge, facial edema/swelling, lethargy, and anorexia. In cases where ORT is a co-infecting pathogen, mortality and morbidity can be extremely high and associated financial losses quite extensive (2, 5). While it is still unclear whether this organism is a primary or secondary pathogen, its prevalence in commercial poultry operations remains a concern. Recently, atypical hemolytic isolates of ORT have been recovered from turkey flocks in the Shenandoah Valley of Virginia. Hemolytic types are identified by alpha-hemolysis on Columbia agar supplemented with 5% sheep blood after 48 hours of incubation at 37^{o}C in microaerophilic environments. Anecdotal information originating from corporate flock supervisors and veterinarians suggested the hemolytic phenotype may be associated with a higher than average mortality in both meat production birds and breeders, but no conclusive evidence has surfaced to support this claim. Traditionally, ORT is non-hemolytic when grown on Columbia agar containing 5% sheep's blood (3), but incomplete hemolysis has been noted after 96 hours of incubation (1). Hemolytic phenotypes have been found in North America (6), but characterization and correlation with altered pathology, morbidity and mortality is lacking.

The main purpose of this research was to characterize, both *in vitro* and *in vivo*, the hemolytic isolates of ORT found in Virginia. We evaluated ORT seroprevalence among turkey flocks in the Shenandoah Valley, its relationship to other respiratory pathogens, its *in vitro* characteristics in comparison to non-hemolytic phenotypes, and differences between the two phenotypes in embryo and live bird experimental models.

We hypothesized the following:

1) The hemolytic and non-hemolytic isolates were, in fact, both ORT.
2) The hemolytic isolate would be the dominant isolate in terms of seroprevalence.
3) Distinct differences between the hemolytic and non-hemolytic phenotypes would be found *in vitro*.
    a. Since other researchers have found a relation between the hemolysis and a cytolytic pore forming protein (6), a difference in protein profiles may exist.
    b. The hemolytic phenotype may be associated with chromosomal mutations or the presence of episomal DNA such as a plasmid. Hemolysin production has been found to correlated with virulence in other Gram-negative bacterial species such as *Escherichia coli* (4).
    c. In turkey embryos, morbidity and mortality would differ between the two phenotypes, with the hemolytic isolate being more virulent
4) *In vivo*, morbidity and mortality would differ between the two phenotypes, with the hemolytic isolate being more virulent (Manuscript in print: Walters, J., R. Evans, T. LeRoith, N. Sriranganathan, A. McElroy, and F. W. Pierson. Experimental comparison of hemolytic and nonhemolytic Ornithobacterium rhinotracheale field isolates in vivo. Avian Dis 58:1:78-82. 2014).

In general, the purpose of this research was to better understand the biological significance of these emergent hemolytic isolates and attempt to determine what organismal changes may be associated with the phenotypic variation.

References:

1. Banani, M., S. A. Pourbakhsh, and P. Khaki. Characterization of Ornithobacterium rhinotracheale Isolates from Commercial Chickens. Arch. Razi Ins. 52:27-36. 2001.
2. Droual, R. Pathogenicity Studies of Ornithobacterium rhinotracheale in Turkeys: Comparisons of Routes of Inoculation and Interaction with Bordetella Avium. In: The Turkey ORT Scientific Symposium. R. A. H. a. Nutrition, ed., Minneapolis, Minnesota. pp 33-36. 1996.
3. Hafez, H. M. Current status on the laboratory diagnosis of Ornithobacterium rhinotracheale "ORT" in poultry. Berl Munch Tierarztl Wochenschr 111:143-145. 1998.
4. Minshew, B. H., J. Jorgensen, G. W. Counts, and S. Falkow. Association of hemolysin production, hemagglutination of human erythrocytes, and virulence for chicken embryos of extraintestinal Escherichia coli isolates. Infect Immun 20:50-54. 1978.
5. Pan, Q., A. Liu, F. Zhang, Y. Ling, C. Ou, N. Hou, and C. He. Co-infection of broilers with Ornithobacterium rhinotracheale and H9N2 avian influenza virus. BMC Vet Res 8:104. 2012.
6. Tabatabai, L. B., M. K. Zimmerli, E. S. Zehr, R. E. Briggs, and F. M. Tatum. Ornithobacterium rhinotracheale North American field isolates express a hemolysin-like protein. Avian Dis 54:994-1001. 2010.
7. VDACS. VAgriculture Facts and Figures. Richmond, VA. 2014.

Chapter One: Review of the Literature

Introduction

In the poultry industry, diseases causing both morbidity and mortality are detrimental to production and can affect consumer prices. This is especially true for the turkey industry where birds are raised longer than other meat birds (i.e. broilers) in confinement operations. Since 1970, an increase in turkey consumption has stimulated the industry to increase production in the United States. In 1970, per capita consumption of turkey meat was 8.1 pounds (lbs). By 2011, this number had increased to 16.1 lbs. In the US alone, turkey production in 2011 totaled 248 million birds (7.319 million lbs of meat). Total exports increased over three million pounds from the year 2000, and Virginia was ranked 4th among turkey producing states in 2013 (72).

Respiratory diseases, particularly bacterial, are some of the most commonly encountered in the poultry industry and have the potential to cause large economic losses (18). While *Escherichia coli* is still the most prevalent secondary cause of mortality (18, 45), other bacterial agents are becoming increasingly important. *Ornithobacterium rhinotracheale* (ORT) is a respiratory agent that emerged in breeder turkeys in the 1980's (65) although identification was not attempted until 1991 (9)**.** More recently, ORT related disease in turkeys has increased, but less so as a primary pathogen and more as a suspected secondary or opportunistic pathogen. Effects causing morbidity and mortality can be amplified when seen with other disease-causing agents (13). Increased mortality, reduced egg production, and loss in weight gain give ORT the potential to have severe economic implications. In older birds, ORT causes more severe lesions and increases in mortality (56), therefore it is of particular concern in the breeder industry because infection and loss of production in breeders impacts all stages of production.

Development of useful vaccines, proper management, and further knowledge is necessary in understanding and implementing control of this disease.

History and Overview

O. rhinotracheale (ORT) is a Gram-negative, pleomorphic rod-shaped bacterium causing respiratory disease in the commercial poultry industry (21). Isolated first in Germany in 1981 from five-week old turkeys showing nasal discharge, facial edema and fibrinopurulent airsacculitis (11), the bacterium was isolated in the United States in 1989 (24). In 1993, it was formally characterized (9). In 1994, after isolating and evaluating 21 strains associated with various respiratory tract infections, *Ornithobacterium rhinotracheale* was given its current name (70). Before this, the organism had been referred to as: *Pasteurella*-like, *Kingella*-like, Pleomorphic Gram-Negative Rod (PGNR), and TAXON 28 (2). After extensive DNA-rRNA hybridization analysis, it was determined that the taxon should be placed on a separate phylogenetic branch within the rRNA superfamily V. Attention was also given to differentiating ORT from *Riemerella anatipestifer* and *Capnocytophaga* species because of the similarities in phenotypic characteristics (71). Although not closely related, *Ornithobacterium*, *Reimerella*, and *Coenonia* are all members of the family Flavobacteriaceae due to their phyolgenetic lineage (69). There are currently 18 known serotypes (A-R) of ORT determined by agar gel precipitation (AGP) serotyping methods (11, 64). A geographical correlation was found associated with serotype. Specifically, van Empel *et al.* found that all serotype C isolates originated in California, and those found in South American and the UK were serotype A. No host specificity was found related to serotype, but researchers have found that most chicken isolates are of serotype A and turkeys have more heterogeneity, most commonly having serotype A, B, and D (64). Serotyping

by AGP is the preferred method, because identifying genetic sequences such as 16S rRNA and a dihydrolipoamide acetyltransferase gene known as OR1 were not able to distinguish serotypes (41). Numee *et al.* found that seven isolates could not be serotyped based on whole cell antigen and antisera from the 12 known serotypes they had available for testing (A-L), suggesting that these isolates either were from the remaining six types (M-R), or other serotypes may be in existence that have not been previously characterized (41).

Clinical signs and lesions of ornithobacteriosis seen in birds by both experimental and field infection are similar to those shown with other respiratory disease-causing agents. Birds affected by the disease will often show clinical signs including weakness, lethargy with ruffled feathers, notable snicking, marked dyspnea, coughing, decreased feed and water intake, and a possible gasping and expectoration of blood stained mucus (20, 56). In older birds, increased mortality may be the only sign of sickness. Just prior to death, slight lethargy, gasping, dypsnea, and blood stained mucus are often observed (10). In general, gross lesions in older birds and breeders may include nasal discharge, facial edema, and swelling in the infra-orbital sinuses. Additionally, airsacculitis, pericarditis, peritonitis, and a mild form of tracheitis are associated with infection (10). Experiments have shown that ORT is pathogenic to both young and old turkeys as well as other bird species (4, 49, 56, 63), but under field conditions, older birds 16-24 weeks of age often show more severe clinical signs than younger birds (47, 56). The range of severity of lesions and clinical signs suggests differences in virulence among the ORT strains tested (49, 67).

Prevalence and Serology

Surveys have shown that the vast majority of turkey flocks in Europe, North America, South America, Africa, and some Asian countries have had contact with ORT (2, 6, 7, 12, 26, 61, 62, 65). The bacterium has been isolated from multiple bird species including chicken, partridge, duck, goose, guinea fowl, ostrich, gull, pheasant, pigeon, quail, rook, and turkey (50), which demonstrates a broad potential reservoir. However, repetitive sequenced based PCR (rep-PCR) fingerprinting of ORT isolates showed that the profiles of strains from passeriform birds, such as rooks, were different than those found in galliforms, such as turkeys, chickens, guinea fowl and partridges (1). Results from both 16S rRNA gene sequence comparison and rep-PCR analysis indicate that there is much broader variation of isolates in wild populations than domesticated birds. This variation suggests that the bacteria may have been introduced into commercial poultry from wild species (1).

The epidemiology and route of infection of ORT are not well understood. In one case report from 1996, a temporal and spatial proximity between two ranches suggested that ORT spread from one to the other. Human traffic between the two farms was non-existent. During the time of the outbreak, weather was cool and foggy which suggests a possibility of transmission via water droplets. Other possibilities include the use of an animal vector or that the disease may have already been present on the farms and a predisposing agent provided opportunity for infection (12). Experiments have shown that vertical transmission circumstantially occurs from hen to progeny (4, 68), but further research is necessary to definitively prove the progression of the organism from hen to progeny. Horizontal transmission can occur in adult birds and is a key source of transmission (65)**.** Both of these findings are important concepts for routes of infection

with regards to the hatching incubator as a potential burden of infection (68) and in confinement operations where disease could spread through birds in close proximity.

Serological testing for other respiratory agents such as paramyxovirus-1 (PMV-1), *Bordetella avium* (BA), and hemorrhagic enteritis virus (HEV) is commonly performed in commercial poultry operations (60, 62). Both PMV-1 and BA are common causes of respiratory disease in poultry (18, 45). Virulent field isolates and vaccine strains of HEV are believed to be immunosuppressive, the effect being greater with the former vs. the latter, and can predispose turkeys to the development of secondary bacterial infections of the respiratory tract such as *E. coli* (55). This immunosuppression can be further enhanced by prior or concomitant exposure to other agents like PMV-1 and BA (44, 55). Infection with these disease-causing agents can amplify the effects and clinical signs observed with ORT (13).

Serological methods are useful in assessing exposure to the bacterium. While they may not help with early detection, many tests are available for use. A serum plate agglutination test (SPAT) has been used for antibody detection using a Minnesota isolate from a turkey with fibrinopurulent exudate as a bacterial whole-cell antigen (3). This test was standardized using known positive and negative turkey serum. The highest dilution of antigen that gave clear agglutination with positive serum and no reaction with negative serum was selected for further use. The SPAT was determined to have good sensitivity and specificity for ORT antibodies (3). An alternate study found this SPAT to detect only 65% of positive sera during the first two weeks of incubation and determined that the use of outer membrane proteins in an indirect ELISA was a better indicator eight weeks post exposure for detection of antibodies (30). A dot-immunobinding assay was also developed as a rapid detection method, but yielded a lower sensitivity than agglutination tests (16).

Multiple ELISAs have been developed for detection of ORT antibodies by the use of varying antigen types including: boiled extract antigen (64), extracted outer membrane proteins (OMP) (30), and SDS-antigen extraction (23). The boiled extract antigen is serotype-specific with regards to antibody detection. As stated previously, there are 18 serotypes of ORT (64) so this can be a disadvantage because of the necessity of multiple separate tests to rule out ORT in a diagnostic setting (64). Additionally, an ELISA using only one serotype as antigen could yield a false negative regarding exposure if alternate serotypes were present. In contrast, ELISAs that employ both the SDS-antigen extraction and OMP extraction allow for detection of multiple serotypes in a single assay, which makes them more useful as diagnostic screening techniques (23, 30).

Growth Characteristics and Biochemical Properties

In vitro, ORT is a slow growing bacterium and is often difficult to isolate. The lungs and trachea are the two most useful sites for isolation (4). The bacterium grows best on Columbia agar supplemented with 5% sheep's blood (CBA) in a candle jar or an environment consisting of approximately 5-10% CO_2. The plates should be incubated at least 48 hours at 37°C, and resulting colonies appear small, circular, and opaque to grayish in color (50). Pure cultures give off a butyric acid-like smell (71). Additionally, primary cultures often show differences in colony size, ranging from 1-3 mm after initial 48-hour incubation period, whereas colonies become more uniform in size when subcultured (64). Traditionally, ORT is non-hemolytic when grown on CBA, although incomplete hemolysis has been noted after 96 hours of incubation (5) and recently, hemolytic field isolates have been identified in North America (58). In addition to blood agar, isolates can grow readily on chocolate agar and tryptose soy agar. An agar that is

commonly used to differentiate Gram-negative bacteria, but on which ORT does not grow is MacConkey's agar. For growth in fluid media, brain heart infusion, *Pasteurella* broth, or Todd Hewitt broth are useful (11). When in contaminated culture, or when overgrowth of ORT colonies is suspected, researchers have found that a combination of 5.2 ug/ml of gentamicin and 5.0 ug/ml of polymyxin B can be added to blood agar media for selection (10).

Key biochemical characteristics that help identify ORT include a positive oxidase and β-galactosidase test, variable urease reactions, and a negative catalase test (22). However, a strain of oxidase negative ORT has been identified from turkeys in Germany (11). Recently, small colony variants (SCVs) of the organism have been identified on CBA where said colonies are seen growing alongside larger more characteristic colonies. These SCVs are oxidase negative, have a higher antimicrobial resistance level, and have a higher survival time in macrophages (73). ORT is commonly misidentified because most commercially available identification systems do not include the organism in their databases, but identification may be done using an API 20NE or an API ZYM strip by using number combinations from referenced literature (Biomerieux, France). The bacterium is often confused with *Pastuerella haemolytica, Weeksella* species, *or Flavobacterium meningosepticum* (10), but with the API 20NE strip, ORT typically codes 0220004 and 0020004 with positive reactions for oxidase, ß-galactosidase, and occasionally urease. The API ZYM system gives fourteen positive reactions, including: alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, napthol-AS-BI-phosphohydrolase, α-galactosidase, ß-galactosidase, α-glucosidase, and N-acetyl- ß-glucosaminidase. It gives five negative reactions including lipase, ß-glucuronidase, ß-glucosidase, α-mannosidase, and α-fucosidase (22). PCR analysis of the 784- bp 16S rRNA sequence has also been proven useful

for identification of ORT and can clearly differentiate it from similar pathogens such as *Riemerella anatipestifer* and *Coenonia anatina.* Primers correspondng to nucleotide positions 8 to 27 and reverse complement, 1467 to 1485 have proven to be the most useful (16S-F, 5'-AGAGTTTGATCCTGGCTCAG and 16S-R, 5'-GGTTACCTTGTTACGACTT) (1, 25).

Virulence Factors

Mechanisms of virulence in bacteria are numerous and include a variety of methods to overcome the host immune system. Iron acquisition mechanisms have been identified, which pathogens use to overcome a host's ability to limit iron available to bacteria during the infection process (57). One of the most common mechanisms of iron acquisition by bacteria, fungi, and some plants is by the use of siderophores. These are low molecular weight structures secreted from an organism with extremely high affinities for Fe(III) ions, which are used to overcome a lack of iron. The ability to secrete these compounds can contribute to an organism's virulence, and to its capacity of overwhelming the host immune system (37). In poultry specifically, *Bordetella avium* produces a dermonecrotoxic toxin and tracheal cytotoxin used to break down the mucociliary escalator and tracheal rings of the avian respiratory system (17). Virulence factors associated with an avian pathogenic *Escherichia coli* include LPS (Gram-negative endotoxin), fimbriae (allows attachment to epithelial surfaces), K1 capsular antigen (reduces association of bacteria with phagocytes), and temperature sensitive hemagglutinin (clumping of red blood cells) (36). *P. multocida* isolates have been shown to produce a capsule, lethal endotoxin, and LPS, all of which contribute to increased virulence in turkey poults (46).

Key bacterial virulence factors have been reported in ORT but do not include the presence of toxins or special structures. Recently, researchers have found that ORT strains in

turkeys and chickens in China have neuraminidase activity used to desialylate glycoproteins of its natural hosts (27). An iron acquisition mechanism has been identified with which the pathogen overcomes a host's ability to limit iron during infection (57). Unlike other microbial species, ORT does not produce siderophores, but utilizes one or more iron-protein receptor mediated uptake systems involving four outer membrane proteins (57). Although a specific receptor protein has not been identified, Tabatabai *et al.* suggested that the organism expressed a receptor for binding and acquiring iron from carrier proteins like sheep and bovine hemoglobin, ovotransferrin, and bovine transferrin (57). The presence of recently discovered small colony variants (SCVs) of ORT that show an increased antimicrobial resistance and increased survival in the presence of macrophages may contribute to pathogenesis of the organism (73). The SCV characteristics suggest a greater ability to survive the host immune system when compared to their wild-type counterparts. Soriano *et al.* found that ORT isolates from Mexico have hemagglutinating ability and the ability for *in vitro* adherence to chicken epithelial cells. Both of these characteristics are virulence factors and contribute to the pathogenicity of the organism (53).

Tabatabai *et al.* characterized hemolytic North American field isolates, noting that the hemolysis factor is a pore forming cytolytic protein (58). Isolates showed beta-hemolytic activity on blood plates after an extended incubation of 48 hours at room temperature. The hemolytic protein was characterized using Western blotting techniques, *in vitro* kinetic assays, and comparative mass spectrometry for an outer membrane protein extract. Similarities between this hemolytic protein and the leukotoxin of both *Mannheimia haemolytica* and *Actinobacillus actinoacetemcomitans* were found with homology of four and two peptides, respectively. This research was the first to describe field isolates from North America with hemolysin activity (58).

While these isolates were not tested regarding virulence capabilities in live birds, in other bacterial species hemolysis is an indicator of increased virulence (38). Additionally, the similarities with leukotoxins indicate an ability to dampen the immune system (leukocyte-toxic) so the presence of hemolysin activity could suggest an increased virulence in ORT.

A plasmid labeled pOR1 was identified in a rare strain of serotype K out of a turkey in Minnesota (25). Isolation was performed by use of a CsCl gradient, and the plasmid was sequenced using a combination of sub-cloning and primer walking. Characteristics show it is circular, supercoiled, and comprises 14,787 bp. Additionally, with a GC content of 35.7%, it is similar to the whole genome of ORT (37-39% GC content) (25, 71). Plasmid pOR1 was detected in two isolates out of 53 analyzed, both of which were serotype K. The rare occurrence of this plasmid suggests that it was recently introduced into the population and its selective advantage remains unclear. Genes that may have an advantage include replication genes and a laccase gene (a virulence factor preventing production of hydroxyl radicals found in *Cryptococcus neoformans*). Plasmid pOR1 also contains a heavy metal ATPase that confers a slightly higher copper resistance to plasmid-carrying strains, but since the effect only became apparent at high concentrations (0.5 mM), it is unlikely to have physiological significance (25). No other plasmids have been identified in this bacterial species (50).

Clinical Signs

Ornithobacteriosis occurs worldwide (2, 5, 12, 26, 53, 74), and the disease causes a variety of clinical signs. In one particular outbreak on a multiage grow-out farm, more severe clinical signs were seen in older turkeys, particularly males, and younger birds showed only subclinical infection with no evidence of respiratory signs (74). Clinical signs observed in older

males included weakness, dyspnea, cyanosis of the head, lethargy, coughing, sneezing, and gasping. Approximately 50% of toms and 20% of hens on the farm showed respiratory signs (74). Other commercial turkey flocks have displayed an increase in mortality and severity of clinical signs associated with age and gender (48). In turkey breeders, clinical signs of infection included nasal discharge, gasping and weakness. Those in egg production or undergoing artificial insemination experienced a higher mortality. Heavier birds in each sex were more severely affected, and mortality in the heavy toms was higher than that of hens, which is likely due to the heavier build and increased weight of toms. Upon necropsy and histopathologic examination, significant pathology was noted in lungs and airsacs (12). In commercial chickens, flocks of broilers, broiler breeders, and layers all showed clinical signs associated with respiratory pathology (5). In commercial meat turkeys, mortality ranged between 1-15% during the acute phase (12).

Although ORT is capable of causing disease as a primary pathogen (67), concomitant exposure to other disease-causing agents can cause higher than average morbidity and mortality, as well as increased severity of lesions. In northern and middle Jordan, *E. coli*, *B. avium*, and ORT were isolated from broilers chickens with airsacculitis. While the largest percentage of airsacculitis was associated with isolation of *E. coli* alone, a high percentage involved various combinations of the three (15). Severe bronchial obstruction coupled with high mortality correlated with concomitant infection of ORT and *Streptococcus zooepidemicus* were discovered in chickens in China. This finding suggested that the combination of these two agents may be associated with the outbreak of bronchial embolization (42). Co-infection with ORT and H9N2 avian influenza virus has been found in chickens in China and is associated with extensive economic losses due to airsacculitis (43).

Experimental Reproduction

Multiple studies have been performed to reproduce a field-type infection in turkeys (49, 56, 63). Sprenger *et al.*, exposed twenty-five 22-week-old commercial male turkeys to both ORT and lung homogenate from turkeys that had been infected with the bacteria (56). Within 24 hours post-inoculation (PI), turkeys inoculated with 2.5×10^{10} CFU/ml intra-tracheally were showing decreased feed intake, lethargy, and coughing. By 48 hours PI, several of the turkeys were producing bloody mucoid expectorates and died within 24 hours. Five days after inoculation with ORT, surviving turkeys were less depressed and clinical signs had subsided. Control birds remained clinically normal throughout the duration of the trial. ORT-infected birds showed a severe weight loss when compared with controls, but statistical analysis was not completed and thus is a limitation of the study. Upon necropsy, gross lesions included airsacculitis, consolidated/edematous lungs, and pericarditis with the intra-tracheal inoculum. Exposure to an aerosolized inoculum produced no clinical signs, but gross lesions were similar to those in the previous trial. Reasons for success in replicating field cases included the use of turkeys typical of the age associated with field outbreaks and those that were known to be bacteriologically and serologically negative prior to exposure. Additionally, intra-tracheal inoculation producing severe pneumonic lesions and mortality as opposed to previous methods of intra-air sac and aerosol inoculation that showed only growth retardation and airsacculitis (63) contributed to the success of the Sprenger *et al.* study. Research has also shown that ORT propagated in live birds, and inoculated as a lung homogenate was capable of causing disease similar to that seen in field outbreaks using fewer CFUs (2 logs less) when compared to inoculum prepared from *in vitro* cultures (56).

Ryll *et al.* demonstrated that younger birds could also be experimentally infected with ORT. A trial using 10-day-old turkey poults yielded huddling, ruffled feather, and slight rhinitis in one group, shivering, crowding, mucosal rhinitis, dyspnea, and depressed activity in another group (49). The severity of gross lesions was strain dependent and ranged from moderate inflammation of thoracic airsacs to a severe generalized serofibrinous airsacculitis and bronchopneumonia (49). Experiments to determine tissue distribution of experimental infection found lungs and trachea to be the most common sites of isolation (4). Other organs from which ORT was isolated included the liver, airsacs, ovaries, oviducts, and spleen (4).

Experiments determining the effect of ORT in broilers have also been well documented. Infection of broilers via intra-air sac inoculation caused a significantly lower mean rate of gain and mean final body weight when compared to those challenged by aerosolization of the organism. Prior administration of both Newcastle Disease Virus and Infectious Bronchitis Virus produced severe lesions in air sacs and trachea whereas sole infection with ORT only yielded growth retardation and decreased feed and water intake (63). However, van Veen demonstrated that ORT alone could cause lesions in broilers following aerosol challenge (67). Infection with ORT via intravenous, intra-air sac, and intra-tracheal routes was used to compare two ORT isolates in Jordan. All three inoculation routes were found to cause a decrease in mean rate of gain and mean final body weight when compared to controls (15). Studies have also suggested that pathogenicity differences between strains and susceptibility of chickens due to production status exist. Prior or concomitant exposure to other pathogens like Infectious Bronchitis Virus, *E. coli* (59), Newcastle disease virus (PMV-1) (67) and avian influenza virus (H9N2) (43) aggravates lesions.

Hafez described a method using chicken embryos to determine differences in virulence among isolates (personal communication-H.M. Hafez, (50)). For infection, specific pathogen free chicken eggs were inoculated via allantoic sac at 11 days of development with a dose of 500 CFU/egg of varying ORT strains. A total of 20 eggs per isolate were used, and eggs were observed 8 days post-inoculation for mortality and cultured for bacterial re-isolation. Isolates causing 10-20% mortality were considered of low pathogenicity, those causing 21-60% mortality were moderately pathogenic, and those causing greater than 60% mortality were considered highly pathogenic. This assay proved beneficial due to its accessibility and low cost as opposed to the cost and many problems associated with experimental infection in live birds (personal communication-H.M. Hafez, (50)).

As previously noted, the presence of concomitant infection with another disease causing agent experimentally often causes an increase in morbidity and mortality. Infection with ORT via intra-tracheal inoculation following exposure to *Bordetella avium* via intra-tracheal inoculation produced severe unilateral pneumonic lesions in turkeys. *B. avium* as a bacterial species is able to produce cytotoxins to destroy tracheal cilia (17), which would allow agents like ORT and *E. coli* to enter the lower respiratory tract. Therefore, this finding may suggest that pneumonic lesions caused by ORT are most likely to occur when local immune defenses are compromised (14). In egg-laying chickens, a study using the combination of ORT with *E. coli* and infectious bronchitis virus yielded exacerbation of clinical signs and significantly higher lesion scores than any agent alone (59). Turkey breeders have also shown a more complicated infection and an increased severity of lesions with a concomitant *E. coli* infection (12). Experimental concurrent infection with Newcastle disease virus aggravated lesions caused by ORT (67). Marien *et al.* demonstrated that combined APV(Avian Pneumovirus)/ORT infections resulted in clinical

respiratory signs, longer persistence of ORT in the respiratory tract, and more severe macroscopic and histological lesions when compared to those infected with a single agent (35).

Economic Implications

Economically, ORT infection has been shown to adversely affect egg production, growth, medication costs, mortality and condemnation rates in commercial turkey flocks (65). van Empel and others have suggested a substantial economic impact of ornithobacteriosis, but have not quantified the cost of disease (50). Disease affects morbidity and mortality (average mortality of ~2.4% in acutely affected ORT hen flocks (12)), which are costly due to loss in meat (pounds) and live birds. In addition to lost pounds, a decreased gain could cause a requirement of birds to remain on the farm longer to achieve goal weight for the plant, adding additional resource costs. Individual flock values may be small, but for a large scale operation with multiple flocks affected, heavy economic losses can be attributable to this disease. Additionally, any changes in factors including prior or concomitant infections, management issues, price fluctuations, or bird type other than light hen could cause increases in the cost of an ORT infection to the producer and the company. Other factors that would also affect the value of losses would include such things as feed conversion ratio, medications costs and most notably, a decrease in line speeds at the processing plant due to lack of bird size uniformity. Each of these is directly related to the economic damages seen with any disease and can be detrimental for production. In turkey breeder hens, broiler breeder hens, and table egg layers, the impact would be on egg production. Thus, the cost of eggs would increase as number produced declined. Additionally, each potential egg lost from a breeder hen with decreased production indicates lost pounds of meat that could

have been sent to slaughter. When these additional losses are considered, ornithobacteriosis has the potential to be extremely costly.

Increased condemnation of broilers at slaughter was found to be associated with ORT (66). One case report indicated that of the birds condemned at slaughter, airsacculitis was found in 84% of birds examined and ORT was cultured from lungs, airsac, bone marrow, tendons; the latter being quite unusual (66). Although less of a problem in broiler chickens, the economic consequences on a per flock basis could be just as detrimental as for turkeys.

Control

Antibiotic resistance

Resistance to antimicrobials among ORT isolates from chickens and turkeys has been noted and is dependent on geographical location and strain. In the United States, a study was performed that focused on the antibiotic resistance profiles of 125 ORT isolates from turkeys raised in Minnesota between 1996 and 2002. Using the Kirby-Bauer methodology as described by the National Committee for Clinical Laboratory Standards for fastidious Gram-negative organisms, the number of isolates resistant to spectinomycin, ampicillin, sulfadimethoxine, and ceftiofur varied from year to year, while the number resistant to penicillin remained low and consistent throughout the study. Those demonstrating resistance to gentamicin, trimethoprim sulfa, and tetracycline increased over the six year period. None of the isolates were resistant to clindamycin, and only one was resistant to erythromycin (33). In Mexico, a broth micro-dilution method was used to determine antimicrobial resistance of ORT reference and field strains. The study found variable susceptibility to amoxicillin, enrofloxacin, and oxytetracycline and a marked resistance to both fosfomycin and gentamicin (54). Marien *et al.* compared the efficacies

of enrofloxacin, florfenicol, and amoxicillin treatments against a combined infection with APV/*E. coli*/ORT in three-week-old turkey poults. Results indicated recovery was most successful after treatment with enrofloxacin followed by florfenicol, whereas amoxicillin was completely ineffective (34).

Vaccination

Vaccination has been mostly unsuccessful in the U.S., but because of the increasing resistance to antimicrobials and decrease of available medicines in food animal species, vaccination with autogenous bacterins have been used frequently (19). Researchers in India found that the vaccination of layer chickens with a killed bacterin at eight weeks followed by a booster at twelve weeks can be effective in protecting against ORT and minimizing pathological changes such as airscacculitis and pneumonia (39). Schuijffel *et al.* showed that vaccination of chickens with an injectable, multi-component subunit vaccine consisting of eight protein antigens mixed in a water-in-oil emulsion was able to produce a high level of protection against an aerosol ORT challenge (51). Furthermore, the group studied potential cross-protectivity across ORT serotypes by the use of each individual recombinant antigen. The study found that only an Or77 antigen conferred cross-protective immunity when administered as a single component, and they suggested its potential as a vaccine candidate (52).

Due to problems associated with the use of injectable vaccines in market birds i.e., the need for handling each bird individually, the short-lived protection afforded by killed products, and injection site reactions in tissues destined for consumption, attempts have been made to create a live vaccine for mass administration. A temperature sensitive mutant strain (Ts) was created by subjecting ORT to *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and subculturing as

incubation temperature was gradually reduced from 41℃ to 31℃. With the creation of a stable mutant that was able to grow at 31℃ but unable to do so at 41℃, colonization of the upper respiratory tract was possible without progression to lower respiratory tract disease. No other biochemical or enzymatic differences between field and Ts strains were found. Following infection of poults with the mutant, a humoral response was detected using an outer membrane protein ELISA three weeks post-vaccination. The Ts strain could be recovered from both tracheas and choanae 13 days after oculonasal or drinking water administration (31). Both laboratory and field evaluations showed a reduction in the severity of gross lesions after vaccination and subsequent challenge (29). While reasoning is unknown as to why this vaccine is not used commercially, one possibility may include the fear of a reversion to virulence sometimes seen with live vaccination. This may be evaluated by repeated passage through birds to see if the vaccine's efficacy and virulence are affected.

With regards to maternal transmission of antibodies, vaccination against ORT in breeders can impact protection levels in progeny. In turkeys, vaccination has been circumstantially shown to reduce the vertical transmission of the organism and protect poults from infection until six weeks of age (68). However, this trial did not examine bacterial survival in every stage of production (i.e. hen, hen's reproductive tract, egg, embryo before pip, embryo at pip, and progeny), so further studies are necessary to determine the true impact of vaccination on vertical transmission. In chickens, vaccination of broiler breeders with an inactivated ORT serovar A bacteria in a water-in-oil emersion (Nobilis ORT inac-from Intervet International B.V., Boxmeer, The Netherlands) caused a rise in antibody titers, and titers remained high during the production period. While there was no significant difference in production between unvaccinated and vaccinated breeder flocks, maternal (IgM) antibody titers were significantly higher, mean

mortality was lower, and mean production index (a factor of weight gain and feed conversion measured in poultry production (28)) was higher in progeny from vaccinated breeders (8).

Management/Environmental

As with many poultry diseases, management problems can contribute to the severity of an ORT infection in a flock. Poor ventilation and high ammonia levels can exacerbate clinical signs associated with infection (45). Ammonia can cause sufficient damage to the tracheal epithelium to predispose birds to infection, as seen with *E. coli* infection. During the winter months, ammonia levels in houses rise and ventilation quality decreases due to efforts to conserve heat and decrease fuel costs. Decreased air quality and toxic levels of ammonia can allow breakdown of respiratory defenses and infection by opportunistic pathogens (40). Serologic surveillance has indicated a seasonal influence of ORT infection and clinical signs related to the disease (32). A study by Lopes *et al.* determined that the organism could withstand environmental temperatures in litter as low as -12°C for up to 150 days, but could not withstand elevated temperatures of 22°C, 37°C, and 42°C for long periods of time (32). This finding contributes support to an increased prevalence of ORT infection during the winter months.

References:

1. Amonsin, A., J. Wellehan, L. Li, P. Vandamme, C. Lindeman, M. Edman, R. Robinson, and V. Kapur. Molecular epidemiology of Ornithobacterium rhinotracheale. J. Clin. Microbiol. 35:2894-2898. 1997.

2. Asadpour, Y., M. H. Bozorgmehrifard, S. A. Pourbakhsh, M. Banani, and S. Charkhkar. Isolation and identification of Ornithobacterium rhinotracheale in broiler breeder flocks of Guilan province, north of Iran. Pak J Biol Sci 11:1487-1491. 2008.

3. Back, A., D. Halvorson, G. Rajashekara, and K. V. Nagaraja. Development of a serum plate agglutination test to detect antibodies to Ornithobacterium rhinotracheale. J Vet Diagn Invest 10:84-86. 1998.

4. Back, A., G. Rajashekara, R. B. Jeremiah, D. A. Halvorson, and K. V. Nagaraja. Tissue distribution of Ornithobacterium rhinotracheale in experimentally infected turkeys. Vet Rec 143:52-53. 1998.

5. Banani, M., S. A. Pourbakhsh, and P. Khaki. Characterization of Ornithobacterium rhinotracheale Isolates from Commercial Chickens. Arch. Razi Ins. 52:27-36. 2001.

6. Canal, C. W., J. A. Leao, D. J. Ferreira, M. Macagnan, C. T. Pippi Salle, and A. Back. Prevalence of antibodies against Ornithobacterium rhinotracheale in broilers and breeders in Southern Brazil. Avian Dis 47:731-737. 2003.

7. Canal, C. W., J. A. Leao, S. L. Rocha, M. Macagnan, C. A. Lima-Rosa, S. D. Oliveira, and A. Back. Isolation and characterization of Ornithobacterium rhinotracheale from chickens in Brazil. Res Vet Sci 78:225-230. 2005.

8. Cauwerts, K., P. De Herdt, F. Haesebrouck, J. Vervloesem, and R. Ducatelle. The effect of Ornithobacterium rhinotracheale vaccination of broiler breeder chickens on the performance of their progeny. Avian Pathol 31:619-624. 2002.

9. Charlton, B. R., S. E. Channing-Santiago, A. A. Bickford, C. J. Cardona, R. P. Chin, G. L. Cooper, R. Droual, J. S. Jeffrey, C. U. Meteyer, H. L. Shivaprasad, and R. Walker. Preliminary characterization of a pleomorphic gram-negative rod associated with avian respiratory disease. J Vet Diagn Invest 5:47-51. 1993.

10. Chin, R. P., and B. R. Charlton. Ornithobacteriosis A Laboratory Manual for the Isolation and Identification of Avian Pathogens 4:89-91. 1998.

11. Chin, R. P., C. P. van Empel, and H. M. Hafez. Ornithobacterium rhinotracheale infection. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan and D. E. Swayne, eds. Wiley-Blackwell, Ames, Iowa. pp 765-774. 2008.

12. De Rosa, M., R. Droual, R. P. Chin, H. L. Shivaprasad, and R. L. Walker. Ornithobacterium rhinotracheale infection in turkey breeders. Avian Dis 40:865-874. 1996.

13. Droual, R. Pathogenicity Studies of Ornithobacterium rhinotracheale in Turkeys: Comparisons of Routes of Inoculation and Interaction with Bordetella Avium. In: The Turkey ORT Scientific Symposium. R. A. H. a. Nutrition, ed., Minneapolis, Minnesota. pp 33-36. 1996.

14. Droual, R. Pathogenicity Studies of Ornithobacterium rhinotracheale in Turkeys: Comparisons of Routes of Inoculation and Interaction with Bordetella Avium. In: The Turkey ORT Scientific Symposium. S. Clark, ed. Roche Animal Nutrition and Health, Minneapolis, Minnesota. 1996.

15. El-Sukhon, S. N., A. Musa, and M. Al-Attar. Studies on the bacterial etiology of airsacculitis of broilers in northern and middle Jordan with special reference to Escherichia coli, Ornithobacterium rhinotracheale, and Bordetella avium. Avian Dis 46:605-612. 2002.

16. Erganis, O., H. H. Hadimli, K. Kav, M. Corlu, and D. Ozturk. A comparative study on detection of Ornithobacterium rhinotracheale antibodies in meat-type turkeys by dot immunobinding assay, rapid agglutination test and serum agglutination test. Avian Pathol 31:201-204. 2002.

17. Gentry-Weeks, C. R., B. T. Cookson, W. E. Goldman, R. B. Rimler, S. B. Porter, and R. Curtiss, 3rd. Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of Bordetella avium. Infect Immun 56:1698-1707. 1988.

18. Glisson, J. R. Bacterial respiratory disease of poultry. Poult Sci 77:1139-1142. 1998.

19. Gornatti Churria CD, V. G., Machuca MA, Nievas VF, Nievas WD, et al. Vaccines against Ornithobacterium rhinotracheale: A Review. J. Vet Med Sci Med Diagn 2. 2013.

20. Hafez, H. M. Current Status on the Role of Ornithobacterium rhinotracheale (ORT) in Respiratory Disease Complexes in Poultry. In: The Turkey ORT Scientific Symposium. R. A. N. a. Health, ed., Minneapolis, Minnesota. 1996.

21. Hafez, H. M. Current status on the laboratory diagnosis of Ornithobacterium rhinotracheale "ORT" in poultry. Berl Munch Tierarztl Wochenschr 111:143-145. 1998.

22. Hafez, H. M. Diagnosis of Ornithobacterium rhinotracheale. International Journal of Poultry Science 1:114-118. 2002.

23. Hafez, H. M., A. Mazaheri, and R. Sting. Efficacy of ELISA for detection of antibodies against several Ornithobacterium rhinotracheale serotypes. Dtsch Tierarztl Wochenschr 107:142-143. 2000.

24. Hinz, K. H., and H. M. Hafez. The early history of Ornithobacterium rhinotracheale (ORT). Arch Geflùgelkd 61:95-96. 1997.

25. Jansen, R., N. Chansiripornchai, W. Gaastra, and J. P. van Putten. Characterization of plasmid pOR1 from Ornithobacterium rhinotracheale and construction of a shuttle plasmid. Appl Environ Microbiol 70:5853-5858. 2004.

26. Joubert, P., R. Higgins, A. Laperle, I. Mikaelian, D. Venne, and A. Silim. Isolation of ornithobacterium rhinotracheale from turkeys in Quebec, Canada. Avian Dis 43:622-626. 1999.

27. Kastelic, S., R. L. Bercic, I. Cizelj, M. Bencina, L. Makrai, O. Zorman-Rojs, M. Narat, M. Bisgaard, H. Christensen, and D. Bencina. Ornithobacterium rhinotracheale has neuraminidase activity causing desialylation of chicken and turkey serum and tracheal mucus glycoproteins. Vet Microbiol 162:707-712. 2013.

28. Lima, A., and I. Naas. Evaluating Two Systems of Poultry Production: Conventional and Free-Range. Brazilian Journal of Poultry Science 7:215-220. 2005.

29. Lopes, V., A. Back, D. A. Halvorson, and K. V. Nagaraja. Minimization of pathologic changes in Ornithobacterium rhinotracheale infection in turkeys by temperature-sensitive mutant strain. Avian Dis 46:177-185. 2002.

30. Lopes, V., G. Rajashekara, A. Back, D. P. Shaw, D. A. Halvorson, and K. V. Nagaraja. Outer membrane proteins for serologic detection of Ornithobacterium rhinotracheale infection in turkeys. Avian Dis 44:957-962. 2000.

31. Lopes, V. C., A. Back, H. J. Shin, D. A. Halvorson, and K. V. Nagaraja. Development, characterization, and preliminary evaluation of a temperature-sensitive mutant of Ornithobacterium rhinotracheale for potential use as a live vaccine in turkeys. Avian Dis 46:162-168. 2002.

32. Lopes, V. C., B. Velayudhan, D. A. Halvorson, and K. V. Nagaraja. Survival of Ornithobacterium rhinotracheale in sterilized poultry litter. Avian Dis 46:1011-1014. 2002.

33. Malik, Y. S., K. Olsen, K. Kumar, and S. M. Goyal. In vitro antibiotic resistance profiles of Ornithobacterium rhinotracheale strains from Minnesota turkeys during 1996-2002. Avian Dis 47:588-593. 2003.

34. Marien, M., A. Decostere, L. Duchateau, K. Chiers, R. Froyman, and H. Nauwynck. Efficacy of enrofloxacin, florfenicol and amoxicillin against Ornithobacterium rhinotracheale and Escherichia coli O2:K1 dual infection in turkeys following APV priming. Vet Microbiol 121:94-104. 2007.

35. Marien, M., A. Decostere, A. Martel, K. Chiers, R. Froyman, and H. Nauwynck. Synergy between avian pneumovirus and Ornithobacterium rhinotracheale in turkeys. Avian Pathol 34:204-211. 2005.

36. Mellata, M., M. Dho-Moulin, C. M. Dozois, R. Curtiss, 3rd, B. Lehoux, and J. M. Fairbrother. Role of avian pathogenic Escherichia coli virulence factors in bacterial interaction with chicken heterophils and macrophages. Infect Immun 71:494-503. 2003.

37. Miethke, M., and M. A. Marahiel. Siderophore-based iron acquisition and pathogen control. Microbiology and molecular biology reviews : MMBR 71:413-451. 2007.

38. Minshew, B. H., J. Jorgensen, G. W. Counts, and S. Falkow. Association of hemolysin production, hemagglutination of human erythrocytes, and virulence for chicken embryos of extraintestinal Escherichia coli isolates. Infect Immun 20:50-54. 1978.

39. Murthy, T. R., N. Dorairajan, G. A. Balasubramaniam, A. M. Dinakaran, and R. Kalaimathi. The effect of vaccination of pullets against Ornithobacterium rhinotracheale infection. Avian Pathol 36:481-485. 2007.

40. Nagaraja, K. V., D. A. Emery, K. A. Jordan, J. A. Newman, and B. S. Pomeroy. Scanning electron microscopic studies of adverse effects of ammonia on tracheal tissues of turkeys. Am J Vet Res 44:1530-1536. 1983.

41. Numee, S., R. Hauck, and H. M. Hafez. Detection and typing of Ornithobacterium rhinotracheale from German poultry flocks. Avian Dis 56:654-658. 2012.

42. Pan, Q., A. Liu, and C. He. Co-infection of Ornithobacterium rhinotracheale with Streptococcus zooepidemicus in chickens. Avian Dis 56:680-684. 2012.

43. Pan, Q., A. Liu, F. Zhang, Y. Ling, C. Ou, N. Hou, and C. He. Co-infection of broilers with Ornithobacterium rhinotracheale and H9N2 avian influenza virus. BMC Vet Res 8:104. 2012.

44. Pierson, F. W., V.D. Barta, D. Boyd, and W.S. Thompson. . The association between exposure to multiple infectious agents and the development of colibacillosis in turkeys. J. Appl. Poult. Res 5:347-357. 1996.

45. Poss, P. E. Turkey industry strategies for control of respiratory and enteric diseases. Poult Sci 77:1181-1185. 1998.

46. Rhoades, K. R., and R. B. Rimler. Effects of Pasteurella multocida endotoxins on turkey poults. Avian Dis 31:523-526. 1987.

47. Roepke, D. C. Field Observations of ORT Infection in an Integrated Turkey Operation. In: The Turkey ORT Scientific Symposium. R. A. N. a. Health, ed., Minneapolis, Minnesota. 1996.

48. Roepke, D. C., A. Back, D. P. Shaw, K. V. Nagaraja, S. J. Sprenger, and D. A. Halvorson. Isolation and identification of Ornithobacterium rhinotracheale from commercial turkey flocks in the upper midwest. Avian Dis 42:219-221. 1998.

49. Ryll, M., K. H. Hinz, H. Salisch, and W. Kruse. Pathogenicity of Ornithobacterium rhinotracheale for turkey poults under experimental conditions. Vet Rec 139:19. 1996.

50. Saif, Y. M., J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Diseases of Poultry, 12 ed. Blackwell Publishing, Ames, Iowa. 2008.

51. Schuijffel, D. F., P. C. van Empel, A. M. Pennings, J. P. van Putten, and P. J. Nuijten. Successful selection of cross-protective vaccine candidates for Ornithobacterium rhinotracheale infection. Infect Immun 73:6812-6821. 2005.

52. Schuijffel, D. F., P. C. Van Empel, R. P. Segers, J. P. Van Putten, and P. J. Nuijten. Vaccine potential of recombinant Ornithobacterium rhinotracheale antigens. Vaccine 24:1858-1867. 2006.

53. Soriano, V. E., M. G. Longinos, P. G. Navarrete, and R. P. Fernandez. Identification and characterization of Ornithobacterium rhinotracheale isolates from Mexico. Avian Dis 46:686-690. 2002.

54. Soriano, V. E., N. A. Vera, C. R. Salado, R. P. Fernandez, and P. J. Blackall. In vitro susceptibility of Ornithobacterium rhinotracheale to several antimicrobial drugs. Avian Dis 47:476-480. 2003.

55. Sponenberg, D. P., C.H. Domermuth, and C.T. Larsen Field outbreaks of colibacillosis of turkeys associated with hemorrhagic enteritis virus. Avian Dis 29:838-842. 1985.

56. Sprenger, S. J., A. Back, D. P. Shaw, K. V. Nagaraja, D. C. Roepke, and D. A. Halvorson. Ornithobacterium rhinotracheale infection in turkeys: experimental reproduction of the disease. Avian Dis 42:154-161. 1998.

57. Tabatabai, L. B., E. S. Zehr, M. K. Zimmerli, and K. V. Nagaraja. Iron acquisition by Ornithobacterium rhinotracheale. Avian Dis 52:419-425. 2008.

58. Tabatabai, L. B., M. K. Zimmerli, E. S. Zehr, R. E. Briggs, and F. M. Tatum. Ornithobacterium rhinotracheale North American field isolates express a hemolysin-like protein. Avian Dis 54:994-1001. 2010.

59. Thachil, A. J., B. T. Velayudhan, D. P. Shaw, D. A. Halvorson, and K. V. Nagaraja. Pathogenesis of Ornithobacterium rhinotracheale in egg-laying hens with coexisting infectious bronchitis virus and Escherichia coli infections J. Appl. Poult. Res. 18:780-788. 2009.

60. Travers, A. F. Concomitant Ornithobacterium rhinotracheale and Newcastle disease infection in broilers in South Africa. Avian Dis 40:488-490. 1996.

61. Travers, A. F., L. Coetzee, and B. Gummow. Pathogenicity differences between South African isolates of Ornithobacterium rhinotracheale. Onderstepoort J Vet Res 63:197-207. 1996.

62. Turkyilmaz, S., and O. Kaya. Detection of Antibodies Produced against Ornithobacterium rhinotracheale and Bordetella avium by Enzyme-Linked Immunosorbent Assay in Hens and Turkeys in Aydin Province, Turkey. Turk J Vet Anim Sci 29:897-902. 2005.

63. van Empel, P., H. van den Bosch, D. Goovaerts, and P. Storm. Experimental infection in turkeys and chickens with Ornithobacterium rhinotracheale. Avian Dis 40:858-864. 1996.

64. van Empel, P., H. van den Bosch, P. Loeffen, and P. Storm. Identification and serotyping of Ornithobacterium rhinotracheale. J Clin Microbiol 35:418-421. 1997.

65. van Empel, P. C., and H. M. Hafez. Ornithobacterium rhinotracheale: a review. Avian Pathol 28:217-227. 1999.

66. van Veen, L., E. Gruys, K. Frik, and P. van Empel. Increased condemnation of broilers associated with Ornithobacterium rhinotracheale. Vet Rec 147:422-423. 2000.

67. van Veen, L., C. P. van Empel, and T. Fabria. Ornithobacterium rhinotracheale, a primary pathogen in broilers. Avian Dis 44:896-900. 2000.

68. van Veen, L., M. Vrijenhoek, and P. van Empel. Studies of the transmission routes of Ornithobacterium rhinotracheale and immunoprophylaxis to prevent infection in young meat turkeys. Avian Dis 48:233-237. 2004.

69. Vandamme, P., H. M. Hafez, and K. H. Hinz. Capnophilic Bird Pathogens in the Family Flavobacteriaceae: Riemerella, Ornithobacterium, and Coenonia. Prokaryotes 7:695-708. 2006.

70. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, L. A. Devriese, M. Bisgaard, K. H. Hinz, and W. Mannheim. Ornithobacterium rhinotracheale gen. nov., sp. nov., isolated from the avian respiratory tract. Int J Syst Bacteriol 44:24-37. 1994.

71. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, and et al. Ornithobacterium rhinotracheale gen. nov., sp. nov., isolated from the avian respiratory tract. Int J Syst Bacteriol 44:24-37. 1994.

72. VDACS. VAgriculture Facts and Figures. In., Richmond, VA. 2014.

73. Zahra, M., M. Ferreri, R. Alkasir, J. Yin, B. Han, and J. Su. Isolation and Characterization of Small-Colony Variants of Ornithobacterium rhinotracheale. J Clin Microbiol. 2013.

74. Zorman-Rojs, O., I. Zdovc, D. Bencina, and I. Mrzel. Infection of turkeys with Ornithobacterium rhinotracheale and Mycoplasma synoviae. Avian Dis 44:1017-1022. 2000.

Chapter Two: Seroprevalence of *Ornithobacterium rhinotracheale* and Other Select Respiratory Pathogens in Market Turkeys and Effects on Performance

Summary

Ornithobacterium rhinotracheale (ORT) is a bacterial pathogen that causes respiratory disease and economic loss in the commercial poultry industry. This study was initiated after irregular hemolytic ORT isolates were isolated from turkeys in Virginia's Shenandoah Valley. A total of 175 flocks (930 serum samples) were evaluated by Enzyme Linked Immunosorbent Assays (ELISA) for the presence of antibodies to hemolytic and non-hemolytic isolates of ORT, hemorrhagic enteritis virus (HEV), *Bordetella avium* (BA), and paramyxovirus-1 (PMV-1). All turkeys sampled were market hens (light 12-15 weeks-of-age, heavy 16-20 weeks-of-age). The seroprevalence for HEV was 100% due to vaccination at approximately 4 weeks of age. That of PMV-1, BA, and ORT was 6.3%, 74.8%, 70.9% respectively. Effects on performance were seen with relation to infection.

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a Gram-negative, pleomorphic rod shaped bacterium that has been recognized to be of economic importance in the poultry industry (7, 23, 25). This bacterium was first isolated in Germany in 1981 (12), and then in the United States in 1989 (7). Characterization was completed in 1993 (7), and it was given its current taxonomic assignment in 1994 (24). Serological surveys and field isolations have shown that the vast majority of turkey flocks in Europe, North America, South America, Africa, and some Asian countries have been exposed to ORT (1, 4, 5, 9, 13, 21-23).

A respiratory pathogen of birds, ORT may cause clinical signs in older turkeys and breeders including: nasal discharge, facial edema, and swelling in the infraorbital sinuses (2, 8, 9). Airsacculitis, pneumonia, pericarditis, peritonitis, and mild tracheitis are observed on necropsy (17, 24). The trachea and lungs are the most common sites of isolation (2). Most ORT isolates are non-hemolytic when grown on Columbia agar with 5% sheep's blood ((24), but incomplete hemolysis has been noted after 96 hours of incubation (3). Researchers recently identified and partially characterized hemolytic North American field isolates, noting that the hemolysis factor is a pore forming cytolytic protein (18).

Serological testing for respiratory agents such as paramyxovirus-1 (PMV-1), *Bordetella avium* (BA), and hemorrhagic enteritis virus (HEV) is commonly performed in commercial turkey operations (20, 22). Both PMV-1 and BA are common causes of respiratory disease (11, 15), especially in younger poults. Virulent field isolates and vaccine strains of HEV are believed to immunosuppressive, the effect being greater with the former vs. the latter and can predispose turkeys to the development of secondary bacterial infections of the respiratory tract. This effect is enhanced by prior or concomitant exposure to other agents like PMV-1 and BA(14, 16).

Since research has previously shown that mixed infections with other respiratory pathogens worsen the severity of ORT-mediated disease (19) and it has been found that ORT is frequently isolated with other respiratory organisms (6), one objective of this study was to determine whether serology performed just prior to slaughter would confirm these relationships. A second objective was to determine the significance of the irregular hemolytic isolates found in Virginia. We speculated that there may be differences in seroprevalence relative to hemolytic and non-hemolytic isolates.

MATERIALS AND METHODS

Sample Collection.

Blood was obtained by service personnel from 175 hen flocks just prior to market (light: 12-15 weeks-of-age, heavy: 16-20 weeks-of-age). Flocks were vaccinated against HEV, but no other disease causing agents. The number of samples per flock ranged from four to fifteen. Serum was collected at Virginia Department of Agriculture and Consumer Services Laboratory (VDACS, Harrisonburg, VA), stored at -20 ˚C, and then transported to the Avian Disease Research Laboratory at the Virginia-Maryland Regional College of Veterinary Medicine for analysis. Performance data for the same flocks were pulled from company records.

Bacterial Isolates and Culture Methods.

Two ORT isolates obtained from field cases submitted to the VDACS lab were used in this study: ORT 22470-08 lg (designated hemolytic, H, due to presence of alpha-hemolysis on Columbia agar with 5% sheep blood after 48 hours incubation), and ORT 725-09 (designated as non-hemolytic, NH, based on the absence of alpha hemolysis after 48 hours of incubation). Both were originally obtained from the VDACS lab in Harrisonburg, VA and were isolated from turkeys with respiratory disease signs in Virginia. For propagation, isolates were inoculated onto Columbia agar plates containing 5% sheep blood (Remel Products, Lenexa, KS) and incubated in a candle jar at 37 ˚C for 48 hours.

Enzyme Linked Immunosorbent Assay (ELISA).

Coating antigen was prepared for the ORT ELISA from both the non-hemolytic and hemolytic isolates using a modified procedure described by van Empel (21, personal correspondence). Hemolytic and non-hemolytic isolates were tested separately to determine serum cross-reactivity. Bacterial lawns were grown as previously described in culture methods. After incubation,

bacteria were washed from the plate with phosphate buffered saline (PBS) and suspended in a mixture of 3% formalin, 8.5% NaCl, and four ml PBS, boiled for 60 minutes at 100 ˚C, and centrifuged at 13,000 rpm (~17,900 x g) for four minutes in an Eppendorf® 5415 R tabletop microcentrifuge. The supernatant was diluted 1:100 in 0.5 M sodium carbonate coating buffer (pH 9.6). Ninety-Six well ELISA plates (Corning Life Sciences, Lowell, MA) were rinsed with coating buffer and then 200 µl per well of the diluted antigen was added to each well and allowed to incubate overnight at 4 ˚C. After washing with PBS containing 0.5% Tween 80 (PBST-80) solution, blocking was accomplished by adding 250 µl of 0.2% Bovine Serum Albumin in PBST-80 to each well followed by incubation for 45 minutes at 37 ˚C. Plates were again washed with PBST-80, and then test serum was added at a 1:1000 dilution in PBST-80 and incubated for two hours at room temperature. After a third wash with PBST-80, 100 microliters of horseradish peroxidase (HRP) conjugated goat anti-turkey IgG (KPL, Inc., Gaithersburg, MD) at a 1:10,000 dilution in PBST-80 was added to each well and allowed to incubate at room temperature for 45 minutes. Following a final, fourth wash with PBST-80, 100 µl of TMB Microwell Peroxidase Substrate (KPL, Inc., Gaithersburg, MD) was added to each well and incubated for five minutes. The reaction was stopped by the addition of 100 microliters of 1N HCl to each well. Plates were read at λ450 on a SpectraMax340PC^{384} microplate reader and analyzed using SoftMax® Pro Data Acquisition and Analysis Software (Molecular Devices, LLC, Sunnyvale, CA).

ELISA Optimization.

The ELISA was optimized using a standard checkerboard design with known positive and negative sera (26). Optimal dilutions were determined to be a 1:100 for the antigen and 1:1000 for serum. Validation was performed using negative sera (average OD=0.09 @ 450 nm) obtained

from turkeys raised in isolation at the Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech and presumed-positive sera (average OD=0.79 @ 450 nm) obtained from confirmed ORT infected flocks in the field. Seventy-five samples from each were tested. An optical density reading of 0.5 was set as the lower-limit threshold for the positive controls (1x standard deviation below the average positive value). While negative controls only reached an OD reading of 0.25 (max negative control value), anything between 0.25 and 0.5 was considered to be suspect and for the purposes of this serosurvey, not considered positive. Both positive and negative samples were compared on the commercial ORT ELISA developed by BioChek (ER Reeuwijk, The Netherlands) and obtained in the laboratory for research comparisons (product not available commercially in the U.S). A flock was considered positive if one or more of the sera out of the flock sample set had an OD greater than 0.5. Additionally, each plate contained three known positive and three known negative samples for comparison and standardization of plates.

Serology for BA, HEV, and PMV-1.

Serologic testing for BA, HEV, and PMV-1 antibodies was completed using ProFLOK® ELISA kits per the manufacturer's instructions (Synbiotics Corporation, Exton, PA). Briefly, sera was added to pre-coated plates at a dilution of 1:50 and allowed to react for 30 minutes at room temperature. Plates were washed and horseradish peroxidase (HRP) conjugated goat-anti-turkey IgG in HRP stabilizer at a 1:100 dilution was added to each well. Plates were incubated for 30 minutes before another wash procedure. Following the second wash, the commercial substrate solution was added and incubated for 15 minutes before the addition of stop solution. Plates were read at 405 nm and OD values were used to calculate positive and negative values. A flock was considered positive if one or more of the sera out of the sample set was positive.

Statistical Analysis.

Chi-square analyses were used to look for significant ($p \leq 0.05$) interactions relative to disease agents. Odds ratios were also calculated for each interaction. Analysis of variance ($p \leq 0.05$) was used to determine the effect of exposure to disease agents on performance (JMP,SAS Institute, Inc., Cary, NC).

RESULTS

Of the 175 flocks that were sampled, 175 were positive for HEV (100%) due to vaccination, 11 were positive for PMV-1 (6.3%), and 131 were positive for BA (74.8%) (Figure 1). A total of 124 flocks were positive for *O. rhinotracheale* (70.9%). Sera cross-reacted uniformly to the non-hemolytic and hemolytic isolates.

In regards to multiple agent interactions across all flocks, 86 flocks were positive for BA and ORT (49.1%), 2 flocks were positive for both PMV-1 and BA (1.1%), and 8 flocks were positive for PMV-1, BA and ORT (4.6%) (Figure 2). None of the flocks were positive for PMV-1 and ORT. A total of 35 flocks were positive for only BA (20.0%), 30 flocks were positive for only ORT (17.1%), 1 flock was positive for only PMV-1 (0.6%), and 13 flocks had no presence of antibodies to any of the disease agents tested for (7.4%) (Figure 2). None of the interactions were significant ($p > 0.05$).

The 175 flocks were also split into heavy and light hen flocks for comparison. Of the total, 39 were classified as heavy and 136 were considered light. Among heavy hens flocks, 34 were positive for BA (87.2%), 29 were positive ORT (74.3 %), and 3 were positive for PMV-1 (7.7%) (Figure 1). Analyzing for interactions, 24 flocks were positive for BA and ORT (61.5%), 1 was positive for both PMV-1 and BA (2.6%), and 1 was positive for PMV-1, BA and ORT

(2.6%) (Figure 2). A total of 8 heavy hen flocks were positive for BA only (20.5%), 4 were positive for ORT only (10.3%), and 1 was positive for PMV-1 only (2.6%) (Figure 2). All flocks were positive for at least 1 agent (*sic*, 0% had no evidence of exposure). Based on seroprevalence, interactions among the pathogens were not significant. Among light hen flocks, 97 were positive for BA (71.3%), 95 were positive ORT (69.9%), and 8 were positive for PMV-1 (5.9%) (Figure 1). When analyzing the interactions of agents, 62 flocks were positive for BA and ORT (45.6%), 1 was positive for both PMV-1 and BA (0.7%), and 7 were positive for PMV-1, BA and ORT (5.1%) (Figure 2). A total of 27 flocks were positive for BA only (19.9%), 26 were positive for ORT only (19.1%), 0 were positive for PMV-1 only (0%), and 13 had no antibodies to any of the disease agents tested for (9.6%) (Figure 2). Interactions were not significant.

Regarding performance parameters, while there was no significant correlation among the presence of antibodies for the agents tested ($p>0.05$), the presence of antibodies against BA and ORT had an effect on final performance parameters. Flocks with antibodies to ORT alone were significantly ($p<0.05$) lighter (average body weight) at slaughter than those with antibodies to both BA and ORT or BA alone (Tables 1 and 2).

DISCUSSION

Ornithobacterium rhinotracheale is a respiratory disease agent that is becoming more prevalent in Virginia's turkey industry, but exact prevalence and effect of disease was unknown. Previous research has shown that active infection with both BA and ORT causes increased severity of clinical signs and mortality (10), so the finding that the combination of antibodies produced heavier birds was unexpected. One possible explanation for the observation that birds were larger and gained better in flocks exposed to both organisms is that medication given to treat any clinical signs offers protection against other potentially harmful organisms. The combination of antibiotics and the increase in square footage for remaining birds (due to mortality) could result in better weight gain for surviving birds. Another possible explanation is that these birds were exposed to lower doses of both organisms compared to the birds in previous studies, and birds exposed to higher doses of BA and ORT may not have made it to slaughter age. Change in antibody level (OD) and infectious dose are unknown since all sera were collected just prior to slaughter, when birds were tested for avian influenza. This convenience sample does not enable us to establish a temporal or dose relationship between exposures since it represents a point prevalence. Ideally, acute and convalescent sera obtained in the peri-clinical period would be of much greater value. Similarly, it is unknown whether there is a correlation between antibody production, disease outbreaks and respiratory signs because all sera were obtained just prior to slaughter. Since flocks with serologic evidence of exposure to ORT alone had a lower medication:weight sold ratio than those with BA+ORT antibodies (Tables 1 and 2), another possibility is that ORT alone does not produce respiratory signs that would warrant medication (subclinical infection). The increased weight in BA+ORT birds may be related to the

administration of medication for BA associated respiratory signs. In addition, medication may be preventing infection with secondary, opportunistic pathogens like *E. coli.*

In regards to the presence of disease agents, vaccination at four weeks of age resulted in antibody production against HEV in 100% of flocks. No other agents could be accounted for by vaccination. The fact that a high percentage of flocks were seropositive for BA (75%) without a corresponding elevation in overall mortality suggests subclinical or mild clinical disease. The presence of antibodies against PMV-1 was rare, although the agent has been historically problematic in the production region (14). The high level of antibodies against ORT suggests that exposure is very common in commercial turkey flocks. As stated previously, ORT is typically non-hemolytic, and the significance of the atypical hemolytic isolate relative to morbidity, mortality, and performance could not be determined since the isolates were serologically indistinguishable.

References

1. Asadpour, Y., M. H. Bozorgmehrifard, S. A. Pourbakhsh, M. Banani, and S. Charkhkar. Isolation and identification of Ornithobacterium rhinotracheale in broiler breeder flocks of Guilan province, north of Iran. Pak J Biol Sci 11:1487-1491. 2008.
2. Back, A., G. Rajashekara, R. B. Jeremiah, D. A. Halvorson, and K. V. Nagaraja. Tissue distribution of Ornithobacterium rhinotracheale in experimentally infected turkeys. Vet Rec 143:52-53. 1998.
3. Banani, M., S. A. Pourbakhsh, and P. Khaki. Characterization of Ornithobacterium rhinotracheale Isolates from Commercial Chickens. Arch. Razi Ins. 52:27-36. 2001.
4. Canal, C. W., J. A. Leao, D. J. Ferreira, M. Macagnan, C. T. Pippi Salle, and A. Back. Prevalence of antibodies against Ornithobacterium rhinotracheale in broilers and breeders in Southern Brazil. Avian Dis 47:731-737. 2003.
5. Canal, C. W., J. A. Leao, S. L. Rocha, M. Macagnan, C. A. Lima-Rosa, S. D. Oliveira, and A. Back. Isolation and characterization of Ornithobacterium rhinotracheale from chickens in Brazil. Res Vet Sci 78:225-230. 2005.
6. Charlton, B. R., A. J. Bermudez, M. Boulianne, D. A. Halvorson, J. S. Schrader, L. J. Newman, J. E. Sander, and P. S. Wakenell, eds. Ornithobacterium rhinotracheale infection, 6th ed. American Association of Avian Pathologists, Athens, Georgia. 2006.
7. Charlton, B. R., S. E. Channing-Santiago, A. A. Bickford, C. J. Cardona, R. P. Chin, G. L. Cooper, R. Droual, J. S. Jeffrey, C. U. Meteyer, H. L. Shivaprasad, and R. Walker. Preliminary characterization of a pleomorphic gram-negative rod associated with avian respiratory disease. J Vet Diagn Invest 5:47-51. 1993.

8. Chin, R. P., and B. R. Charlton. Ornithobacteriosis A Laboratory Manual for the Isolation and Identification of Avian Pathogens 4:89-91. 1998.

9. De Rosa, M., R. Droual, R. P. Chin, H. L. Shivaprasad, and R. L. Walker. Ornithobacterium rhinotracheale infection in turkey breeders. Avian Dis 40:865-874. 1996.

10. Droual, R. Pathogenicity Studies of Ornithobacterium rhinotracheale in Turkeys: Comparisons of Routes of Inoculation and Interaction with Bordetella Avium. In: The Turkey ORT Scientific Symposium. R. A. H. a. Nutrition, ed., Minneapolis, Minnesota. pp 33-36. 1996.

11. Glisson, J. R. Bacterial respiratory disease of poultry. Poult Sci 77:1139-1142. 1998.

12. Hinz, K. H., C. Blome, and M. Ryll. Acute exudative pneumonia and airsacculitis associated with Ornithobacterium rhinotracheale in turkeys. Vet Rec 135:233-234. 1994.

13. Joubert, P., R. Higgins, A. Laperle, I. Mikaelian, D. Venne, and A. Silim. Isolation of ornithobacterium rhinotracheale from turkeys in Quebec, Canada. Avian Dis 43:622-626. 1999.

14. Pierson, F. W., V.D. Barta, D. Boyd, and W.S. Thompson. . The association between exposure to multiple infectious agents and the development of colibacillosis in turkeys. J. Appl. Poult. Res 5:347-357. 1996.

15. Poss, P. E. Turkey industry strategies for control of respiratory and enteric diseases. Poult Sci 77:1181-1185. 1998.

16. Sponenberg, D. P., C.H. Domermuth, and C.T. Larsen Field outbreaks of colibacillosis of turkeys associated with hemorrhagic enteritis virus. Avian Dis 29:838-842. 1985.

17. Sprenger, S. J., A. Back, D. P. Shaw, K. V. Nagaraja, D. C. Roepke, and D. A. Halvorson. Ornithobacterium rhinotracheale infection in turkeys: experimental reproduction of the disease. Avian Dis 42:154-161. 1998.

18. Tabatabai, L. B., M. K. Zimmerli, E. S. Zehr, R. E. Briggs, and F. M. Tatum. Ornithobacterium rhinotracheale North American field isolates express a hemolysin-like protein. Avian Dis 54:994-1001. 2010.

19. Thachil, A. J., B. T. Velayudhan, D. P. Shaw, D. A. Halvorson, and K. V. Nagaraja. Pathogenesis of Ornithobacterium rhinotracheale in egg-laying hens with coexisting infectious bronchitis virus and Escherichia coli infections J. Appl. Poult. Res. 18:780-788. 2009.

20. Travers, A. F. Concomitant Ornithobacterium rhinotracheale and Newcastle disease infection in broilers in South Africa. Avian Dis 40:488-490. 1996.

21. Travers, A. F., L. Coetzee, and B. Gummow. Pathogenicity differences between South African isolates of Ornithobacterium rhinotracheale. Onderstepoort J Vet Res 63:197-207. 1996.

22. Turkyilmaz, S., and O. Kaya. Detection of Antibodies Produced against Ornithobacterium rhinotracheale and Bordetella avium by Enzyme-Linked Immunosorbent Assay in Hens and Turkeys in Aydin Province, Turkey. Turk J Vet Anim Sci 29:897-902. 2005.

23. van Empel, P. C., and H. M. Hafez. Ornithobacterium rhinotracheale: a review. Avian Pathol 28:217-227. 1999.

24. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, and et al. Ornithobacterium rhinotracheale

gen. nov., sp. nov., isolated from the avian respiratory tract. Int J Syst Bacteriol 44:24-37. 1994.

25. Vargas, E., M. Garduno, P. Rosas. Isolation and Characterization of *Ornithobacterium rhinotracheale* from respiratory diaseased turkeys. Veterinaria Mexico 34:283-288. 2003.

26. Voller, A., D. E. Bidwell, and A. Bartlett. The Enzyme Linked Immunosorbent Assay (ELISA): A guide with abstracts of microplate applications. Regent's Park, London, NW1. 1979.

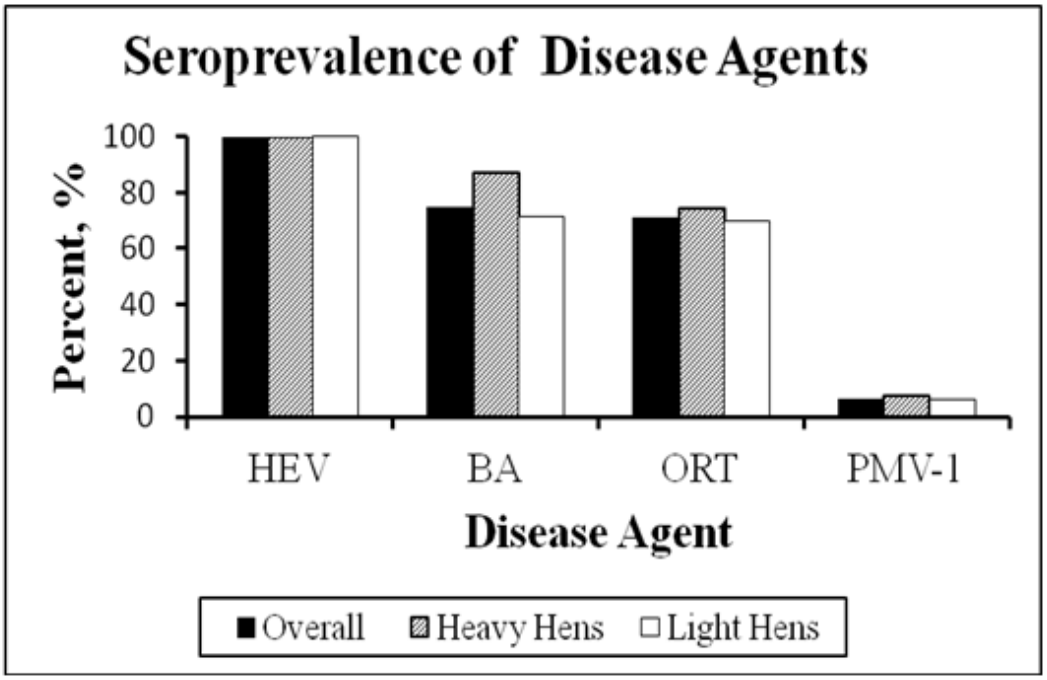

Figure 1. Presence of antibodies to HEV, BA, ORT, and PMV-1 in commercial turkey hens.

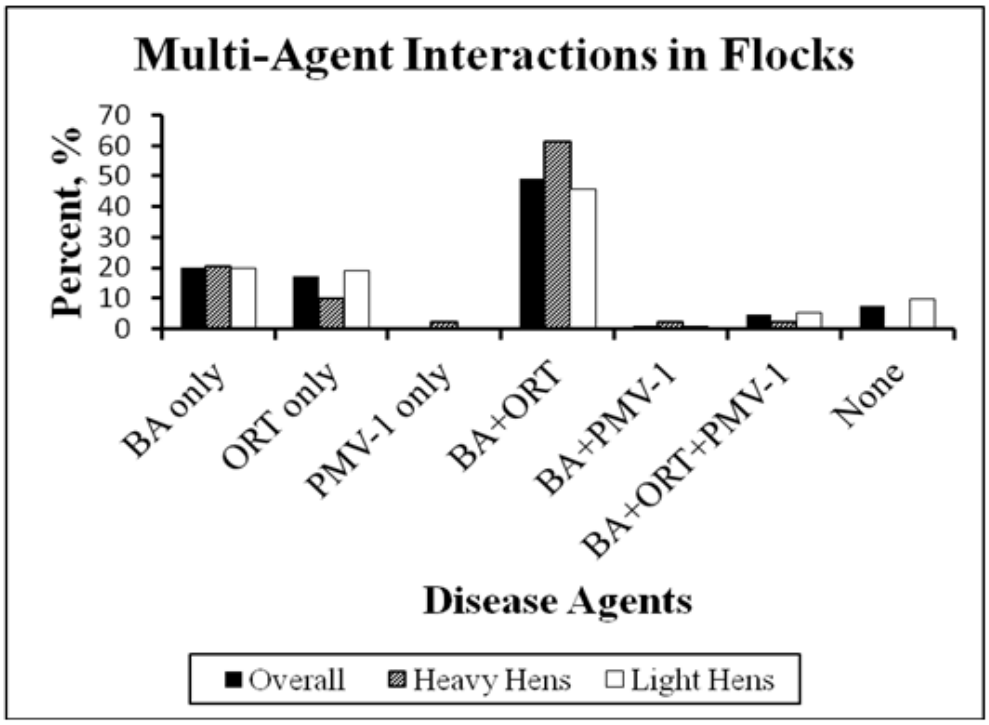

Figure 2. Presence of antibodies to concurrent disease-causing agents in commercial hens.

Agent[A]	Total Number[B] (Flocks)	Average Total Mortality[C] (%)	Average Weight Sold[D] (lbs)	Average Med/Weight Sold[E] (lbs)	Average Livability[F] (%)	Average Weight[G] (lbs)	Average Gain[H] (lbs[4])	Average FCR[I]	Average Adjusted Feed Conversion[J]
BA only	8	6.33	170755	0.16	93.09	22.93	1891.9	2.44	2.33
ORT only	4	7.66	188379	0.228	91.64	22.7	1872.3	2.45	2.36
PMV-1 only	1	4.73	314093	0.27	94.38	23.14	1961	2.42	2.3
BA + ORT	24	6.41	236760	0.246	93.09	24.43	1986.1	2.45	2.33
BA + PMV-1	1	3.49	244456	0.11	96.12	23.12	1911	2.43	2.31
BA + ORT + PMV-1	1	2.36	196242	0.13	97.74	22.18	1929	2.34	2.29
None	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

[A]Agent(s) to which flocks were exposed
[B]Total number of flocks which tested positive for the agent(s)
[C]Total mortality rate of the flock exposed to each agent
[D]Total lbs of bird sold by flock
[E]Lbs of medication given per lb of bird sold by flock
[F]Percentage livability by flock based on start number and number of birds sold
[G]Weight of individual bird
[H]Average daily gain of birds in the flock
[I]Estimated feed conversion ratio of individual birds (lb food/lb gain)
[J]Adjusted feed conversion by bird to account for external factors (feed waste, etc)

Table 1. Performance parameters in heavy hens.

Agent[A]	Total Number[B] (Flocks)	Average Total Mortality[C] (%)	Average Weight Sold[D] (lbs)	Average Med/Weight Sold[E] (lbs)	Average Livability[F] (%)	Average Weight[G] (lbs)	Average Gain[H] (lbs[4])	Average FCR[I]	Average Adjusted Feed Conversion[J]
BA only	27	5.52	238199	0.247	94.57	15.85	1743.9	2.11	2.04
ORT only	26	5.89	185636	0.203	93.84	15.17	1701.5	2.1	2.08
PMV-1 only	0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
BA + ORT	62	6.29	197747	0.308	93.4	16.14	1754.6	2.12	2.04
BA + PMV-1	1	2.91	379508	0.15	95.52	16.84	1830	2.1	1.97
BA + ORT + PMV-1	7	7.93	228051	0.411	90.57	15.64	1710.9	2.13	2.07
None	13	5.64	188898	0.228	93.92	16.14	1784.8	2.1	2.02

[A]Agent(s) to which flocks were exposed
[B]Total number of flocks which tested positive for the agent(s)
[C]Total mortality rate of the flock exposed to each agent
[D]Total lbs of bird sold by flock
[E]Lbs of medication given per lb of bird sold by flock
[F]Percentage livability by flock based on start number and number of birds sold
[G]Weight of individual bird
[H]Average daily gain of birds in the flock
[I]Estimated feed conversion ratio of individual birds (lb food/lb gain)
[J]Adjusted feed conversion by bird to account for external factors (feed waste, etc)

Table 2. Performance parameters in light hens.

Chapter Three: Characterization of Atypical Hemolytic Isolates Compared to Non-Hemolytic Isolates of *Ornithobacterium rhinotracheale* Using *In Vitro* and Turkey Embryo Lethality Assays

Summary

Ornithobacterium rhinotracheale (ORT) is a bacterial pathogen that causes respiratory disease and economic loss in the commercial poultry industry. This study was initiated after atypical hemolytic isolates of ORT were recovered from turkeys in the Shenandoah Valley of Virginia. The primary objective was of this work was to determine what differences may exist *in vitro* between hemolytic and non-hemolytic isolates. Comparison was based on biochemical profiles protein profiles (SDS-PAGE), plasmid identification, PCR, antimicrobial resistance patterns, and virulence in experimentally inoculated turkey embryos. No differences were found in biochemical characteristics or the protein profiles. A 4000 bp plasmid was found in the hemolytic isolate. The non-hemolytic strain contained no plasmids. BLAST analysis and comparison of the plasmid sequence shows similarities with *Riemerella anatipestifer* plasmid pCFC1 (213/282 bp-76%), *Flavobacterium sp.* KP1 plasmid pFL1 DNA- (306/446 bp-69%), *Ornithobacterium rhinotracheale* plasmid pOR1 (83/87 bp-95%), *Helicobacter pylori* Puno135-complete genome (134/176 bp-76%), and the *Helicobacter pylori* B8 complete genome (132/175-75%). PCR results indicated that the ATCC Type Strain ORT 51463, non-hemolytic ORT 725-09 and hemolytic ORT 22470-08 lg all share the common 784-bp 16S rRNA fragment specific for ORT. Antimicrobial resistance patterns differed between the two isolates with the hemolytic isolate demonstrating resistance to tetracyclines as a class (tetracycline, oxytetracycline, and doxycycline), while the non-hemolytic type was pan-sensitive. No significant mortality or developmental differences between isolates were found in turkey embryos.

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a Gram-negative, pleomorphic rod shaped bacterium that causes respiratory disease of economic significance in poultry (5, 27, 31). ORT was first isolated in Germany in 1981 (12), and in the United States in 1989 (5). Characterization was completed in 1993 (5), and ORT was given its current name in 1994 (30). A respiratory pathogen of birds, ORT may cause nasal discharge, facial edema, and swelling in the infraorbital sinuses in older turkeys and breeders (2, 6, 8). Airsacculitis, pneumonia, pericarditis, peritonitis, and mild tracheitis are observed on necropsy (25, 30). The trachea and lungs are the most common sites of isolation (2). Most ORT isolates are non-hemolytic when grown on Columbia agar with 5% sheep's blood (30), but incomplete hemolysis has been noted after 96 hours of incubation (3). Researchers recently characterized fifteen hemolytic North American field isolates, noting that the hemolysis factor is a pore forming cytolytic protein (26). Resistance to antimicrobials among ORT isolates from chickens and turkeys has been noted and is dependent on geographical location and strain (17, 18, 23). More recently, atypical field isolates have been identified with increasing frequency in the Shenandoah Valley of Virginia.

Biochemical characteristics and genomic analysis are standard approaches to isolate identification. Key biochemical characteristics that help identify ORT include positive oxidase and β-galactosidase tests, variable urease reactions, and a negative catalase test (10). Identification may be done using API 20NE or API ZYM strips (Biomerieux, France). PCR analysis of a 784- bp 16S rRNA sequence has also been proven useful for identity confirmation, clearly differentiating it from other similar pathogens like *Riemerella anatipestifer* and *Coenonia anatina.* Primers correspondng to nucleotide positions 8 to 27 and reverse complement, 1467 to

1485 have proven to be the most useful (16S-F, 5'-AGAGTTTGATCCTGGCTCAG and 16S-R, 5'-GGTTACCTTGTTACGACTT) (1, 14).

Embryo lethality assays were developed by Dr. H.M. Hafez (personal communication,(7)). The use of embryos provided a less expensive alternative to live bird trials and isolates could be classified based on percent mortality in inoculated chicken eggs. Specific pathogen free eggs, 20 per isolate, were inoculated via allantoic sac at 11 days of development with a dose of 500 CFU/egg. Mortality was determined 8 days post-inoculation and embryos were cultured to insure ORT re-isolation. Isolates causing 10-20% mortality were classified as being of low pathogenicity, whereas those causing 21-60% mortality were classified as moderately pathogenic and those causing greater than 60% mortality, highly pathogenic. This assay proved beneficial due to ease of use and low cost as opposed to experimental infection of live birds (7).

The purpose of this study was to determine biochemical, molecular, genetic and pathotypic differences between atypical hemolytic and non-hemolytic ORT isolates using *in vitro* and embryo lethality assays.

MATERIALS AND METHODS

Bacterial Isolates and Culture Methods.

O. rhinotracheale isolates obtained from 2008-2009 field cases submitted to the Virginia Department of Agriculture and Consumer Services (VDACS) were used in this study: ORT 22470-08 lg (designated hemolytic, H, due to presence of consistent alpha-hemolysis after initial 48 hours incubation), and ORT 725-09 (designated as non-hemolytic, NH, based on the consistent absence of alpha hemolysis after 48 hours of incubation), ORT 16744-08sm, ORT 15286-08lg, ORT WR 3-1, ORT 16744-08lg, and ORT 9436. Recent isolates from 2012 included ORT 682 and ORT 691. All isolates originated from turkeys with respiratory disease. A *Bordetella avium* isolate, BA 636, was also received from VDACS and was used for comparison because of its similar respiratory disease-causing ability in birds. For propagation, isolates were inoculated onto Columbia blood agar (CBA) plates containing 5% sheep's blood (Remel, Thermo Fisher Scientific, Lenexa, KS USA) and incubated in reduced oxygen using a candle jar at 37˚C for 48 hours. For comparison, an isolate of *Escherichia coli* (*E. coli* V517) carrying known plasmids (21) from the laboratory of Dr. Nammalwar Sriranganathan (Virginia-Maryland Regional College of Veterinary Medicine, Center for Molecular Medicine and Infectious Diseases, Blacksburg, Virginia, USA) was grown on CBA in an aerobic environment at 37˚C for 12 hours.

Biochemical Testing.

Preliminary tests included oxidase, catalase, and 3% KOH to verify a negative Gram's reaction. In addition, identification of both isolates was performed using commercial API 20NE and API ZYM strips (bioMérieux, Inc., Durham, NC, USA). The reference numbers for ORT are 0020004 and 0220004 respectively (9).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Bacterial cultures were grown as previously described in bacterial culture and methods for 48 hours and re-suspended in 500 uL 0.85% saline. Cells were pelleted by centrifugation at 17,900 x g and supernatant was removed. Post-pelleting, 80 uL ß-mercapto-ethanol Laemmli buffer was added to each pellet and boiled for 5 minutes. Samples (5-10ul) were loaded into a 12% TRIS-HCL gel (Bio-Rad, Hercules, CA USA). The gel was immersed in buffer consisting of 1% SDS, 14g glycine, 3g Tris base, and 1L ddH_2O and allowed to run for approximately 30 minutes at 200V. The gel was stained for 30-45 minutes using Coomassie blue (1g Coomassie Brilliant Blue R-250, 450 ml methanol, 450 ml ddH_2O, and 100 ml glacial acetic acid). De-staining of non-proteinaceous material was performed using 450 ml methanol, 450 ml ddH_2O, and 100 ml glacial acetic acid until the background was clear. Following de-staining, the gel was placed in ddH_2O for 30 minutes, and then digitally imaged using a Gel Logic 200 Imaging System and the KODAK1D 3.6 Imaging software.

Plasmid Comparisons.

Lawns of ORT were grown on Columbia Blood Agar as previously described in bacterial culture and methods. Each plate was re-suspended in 1700 ul PBS, and cells were harvested post-centrifugation. Plasmid DNA was purified and eluted using the QIAprep Spin Miniprep Kit (Qiagen, Inc. Valencia, CA USA) per manufacturer instructions. For DNA elution, 50 ul of ddH_2O purified for molecular diagnostics (Invitrogen, Life Technologies, Carlsbad, California, USA) (55˚C) was used. Samples were stored at -20˚C. To verify the presence of plasmids, samples were run on a 1% agarose gel containing 5 ul Ethidium Bromide (EtBr) for viewing purposes. Briefly, purified plasmid at a concentration of 200 ng/ul was added to ddH_20 to obtain a total volume of 15 ul per well (+ 1 ul 6X loading dye (Thermo Scientific, Pittsburgh,

Pennsylvania, USA) in agarose gel. Following loading, the gel was allowed to run for one hour at 140V in an immersion buffer of 1X Tris/Borate/EDTA (TBE) (Thermo Scientific, Pittsburgh, Pennsylvania, USA). After electrophoresis, the gel was then digitally imaged using a Gel Logic 200 Imaging System and the KODAK1D 3.6 Imaging software.

Restriction Enzyme Digestion.

Isolated plasmid DNA was linearized by single restriction enzyme digestion with the following enzymes: *BamH*I, *EcoR*1, *Sac*I, *Bgl*II, *Xho*1, and *EcoR*V (Promega Corporation, Madison, Wisconsin, USA). Briefly, 27 ul dH_20 was added to 31 ul of plasmid DNA prepared as described previously for a total volume of 58 ul. This was divided into 7 reactions of 8 ul/reaction (6 enzymes, plus a negative control) to which 1 ul of a 10X buffer was added. Reactions were incubated at 37°C for 30 minutes. Products of restriction digest were run on a 0.6% gel at 100V for 90 minutes.

Plasmid Sequencing.

Plasmid sequencing was performed by ACGT, Inc. (Wheeling, Illinois, USA) *de novo* following digestion with Xho1. Quantified DNA was used for fragmentation according to the protocol formulated for the NexteraXT DNA sample preparation kit (Illumina, Inc., San Diego, California, USA). Nextera primers and index adaptors were used to develop a DNA fragment library. A Bioanalyzer DNA HS kit was used for quantification. The pooled library was loaded at 8 pmol into a MiSeq flow cell and paired-end sequencing was performed. Genomic and plasmid sequences were identified using the reporter software and plasmids were then assembled de novo using ABySS (Michael Smith Genome Science Center, Canada). Sequence was compared to other known sequence by use of BLAST software Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information, USA).

Polymerase Chain Reaction (PCR).

Isolates of ORT were grown on CBA plates as previously described. Bacteria were scraped from the plates and re-suspended in buffer before lysing with protease in a commercial lysis buffer (Qiagen, Inc., Valencia, California, USA). DNA was purified and eluted using the QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, California, USA). Primers used for PCR were those described by van Empel and Hafez: OR16S-F1 (5'-GAG AAT TAA TTT ACG GAT TAAG-3') and OR16S-R1 (5'-TTC GCT TGG TCT CCG AAG AT-3'). These were commercially ordered and constructed by Invitrogen (Invitrogen, Grand Island, New York, USA) (27). These primers amplify a 784-bp fragment in the 16S rRNA of the bacterial genome. Primers were diluted to a stock concentration of 100 uM for storage and a working concentration of 10 uM. Reactions were performed with a final volume of 25 uL containing 22.5 uL Platinum PCR SuperMix (Invitrogen, Grand Island, NY, USA), 0.5 ul of forward primer, 0.5 uL of reverse primer, and 1.0 uL of DNA. Initial denaturation was at 94° C for 5 min, followed by 45 cycles of denaturation at 94° C for 30s, annealing at 52° C for 60s, and extension at 72° C for 90s. A final extension was performed at 72° C for 7 min, and resting state was at 4° C (11). The ATCC Type strain ORT 51463 (American Type Culture Collection, Manassas, VA, USA) and ddH_20 purified for molecular diagnostics (Invitrogen, Life Technologies, Carlsbad, California, USA) were used as positive and negative controls, respectively.

Antimicrobial Resistance Profiles.

Isolates were grown in lawns on CBA. *Bordetella avium* (BA 636) was used for comparison as a control. Antibiotic disks (four per plate) were placed manually using sterile forceps on inoculated, un-incubated plates. A panel of common antimicrobials in poultry was used: tetracycline (TE30), penicillin (P10), bacitracin (B10), neomycin (N30), oxytetracycline (T30),

triple sulfa (SSS), chlorotetracycline (aureomycin), sulfamethoxazole trimethoprim (SXT), streptomycin (S10), doxycycline (D30), ceftiofur (XNL 30), tilmicosin (TIL 15), and sulfamethizole (TH 25) (BD Diagnostics. Sparks, MD, USA). Plates were allowed to incubate 24-48 hours at 37˚C in reduced oxygen using a candle jar. Measurements of zones of inhibition were recorded in millimeters. Cutoffs to determine resistance were to be based on those assigned by the Clinical and Laboratory Standards Institute (CLSI) for fastidious Gram-negative organisms. However, not all antimicrobials were available for reference on CBA so quantified clearance zones were used for direct comparison between isolates.

Embryo Lethality Assay.

*Turkey Embryos***.** Fertilized eggs were obtained from Aviagen Turkeys, Inc. (Lewisburg, West Virginia USA). Eggs were incubated on site until approximately 13 days of age, when they were moved to the Virginia-Maryland Regional College of Veterinary Medicine, Center for Molecular Medicine and Infectious Diseases, Avian Medicine Laboratory. Live embryos were identified by candling before transport. All procedures were reviewed and verbally approved by the university veterinarian, since use of embryos is not regulated by the Institutional Animal Care and Use Committee.

Assay Optimization for Turkeys. An embryo lethality model used to compare ORT pathogenicity in European strains was developed by Hafez ((7), personal communication) using 11 day-old SPF chicken embryos and recording mortality 8 days post-inoculation with ~500 CFUs/embryo (~2500 CFUs/mL). To replicate this developmental stage, both 14 and 15 day-old turkey embryos were used to determine optimal assay conditions. The first 15 day-old embryos were held for 10 days post inoculation to attempt to obtain higher embryo mortality. In subsequent assays the 14-15 day-old embryos were held for 8 days post-inoculation.

Embryo Challenge. Inoculation of the allantoic sac was performed by nicking the shell slightly above a marked line distinguishing the location of the air cell. A syringe fitted with a 25-gauge 5/8-inch needle was inserted to its full length vertically through the hole and a volume of 0.2 ml of inoculum was injected. Treatments included: un-inoculated, sham inoculated (PBS), NH in PBS, and H in PBS. Embryos were kept at 37° C in a Humidaire (Humidaire Incubator Company, New Madison, Ohio, USA) incubator and candled daily until 23 days of age. Embryo mortality based on breakdown of vascular structure, coagulation of suspending liquids, or absence of embryonic movement were recorded daily (20) (Figure 1).

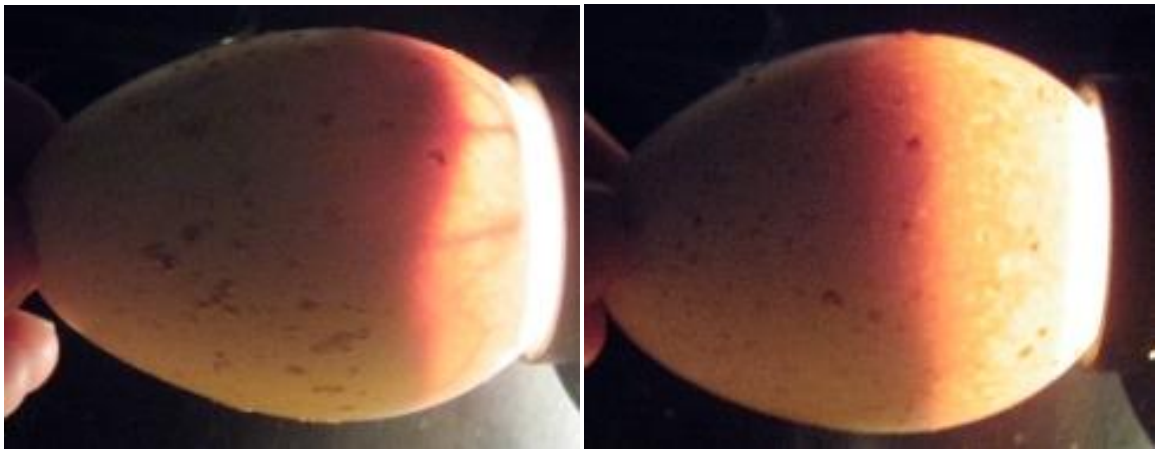

Figure 1. Live embryo at 19 days of age showing prominent vascularization (top) versus dead embryo showing vascular breakdown (bottom).

Embryo LD_{50} Determination. Following the technique outlined previously, 15 day-old embryos were inoculated with 5 different concentrations of either NH or H. Groups of ten embryos receiving either the NH or H were inoculated with: 10^3, 10^4, 10^5, 10^6, or 10^7 CFUs/mL. Un-inoculated controls and those inoculated with PBS were used for comparison (n=10 eggs/group). After inoculation, eggs were candled daily for 8 days to determine embryo viability.

Embryo Passage of Isolates to Determine Effect on Virulence. As previously described, 15 day-old embryos were inoculated with 10^3 CFUs/ml of either NH (n=10) or H (n=10). Two days post-inoculation, allantoic fluid from dead embryos was collected and CFU counts performed.

Allantoic fluid was diluted to 10^3 CFUs/ml and inoculated into another set of 15 day-old embryos (n=10 per isolate). Subsequent passages of ORT were repeated four times in an attempt to amplify virulence in embryos. Each passage was frozen back in 50% glycerol + 50% allantoic fluid for future inoculations.

Infection through Pip. As previously described, 15 day-old embryos (n=10/group) were inoculated with 50-150 organisms of NH (original received isolate), H (original received isolate), NH (5X embryo passage), and H (5X embryo passage). Non-inoculated and PBS-inoculated sham controls (n=10/group) were used for comparison. Mortality was checked daily, dead embryos were cultured, and CFU counts performed. Live embryos were carried through pip, sacrificed by cervical dislocation, weighed, and cultured. CFU determinations were made on 1 gram of yolk vortexed in 200 ul PBS.

Statistical Analysis.

Embryo mortality rates, as well as surviving embryo weights, were compared via Analysis of Variance (ANOVA) using JMP (SAS Institute, Inc.) where p-values ≤ 0.05 were considered significant. Differences were separated using Tukey's HSD.

RESULTS

Biochemical Testing.

Consistent with published characteristics, NH and H were identified as ORT. Preliminary tests indicated that both were oxidase-positive, catalase-negative, and Gram-negative. The API 20NE identification code (0020004) reflected positive reactions for ß-galactosidase and oxidase. Five API ZYM reactions were negative including lipase, ß-glucuronidase, ß-glucosidase, α-mannosidase, and α-fucosidase. Fourteen were positive including alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, napthol-AS-BI-phosphohydrolase, α-galactosidase, ß-galactosidase, α-glucosidase, and N-acetyl- ß-glucosaminidase (9).

SDS-PAGE.

SDS-PAGE indicated that NH and H isolates had similar protein profiles (Figure 2).

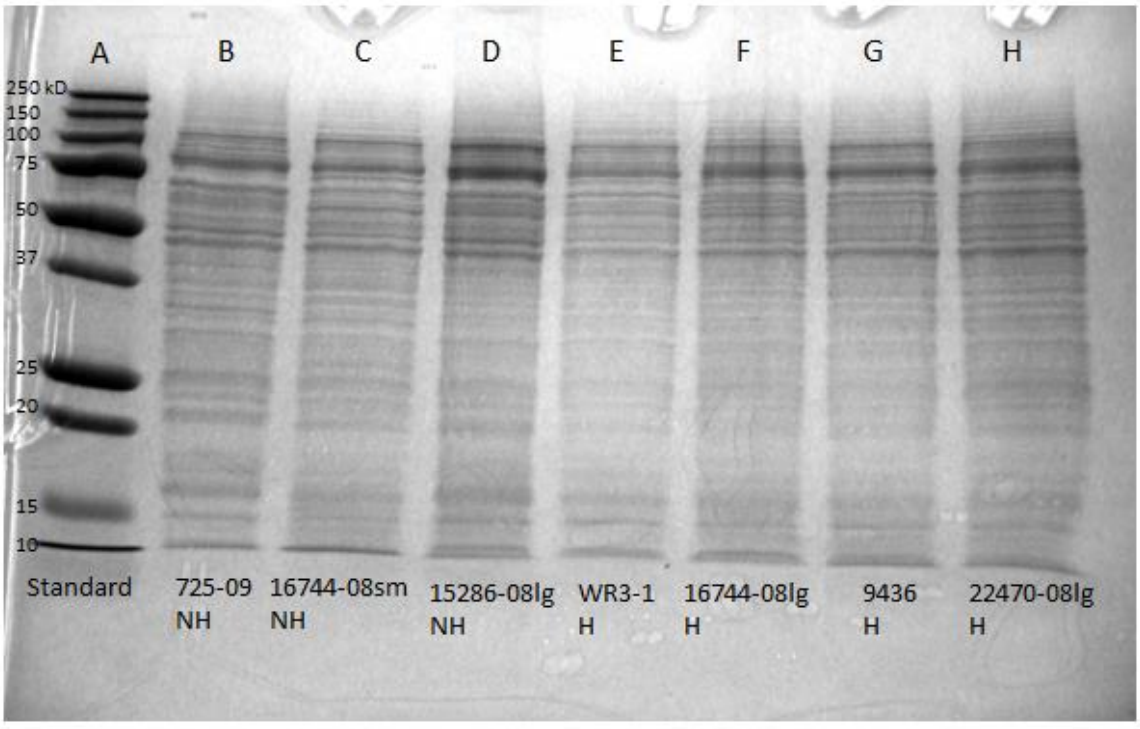

Figure 2. Comparison of non-hemolytic (NH) and hemolytic (H) isolates using SDS-PAGE.

Plasmid Comparisons.

Noticeable differences were found in NH and H isolates relative to presence of plasmids (Figure 3). Plasmid OR1 was characterized in the ATCC Type strain ORT 51463 as a 14,787 bp DNA fragment (14). Three plasmid bands were found in the H isolate: 7kb, 3.5 kb, and 2.7 kb. These were compared to *E. coli* V517 as a standard.

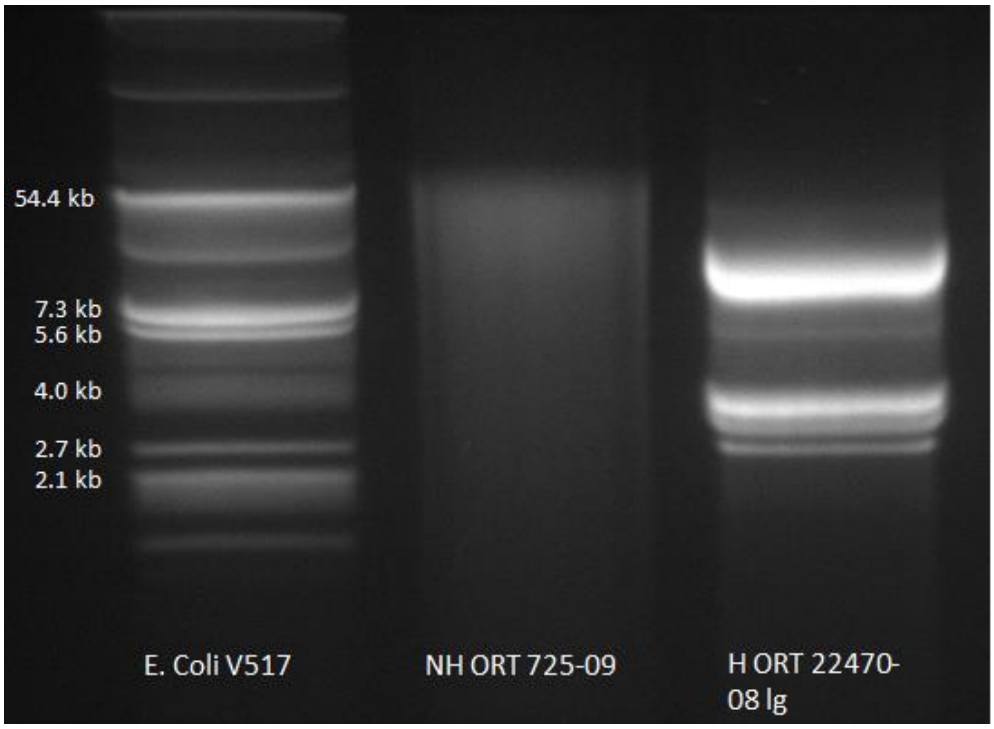

Figure 3. Comparison of plasmids present in non-hemolytic and hemolytic ORT isolates with *E. coli* V517.

Restriction Enzyme Digestion.

Restriction enzyme digestion with *Xho*1 or *Bgl*II linearized the plasmid, revealing it to be approximately 4000 bp in length. This suggested that the multiple plasmid bands observed on the routine extraction were derived from a single coiled plasmid.

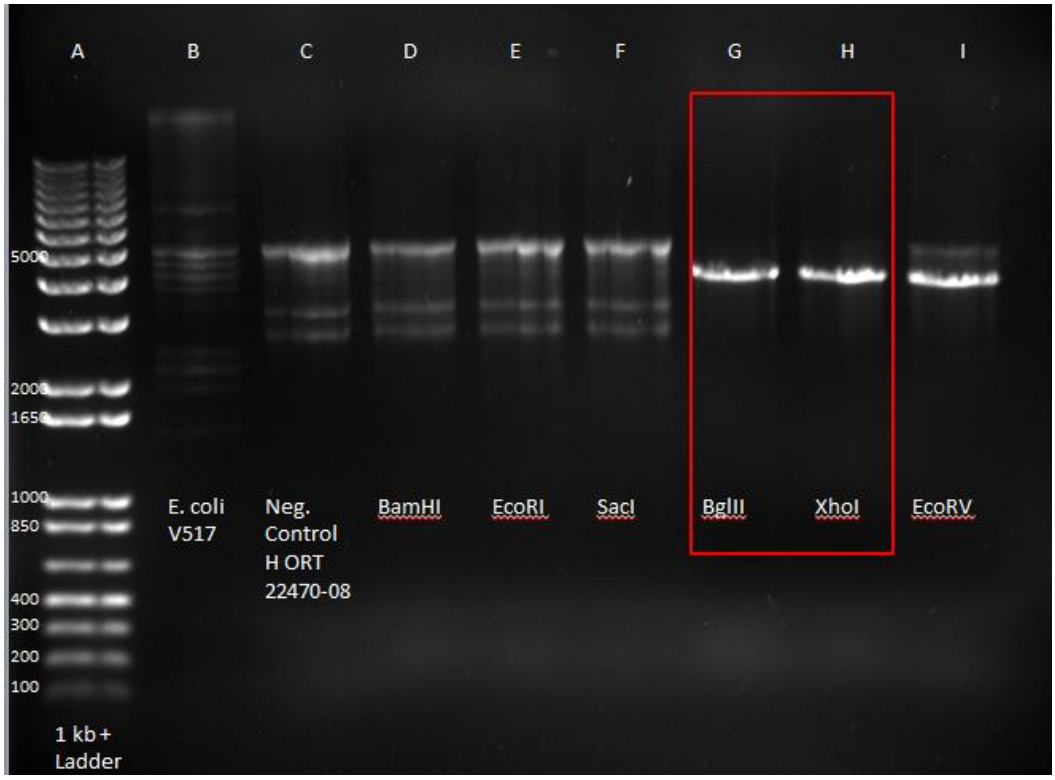

Figure 4. Restriction enzyme digest showing single bands formed with *Bgl*II and *Xho*1.

Plasmid Sequencing.

De novo sequencing indicated that the suspected plasmid from the H isolate was 3942 bp in length (Table 1). The GC content of the sequence was 33.8%. Additionally, while the entire sequence is AT rich, a region which seems to be more AT rich than the remaining sequence was noted between 1,140 and 1,740 bp. This region could be speculated as an origin of replication.

5′-ACTTTCTCTTTTTTGCGCCTCCTCTCGATGTCTGCGGTACCATTTTATCGTTTCTTC
TTTCTCCGCTTTAAAATTAGCTTTTAATGCTCCTGCTATGCTTACTCTATCTTTCATCTT
GAGTATTTGTATCTCTTTATTTTTTATCTCATTCTCCAAATAAGAGACTTTTGCGAGCAC
TTTCTCCTCATCTAGTGGTTTTGATAGTCCAAATAGTTTAGATTGCCTTAAATCGTCTAT
ATTCTCCCTCTTCGGCGATATTCTAGCATAATATTCTCTAGCACTTTCGTGTTTGATGCC
AGTACTCCTAATGCCTCTTTTTAGTCCAAATTTCTCCATTTCTTCAGCATAGCGATCCTG
TCGCTCCTGCATTTCTTTTTTGTTTCCCATCACTTCCTTAGCTGACAATCTTCCATCTTC
GGTCAATGGAACAATGACAGCATGCATATGCGGAGTCTTCTCATCTAAATGAACTGTAGC
TCTTACTATATTTTCTCTTCCAAATTCATCTATCAAGAATTTTACATTTGAAATAATCCA
AGCGTCTTTTTTCTCTTCATCTTTGAAAATTTCCACCATATCTTCGTGGGTTCCCGTAAG
GACATGAGTCATATACTTTACGGCGTCTTTGCGAATGGCTTTTTTACCCTTATACCCTTC
TTCTATTCTTTCTGCAATAGCTTGTTTCAGCGGTTTTTTATGGTAGTCATTTAATAGACC
GATATTCACATTTAGATGTGTTCTGCTTTTATCAGCGTGCAGATAAGTGTGTTCAGCTCC
AGCTACTCGGTCAATATGATTACCAATTCCTCCGCTCGATGTTTTGCCCTTTTCAGAATG
TAAAACTGCGTATCCCATAATTTTATTTTTTGAAGAAAAATAACTAAAAAAATACCTTTT
AAAAATAGCCTCGTAGAGCCCTCGGAGAGCCCACGACTTAACTAATTTTGGTTTTCAAAT
TTACGGAGTAATGCAGAAATAACAAAATGTTAAGTCTAGTGGGCTATACTTTTAAGCGAA
AAAAAACATAAGTTTTAACGTTTATTTTTTTTTCTTTTTCAAAAAAGACTTATATTTGTA
GAGCAATTAGGAAGGAGTTTGGCTCTGCTGAGGAGCAACCAAACAAGCGCCGAAGTTGTG
CAATAAACGCAAGTTTAAATCCACTTTTTTAAAGTGGATTATTTTTAATTATAATTATTA
TGAAAATACAACTATTCCAAAAGATATTATTGATGTTATTTTTATCATTTTTTATTAGTT
GTAATAATAATGATGATGATAAAACAGAAAATATAAAAGTATCTTCTTTTTTCAAAGAAG
ATCTTTCTAAAAACAATAAAATTTATACCAGTCCTATTTTTATTAATAGTAGTGATTTAA
TTAAATCAAACACAAATATAAATTTTATAGAAAATAAAGTTTTACTCTTTACTTCGACCT
ACGATATTCATTACTTAAAATTATACAGATTTAACAATAGCTTATTAGTTTATACTTATT
CTTATACCTCACCAAATAGTAATTCCTCTTTTAATTTGAAGAATTATTTTGTAGTATTTG
ATAAAAAGTTACTATTAAAAAACCCAGATAATATATAAAATTAGTAAACAATATTATAAATA
AATAATTATGAAAATTAAATTCTTTTTAATCGGCATTTTTTCTATTATTTTCTGTTCTTC
TTGTGAAGATTCAGATAGTTCTATGGATTTCAATAATGAAAACCCTTCTTTGAAAAATTT
TGAGAGCTACTATACTAAAGAGGATAATGATAAAAATATCAAAGCAATGGAAGACTATAA
CTTGTCTTTTTTTTGGAGAAAAGAAAGTTGGAGTTATAGAAATGAATGGAAGAACTATTGA
TGATATTCTCAATGAAATAAAAAAAAGAATGGGAAATCAGTATAGACGTGGATTTAATAC
AATTTCCATAACTCAAAATCCTTTTGCTAATGGACCATTTTATGCAGAAGCAAAAAAAGT
TAGAAATTTTAAAGATATAAAAATATTCTTTAAAGGACATGGTTTTCCTCGAGGTGTTTA
TATTGCAGATATATATAGTTTTCCTGTTAAAGTAGATATTCCTAAAGGTGTGTCATTTGT
TGTTTTAGATAAAGAAGAACCTGCGGATCATATTGGTTATATAGATTATAATGAACAAAA
TATAGGTATATCTAGTAGTATAATAAGTAAAGGAAATGGTAAAACTCAATTAGCTGCTAA
TACTATGGCAGTAGATATAAAAAATAATATGCTAGGGCAACTGATTGTTGGTGGTTATTA
CCCTTTTTATTCAGAAAAGATAACATATAAATATTATACTATAACTATTCCTGGATTATA
ATTTTTTTTCAATTAAAATAACAATTATGTACGCAGTATTATTTGATTTAGACACGAATT
GTTTAAGTGTAAATTACGAGGGAAACGCTTACAATAATGCATATAAATTAATCAAAGATT
TTATGCTAAAAAATGGCTTTCAATGGAAACAAGGCAGTGTTTATTTTGGTGATGAAACAA
TAAACGCAGTTAATTGTGTGGTTATAGTCCAAAAGTTAGCTAAAGAATATCCTTGGTTTT
CGACTTGTTGCAAAGATGTTAGAATGCTACGAATTGAAGAGAACAACGATTTATTATCTG
CTATTTCTATATAAAGCAAATTAAATTTAAAGTCCCCCCGAATGGGGGATTTTTTTTGA
ATAAACACCTCCTAAATAGATTGACATGCGGAATTGCTACAAAGCTAAAAAAACCTACAG
GATTATTCTTCTTCTATTGAATGAATATTCTCTATCATCATTTTGTTAGCTTGTTTTTCT
GAATAAGGGTTTGAGATTGTTTTTATTATTTGCCCTGTATTACTATTAATTAAATCGATA
TTTCCGTCTAGATTGTATTGGCTAATATAGGGAGTTTCCATTTTGATTAATTTCTTATGC
TCTTGGACATTTTGTTTATTTAGCTTACCCTTAAATGCTAGTTGAGGATTTAGTAAATCA
TAGTTAAATGAAACCGCATATTTGGCAACATTTTCTTTTTTCTCGATTATGATTTTCTTT
TCTCTTAATTTCTTCAAGGCTTTTGAAACTTGAGCTTGAGTCATACTAAGTTCTCGCATT
AAAATTTGTTGATTTACCAAAATCCTATTCCCATAATCCATAACGGACATAAACCACATG
AAAACCTCCCATTCTCTGCCTTTTAAACCACTACACTTGAGAACATAGCTTAAAAACTGT
TGATTTAGGGTAAAAAACTCATCATATAGACCTTTAGCGTGATTAACTTTGATTAATCCA
GCCGTAACATTTTCCCTTTCATTATCTGGGATATCAAATTCTATTTTTTGAGTTTCTTGA
GTTTTAGTCAGTGTTATTTTTGACTTTGAATAATGTCCATTTTTATTCATATATGAATGT
TTTTCTTTTGGCAAATATATAAAAAATATTCATATATGAATAATTCTATATCAAATAAGA
ATATTGTTATATCAAATAAGAATATTGAATCCCCATTTTATGCGGAGTACAGACCTTTTC
TTATTATATTAGTTAATTGCAGTGCCTTGTCAAGGGATTTTTTCAATATCTGTGCTTAAC
TCAACTAATAGCTTTAGGTTTTTTAAGCATTAAGGCAATTCCGCAAGTTTATCTTCTGAA
CCCTCTTGGTTTGTTTTGCTCTTGTTCCCTGTTCTGTCTAGCTTTTAGTATTTTTTCTGC
CTGCTCTTTAGCTTGAGCTTTTATTTTATCCATTCTTTCCATTTCTTCACTCTTTTGCTG
TTGTTGCAGATATTCATTTCTAAGCTGTTTGTATATCTCGGGATTTCTCGTTAAAATTTT
CGTCGTATTTTCACTTGCCTGTTTTCCGACTTTCTTTATGTTATACCTGTATTCTTCTTT
GAGTTTAGACATATCTGCATTAAGTGATGAAATTTCTCTTTCTAA-3′

Table 1. 5’-3’ Sequence of 3942 bp H plasmid. Grey highlighting indicates an AT rich area which may be speculated as an origin of replication.

PCR Analysis.

Using primers OR16S-F1 and OR16S-R1 corresponding to a 784bp segment of the 16s rRNA of the ATCC type strain, NH and H isolates were verified as ORT (Figure 5).

Figure 5. Amplification 784-bp 16S rRNA fragment of the ATCC type strain, NH, and H isolates. Standards: 100 kb (left) and 1000+ kb (right).

Antimicrobial Resistance.

Antimicrobial resistance patterns differed between NH and H isolates (Table 2). Resistance to tetracyclines as a class (TE30, T30) was evident with the H isolate. Isolates obtained more recently appear to show a greater degree of resistance. Bacitracin was the only antimicrobial that consistently resulted in zones of inhibition. NH and H resistance patterns were dissimilar from *B. avium*.

	Isolate				
Antibiotic	NH ORT 725-09 (2009)	H ORT 22470-08lg (2008)	ORT 682 (2012)	ORT 691 (2012)	B. avium 636
Tetracycline (TE30)	15mm	no inhibition	no inhibition	no inhibition	14 mm
Penicillin (P10)	18 mm	17 mm	21 mm	18 mm	23 mm
Sulfamethoxazole trimethoprim (SXT)	no inhibition	slight inhibition but no clear zone	no inhibition	no inhibition	29 mm
Streptomycin (S10)	no inhibition	12 mm	no inhibition	no inhibition	15 mm
Doxycycline (D30)	16 mm	9 mm	12 mm	12 mm	18 mm
Bacitracin (B10)	21 mm	19 mm	20 mm	23 mm	no inhibition
Neomycin (N30)	8 mm	19 mm	no inhibition	no inhibition	19 mm
Oxytetracycline (T30)	17 mm	7 mm	8 mm	no inhibition	14 mm
Ceftiofur	24 mm	22 mm	24 mm	28 mm	30 mm
Tilmicosin	26 mm	30 mm	30 mm	34 mm	14 mm
Sulfamethizole	no inhibition	25 mm	16 mm	16 mm	unclear zone

Table 2. Antimicrobial resistance patterns for NH and H isolates.\

Embryo Lethality Assay.

Assay Optimization. The study determined that 15 day-old turkey embryos would be the most appropriate substitution for the 11 day-old chicken embryos used in the Hafez assay (7) based on consistency of differentiation of isolates. Additionally, there were no significant differences seen in embryo mortality between the time frames of 8 and 15 days post-inoculation. Therefore, all subsequent trials used a 15 day-old embryo and an observation period of 8 days.

LD_{50} Determination. Eggs inoculated with 10^3, 10^4, 10^5, 10^6, and 10^7 CFUs/mL of NH or H did not produce typical dose titration responses i.e., mortality did not increase with inoculum concentration (Figure 6). Higher mortality was seen with the H isolate at 10^3-10^5 CFU / ml, but differences were not significant ($p>0.05$). All subsequent embryo trials used a 10^3 CFU / ml.

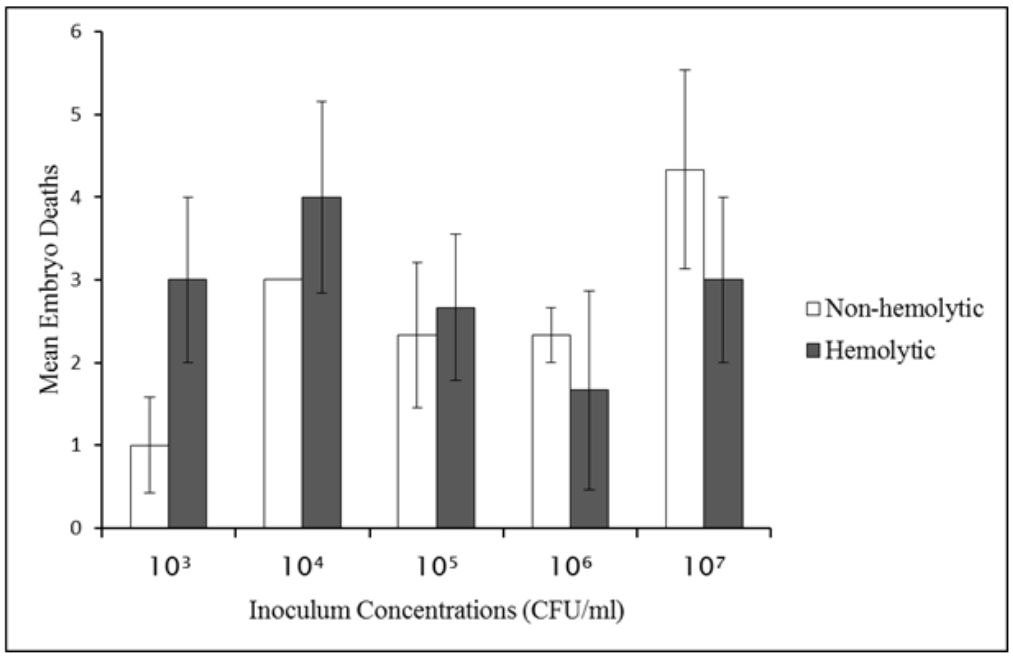

Figure 6. Mean number of embryo deaths (+/- SEM) produced by NH and H isolates as a function of increasing inoculum concentration.

Serial Passage of Isolates. Serial passage of NH and H isolates in turkey embryos did not alter virulence (Table 3). Comparisons were made by passage for the same isolate and between isolates.

Isolate	Mortality (n=10)
NH Stock Culture	2/10
H Stock Culture	4/10
NH 5X Embryo Passage	2/10
H 5X Embryo Passage	3/10

Table 3. Total embryo mortality comparing NH and H stock cultures to isolates that had been consecutively passed through embryos (5X)

Infection through Pip. At pip (28 days of incubation), ORT was re-isolated from all surviving embryos in pure culture. However, the number of CFUs recovered per gram of yolk and poult weights were not significantly affected by isolate type or serial passage ($p>0.05$) (Table 4).

Isolate	Actual Inoculum	Concentration at Pip (CFU/ml)	Poult Weight at Pip (g)	Average Weight at Pip (g)
NH SC	5.0×10^1	9.50×10^3	58.65	
NH SC	5.0×10^1	3.25×10^3	59.75	
NH SC	5.0×10^1	4.67×10^3	65.72	
NH SC	5.0×10^1	5.00×10^4	64.24	64.83
NH SC	5.0×10^1	2.00×10^6	67.62	
NH SC	5.0×10^1	4.50×10^4	75.91	
NH SC	5.0×10^1	3.00×10^4	61.26	
NH SC	5.0×10^1	4.25×10^5	65.50	
H SC	1.0×10^2	4.50×10^5	58.45	
H SC	1.0×10^2	8.25×10^4	69.27	
H SC	1.0×10^2	2.25×10^8	65.49	65.44
H SC	1.0×10^2	4.00×10^4	64.07	
H SC	1.0×10^2	4.50×10^4	66.53	
H SC	1.0×10^2	7.25×10^3	68.86	
NH 5X	1.5×10^2	3.00×10^5	61.71	
NH 5X	1.5×10^2	2.75×10^4	62.98	
NH 5X	1.5×10^2	1.25×10^8	55.46	
NH 5X	1.5×10^2	2.50×10^4	71.60	66.13
NH 5X	1.5×10^2	8.50×10^3	66.71	
NH 5X	1.5×10^2	1.23×10^5	70.07	
NH 5X	1.5×10^2	6.25×10^4	68.36	
NH 5X	1.5×10^2	3.25×10^4	72.18	
H 5X	5.0×10^1	3.50×10^5	60.36	
H 5X	5.0×10^1	6.50×10^6	67.39	
H 5X	5.0×10^1	2.15×10^4	51.28	
H 5X	5.0×10^1	1.48×10^4	65.92	61.76
H 5X	5.0×10^1	1.10×10^5	65.94	
H 5X	5.0×10^1	1.23×10^5	62.14	
H 5X	5.0×10^1	6.25×10^6	59.29	

Table 4. The effect of isolate type, passage, and inoculum concentration on ORT recovery and poult weight. Legend: NH SC- Non-Hemolytic ORT from stock (original) culture, NH 5X- Non-Hemolytic ORT after five consecutive passages through embryos, H SC- Hemolytic ORT from stock (original) culture, H 5X- Hemolytic ORT after five consecutive passages through embryos,

DISCUSSION

The discovery of atypical, hemolytic isolates of ORT is a relatively recent occurrence. Incomplete hemolysis has been noted (3), and Tabatabai et al. identified a hemolysin-like cytolytic pore-forming protein in some North American isolates (26). However, very little work has been done to further characterize isolates of this phenotype, *in vitro* or *in vivo.*

While protein profiles and biochemical characteristics appear to be similar, a 4 kb band suggestive of a plasmid was found in the H isolate. The NH isolate did not possess a plasmid. Further analysis to determine open reading frames and an origin of replication is necessary before definitive labeling of this suggested plasmid can be completed. However, upon initial examination, we identified three plasmid-like bands of 7 kb, 3.5 kb, and 2.7 kb in the H isolate. Linearization of the plasmid DNA with *Xho*1 or *Bgl* II produced a single band, suggesting that the potential plasmid was likely super-coiled, singular, and α-linearized. Sequencing followed by a BLAST search revealed that the DNA sequence did not map to the genome of *Ornithobacterium* (BLAST Analysis, (16)). When the search included somewhat similar sequences, the top five matches included *Riemerella anatipestifer* plasmid pCFC1 (213/282 bp-76%), *Flavobacterium sp.* KP1 plasmid pFL1 DNA (306/446 bp-69%), *Ornithobacterium rhinotracheale* plasmid pOR1 (83/87 bp-95%), *Helicobacter pylori* Puno135-complete genome (134/176 bp-76%), and *Helicobacter pylori* B8 complete genome (132/175 bp-75%). Interestingly, all of these organisms are found in poultry, but no single match was sufficient to clearly identify the source of the plasmid. The *Riemerella* insert was found to carry four open reading frames (ORFs) corresponding to virulence-associated proteins from external DNA sources. Two of these ORFs contain chromosomal DNA homologous to virulence proteins found in *Dichelobacter nodosus* (a pathogen associated with footrot in sheep) (4). Additionally, during

original characterization of ORT, the organism was classified as a member of the *Reimerella/Flavobacterium* group and efforts were made to distinguish it from *R. anatipestifer* because of similar phenotypes (29).This suggests the close taxonomic relationship of the two organisms and may propose a mechanism of plasmid transfer if *R. anatipestifer* is a definitive source of genetic material in the H ORT plasmid.

Antimicrobial resistance patterns suggest the H isolate has acquired resistance to tetracyclines as a class, which are antimicrobials commonly used to treat poultry respiratory infections. Attempts to cure the plasmid to determine if it carries antimicrobial resistance genes and putative pathogenicity islands were unsuccessful (Appendix 1), but data from other experiments suggest that the plasmid found in this hemolytic isolate does not correlate to pathogenicity (32). Plasmids found in other respiratory-disease causing bacterium such as *Bordetella avium* are known to confer antimicrobial resistance (13) while not affecting virulence (22). Resistance to tetracyclines in particular has been determined to be plasmid-derived in bacterial species including *Staphylococccus aureus, Enterococcus, Enterobacteriacae, Vibrio, Streptococcus* and *Bacillus* (19, 24). Using this evidence, we suspect that the tetracycline resistance mechanism may be found on the plasmid in the H phenotype. Since tetracyclines are commonly used in the poultry industry, resistance to this class of drugs is problematic since they are one of the few classes that remain available for treatment of food animal species. The sensitivity to sulfas is for the time being useful, but these drugs are receiving increased scrutiny due to concern over residues in other food animals (Food Animal Residue Avoidance Databank, 2014). Likewise, sensitivity to bacitracin methylene-disalicylate is interesting, but of little practical value with regard to the treatment of respiratory disease since it is not absorbed from the gastrointestinal tract (15).

Regarding embryo work as a viable alternative to live bird studies to compare virulence of ORT isolates, we determined that it was not an appropriate method to differentiate the NH and H isolates. Research with varying inoculum levels determined that a 10^3 CFU/ml inoculum would be the most appropriate for comparison, which is consistent with work of Hafez (7). However, this inoculum did not produce significant differences in mortality between NH and H.

Survivability of inoculated embryos through pip suggested that it was possible for ORT to pass vertically from hen to egg, and poults could carry low levels of bacteria onto the farm. Experiments have shown that vertical transmission circumstantially occurs from hen to progeny (2, 28), but these experiments did not confirm ORT infection through every level of the breeder to growout process (breeder, reproductive tract, egg, embryo, poult, young bird). Further research is necessary to definitively prove the progression of the organism from hen to progeny, and to therefore determine the mechanism of vertical transmission, but we have shown here that inoculums as low as 50 organisms can replicate in the egg and be carried through pip.

In conclusion, we have shown that the H isolate carries a plasmid that is not present in NH isolate. While the two are biochemically and molecularly identical, the plasmid may confer some differences like antimicrobial resistance and possibly code for a hemolysin-like protein. However, the embryo lethality assay suggests that the plasmid does not encode for virulence factors. Unlike other bacteria where hemolytic activity is associated with virulence, this does not appear to be the case with ORT.

References:

1. Amonsin, A., J. Wellehan, L. Li, P. Vandamme, C. Lindeman, M. Edman, R. Robinson, and V. Kapur. Molecular epidemiology of Ornithobacterium rhinotracheale. J. Clin. Microbiol. 35:2894-2898. 1997.
2. Back, A., G. Rajashekara, R. B. Jeremiah, D. A. Halvorson, and K. V. Nagaraja. Tissue distribution of Ornithobacterium rhinotracheale in experimentally infected turkeys. Vet Rec 143:52-53. 1998.
3. Banani, M., S. A. Pourbakhsh, and P. Khaki. Characterization of Ornithobacterium rhinotracheale Isolates from Commercial Chickens. Arch. Razi Ins. 52:27-36. 2001.
4. Chang, C. F., P. E. Hung, and Y. F. Chang. Molecular characterization of a plasmid isolated from Riemerella anatipestifer. Avian Pathol 27:339-345. 1998.
5. Charlton, B. R., S. E. Channing-Santiago, A. A. Bickford, C. J. Cardona, R. P. Chin, G. L. Cooper, R. Droual, J. S. Jeffrey, C. U. Meteyer, H. L. Shivaprasad, and R. Walker. Preliminary characterization of a pleomorphic gram-negative rod associated with avian respiratory disease. J Vet Diagn Invest 5:47-51. 1993.
6. Chin, R. P., and B. R. Charlton. Ornithobacteriosis A Laboratory Manual for the Isolation and Identification of Avian Pathogens 4:89-91. 1998.
7. Chin, R. P., C. P. van Empel, and H. M. Hafez. Ornithobacterium rhinotracheale infection. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan and D. E. Swayne, eds. Wiley-Blackwell, Ames, Iowa. pp 765-774. 2008.

8. De Rosa, M., R. Droual, R. P. Chin, H. L. Shivaprasad, and R. L. Walker. Ornithobacterium rhinotracheale infection in turkey breeders. Avian Dis 40:865-874. 1996.

9. Hafez, H. M. Current status on the laboratory diagnosis of Ornithobacterium rhinotracheale "ORT" in poultry. Berl Munch Tierarztl Wochenschr 111:143-145. 1998.

10. Hafez, H. M. Diagnosis of Ornithobacterium rhinotracheale. International Journal of Poultry Science 1:114-118. 2002.

11. Hassanzadeh, M., Vahid Karrimi, Neda Fallah, Iradj Ashrafi. Molecular characterization of Ornithobacterium rhinotracheale isolated from broiler chicken flocks in Iran. Turk. J. Vet. Anim. Sci. 34:373-378. 2010.

12. Hinz, K. H., C. Blome, and M. Ryll. Acute exudative pneumonia and airsacculitis associated with Ornithobacterium rhinotracheale in turkeys. Vet Rec 135:233-234. 1994.

13. Jackwood, M. W., Y. M. Saif, and D. L. Coplin. Isolation and characterization of Bordetella avium plasmids. Avian Dis 31:782-786. 1987.

14. Jansen, R., N. Chansiripornchai, W. Gaastra, and J. P. van Putten. Characterization of plasmid pOR1 from Ornithobacterium rhinotracheale and construction of a shuttle plasmid. Appl Environ Microbiol 70:5853-5858. 2004.

15. LaVorgna, M., J. L. Schaeffer, D. Bade, J. Dickson, K. Cookson, and S. W. Davis. Performance of broilers fed a broader spectrum antibiotic (virginiamycin) or a narrower spectrum antibiotic (bacitracin methylene disalicylate) over 3 consecutive grow-out cycles. J Appl Poul Res 22:574-582. 2013.

16. Lucas, S., A. Copeland, A. Lapidus, L. Goodwin, S. Pitluck, L. Peters, N. Mikhailova, H. Teshima, N. Kyrpides, K. Mavromatis, I. Pagani, N. Ivanova, G. Ovchinnikova, A.

Zeytun, J. C. Detter, C. Han, M. Land, L. Hauser, and J. A. Eisen. The complete genome of Ornithobacterium rhinotracheale DSM 15997. In., EMBL/GenBank/DDBJ Database. 2012.

17. Malik, Y. S., K. Olsen, K. Kumar, and S. M. Goyal. In vitro antibiotic resistance profiles of Ornithobacterium rhinotracheale strains from Minnesota turkeys during 1996-2002. Avian Dis 47:588-593. 2003.

18. Marien, M., A. Decostere, L. Duchateau, K. Chiers, R. Froyman, and H. Nauwynck. Efficacy of enrofloxacin, florfenicol and amoxicillin against Ornithobacterium rhinotracheale and Escherichia coli O2:K1 dual infection in turkeys following APV priming. Vet Microbiol 121:94-104. 2007.

19. Mojumdar, M., and S. A. Khan. Characterization of the tetracycline resistance gene of plasmid pT181 of Staphylococcus aureus. J Bacteriol. 170:5522-5528. 1988.

20. Pine, L., F. D. Quinn, E. P. Ewing, Jr., K. A. Birkness, E. H. White, D. S. Stephens, and E. Ribot. Evaluation of the chick embryo for the determination of relative virulence of Neisseria meningitidis. FEMS Microbiol Lett 130:37-44. 1995.

21. Sanchez, F., G. Jimenez, A. Aguilar, F. Baquero, and V. Rubio. Plasmid pVA517C from Escherichia coli V517 is required for the expression of an antibiotic microcin. The Journal of antibiotics 39:1028-1030. 1986.

22. Simmons, D. G., L. P. Rose, F. J. Fuller, L. C. Maurer, and G. H. Luginbuhl. Turkey coryza: lack of correlation between plasmids and pathogenicity of Bordetella avium. Avian Dis 30:593-597. 1986.

23. Soriano, V. E., N. A. Vera, C. R. Salado, R. P. Fernandez, and P. J. Blackall. In vitro susceptibility of Ornithobacterium rhinotracheale to several antimicrobial drugs. Avian Dis 47:476-480. 2003.

24. Speer, B. S., N. B. Shoemaker, and A. A. Salyers. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clinical microbiology reviews 5:387-399. 1992.

25. Sprenger, S. J., A. Back, D. P. Shaw, K. V. Nagaraja, D. C. Roepke, and D. A. Halvorson. Ornithobacterium rhinotracheale infection in turkeys: experimental reproduction of the disease. Avian Dis 42:154-161. 1998.

26. Tabatabai, L. B., M. K. Zimmerli, E. S. Zehr, R. E. Briggs, and F. M. Tatum. Ornithobacterium rhinotracheale North American field isolates express a hemolysin-like protein. Avian Dis 54:994-1001. 2010.

27. van Empel, P. C., and H. M. Hafez. Ornithobacterium rhinotracheale: a review. Avian Pathol 28:217-227. 1999.

28. van Veen, L., M. Vrijenhoek, and P. van Empel. Studies of the transmission routes of Ornithobacterium rhinotracheale and immunoprophylaxis to prevent infection in young meat turkeys. Avian Dis 48:233-237. 2004.

29. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, L. A. Devriese, M. Bisgaard, K. H. Hinz, and W. Mannheim. Ornithobacterium rhinotracheale gen. nov., sp. nov., isolated from the avian respiratory tract. Int J Syst Bacteriol 44:24-37. 1994.

30. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, and et al. Ornithobacterium rhinotracheale

gen. nov., sp. nov., isolated from the avian respiratory tract. Int J Syst Bacteriol 44:24-37. 1994.

31. Vargas, E., M. Garduno, P. Rosas. Isolation and Characterization of *Ornithobacterium rhinotracheale* from respiratory diaseased turkeys. Veterinaria Mexico 34:283-288. 2003.

32. Walters, J., R. Evans, T. LeRoith, N. Sriranganathan, A. McElroy, and F. W. Pierson. Experimental comparison of hemolytic and nonhemolytic Ornithobacterium rhinotracheale field isolates in vivo. Avian Dis 58:78-82. 2014.

Chapter Four: Experimental Comparison of Hemolytic and Non-Hemolytic *Ornithobacterium rhinotracheale* Field Isolates *In Vivo*

Walters, J., R. Evans, T. LeRoith, N. Sriranganathan, A. McElroy, and F. W. Pierson. Experimental comparison of hemolytic and nonhemolytic Ornithobacterium rhinotracheale field isolates in vivo. Avian Dis 58:1:78-82. 2014.

Summary

Ornithobacterium rhinotracheale (ORT) is a non-hemolytic, Gram-negative, pleomorphic rod-shaped bacterium that causes upper and lower respiratory tract disease in poultry. Recently, hemolytic strains of ORT have been isolated with increasing frequency from field outbreaks. A study was conducted to determine whether the hemolytic phenotype is associated with any change in virulence. Briefly, 225 turkey poults vaccinated against hemorrhagic enteritis at four weeks-of-age were randomly divided into nine groups: three control (C) (n=25 poults/group), three infected with a non-hemolytic (NH) isolate, and three infected with a hemolytic (H) isolate (n=24 poults/group). Nine days post-vaccination, poults were inoculated intra-tracheally with either 0.2 ml sterile phosphate buffered saline (PBS, sham-control), 2 x 10^8 colony forming units (CFU) of the non-hemolytic strain (NH 725-09) or 2 x 10^8 CFU of a hemolytic strain (H 22470-08 lg). Serum and body weights were obtained at 0, 7, 14, and 21 days post-inoculation (dpi). Tissues were taken for culture and histopathology from 5 randomly selected poults/group at 7, 14, and 21 dpi. A highly significant depression in weight gain was noted for poults in the NH groups at 7 dpi when compared to H and C groups. Poults in the NH groups also had significantly higher antibody levels against ORT as determined by ELISA at 14 and 21 dpi when compared to those in the H group. Re-isolation rates for infected poults decreased over time and by 21 dpi, only the NH phenotype could be isolated from NH inoculated poults. Based on a Likert-type scale, poults in the NH groups had significantly higher histopathological lesion scores in lung tissue at 7, 14, and 21 dpi. Results suggest that non-hemolytic field isolates are more virulent then hemolytic ones. These findings are unusual because hemolytic phenotypes often more virulent in other bacterial species.

INTRODUCTION

Ornithobacterium rhinotracheale (ORT) is a Gram-negative, pleomorphic rod-shaped bacterium that causes respiratory disease in poultry (2, 5, 6). First isolated in Germany in 1981 from five-week old turkeys with nasal discharge, facial edema, and fibrinopurulent airsacculitis (3), it was subsequently found in the United States in 1989 and characterized by Charlton et al., in 1993 (2). Vandamme et al., proposed its current taxonomic classification a year later (14).

Experiments have shown that ORT is pathogenic to both young and old turkeys as well as other avian species. Clinical signs associated with ORT infection include: decreased feed and water intake, weakness, an audible snick, marked dyspnea, coughing, and expectoration of blood stained mucus (4, 10). Older poults, 16-24 weeks of age, often show more severe clinical signs than younger poults (8, 10).

Previous experiments looking at infection with ORT have used typical non-hemolytic isolates. Sprenger *et al*. (9) exposed twenty-five 22-week-old commercial male turkeys to both pure ORT cultures and lung homogenate from turkeys that had been infected with the bacteria. Within 24 hours post-inoculation (PI), those that had been inoculated intra-tracheally exhibited decreased feed intake, depression, and coughing. In several birds, expectorates became mucoid and bloody by 48 hours PI and these subsequently died within another 24 hours. Five days after the inoculation with ORT, surviving birds were less depressed and clinical signs had subsided. Controls remained clinically normal throughout the duration of the trial. In addition, ORT-infected birds, whether infected with pure culture or a lung homogenate, showed significant weight loss when compared to controls (10). ORT has also been shown to cause disease in younger turkeys (9) and decreased weight gain in chickens (10, 12).

More recently, atypical North American field isolates have been exhibiting hemolysis on Columbia Blood Agar supplemented with 5% sheep's blood (11). The purpose of this study was to determine if there were differences between non-hemolytic and hemolytic field isolates *in vivo* since hemolysin production has been shown to enhance virulence in other Gram-negative bacteria such as *E. coli* (7). We sought to test the hypothesis that the presence of hemolysis correlated with a pathotypic difference between isolates.

MATERIALS AND METHODS

Challenge with *Ornithobacterium rhinotracheale*.

A total of 225 turkeys were used for the study. These were obtained at 1 day-of-age from Aviagen Turkeys (Lewisburg, WV) and originated from parent flocks that were determined to be negative for ORT by ELISA. Serum samples from parent flocks were tested on a self-made ELISA coated with both non-hemolytic and hemolytic boiled extract antigens as well as on a commercial ORT ELISA from BioChek (BioChek BV, ER Reeuwijk, The Netherlands). Poults were raised in floor pens (10' x 10') on pine shavings at the Center for Molecular Medicine and Infectious Diseases, Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech. Food and water was provided *ad libitum.* At 28 days of age, they were vaccinated orally with live splenic HEV vaccine, wing-banded, and randomly distributed into 9 rooms (3 replicate rooms/treatment groups). At 37 days of age, sham controls (C, 3 groups, 25 birds/group) were inoculated intra-tracheally with 0.2 ml of sterile PBS. Those in the non-hemolytic (NH, Isolate ORT-725-09) and hemolytic (H, Isolate ORT-22470-081g) groups were inoculated with 2×10^8 CFU of the corresponding isolate (3 groups/treatment, 24 birds/group). ORT isolates were propagated on Columbia agar containing 5% sheep blood (CBA, Remel-Thermo Fisher Scientific, Lenexa, KS) and incubated in a candle jar at 37 ˚C for 24 hours. Plates were scraped and dilutions prepared in PBS. All protocols were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Data and Sample Collection.

Mortality was recorded daily by room and clinical signs were scored using a Likert-based scoring system: 0 - normal, 1 - nasal discharge/sneezing, 2 - nasal discharge, sneezing, coughing, ruffled feathers, lethargy 3: sneezing, coughing, ruffled feathers, somnolence,

reluctance to move when prodded. Birds exhibiting a score of 3 were to be euthanatized by cervical dislocation. Blood and body weights were obtained on the day of inoculation (0 days post-inoculation, dpi) as well as 7, 14, and 21 dpi. Sera were tested using ELISA methodology described by van Empel (13) and antibody responses were compared based on differences in optical density (OD). A total of five birds per group were euthanatized at 7, 14, and 21 dpi to obtain portions of lung and trachea for histopathology and culture.

Histopathology.

Lung tissue was placed in 10% buffered formalin for a minimum of 24 hours and processed. Paraffin imbedded thin sections (4 um) were stained with hematoxylin and eosin and evaluated by standard light microscopy for lesions consistent with pneumonia i.e., the presence of multi-nucleated giant cells, reactive macrophages, large areas of necrosis, and bacteria. Sections were scored using a Likert-based system of 0-4 with 0: within normal limits, 1: minimal inflammation, 2: mild inflammation, 3: moderate inflammation, and 4: severe inflammation (Figure 1).

Statistical Analysis.

Body weights, weight gains, antibody responses, and lung lesion scores were averaged by group and analyzed using one-way Analysis of Variance (ANOVA). Tukey's HSD test was used to compare means and significance was assigned based on $p \leq 0.05$ (JMP, SAS Institute, Inc., Cary, NC)

RESULTS

Challenge with *O. rhinotracheale.*

None of the poults exhibited outward clinical signs following challenge / inoculation (score of 0) and no significant differences were seen in total body weight among groups. However, those inoculated with the NH isolate experienced a highly significant depression in weight gain ($p=0.0032$) when compared to birds the H and C groups between 0 and 7 dpi. There was no significant difference in weight gains during remaining weeks of the study, suggesting compensatory gain (Table 1).

Antibody responses were compared with both positive control serum (pooled) from an endemically infected ORT farm and negative control serum (pooled) from poults raised in isolation at the university. Serum samples were tested on an in-house ELISA coated with both non-hemolytic and hemolytic boiled extract antigens. Responses (OD value at 405 nm) from all birds were averaged by room and statistical analysis was completed on treatment group averages (n=3 rooms/treatment group). Responses were seen in both NH and H inoculated groups 7-21 dpi, but a significantly higher antibody response was seen in the birds inoculated with the NH isolate (7 dpi-$p<0.0001$, 14 dpi $p=0.0003$, 21 dpi $p<0.0001$) (Table 2). Antibodies to ORT were not detected in the controls.

Re-isolation from lung and trachea served as a means to determine whether there was a change in phenotype to detect cross-contamination. Upon re-isolation, both isolates retained their original phenotypic characteristics on CBA. ORT could be re-isolated from poults in the NH groups through 21 dpi, whereas isolation in the H groups was only possible through 14 dpi (Table 3). All isolates were verified using the API ZYM identification system (BioMérieux, Inc. Durham, NC USA). There was no evidence of cross-contamination.

Histopathological lesion scores were significantly higher in poults inoculated with the NH isolate 7, 14, and 21 dpi (Figure 2) when compared to H and C poults.

DISCUSSION

Although typically non-hemolytic, ORT isolates exhibiting varying degrees of hemolysis have begun to appear more frequently in the field (1, 11). A hemolysin-like cytolytic pore-forming protein was identified (11), but very little work has been done to investigate the clinical effects of this atypical phenotype *in vivo*. This is the first study conducted in live birds to compare non-hemolytic and hemolytic ORT field isolates.

Anecdotal information supplied by field personnel suggested that mortality may be higher in flocks infected with hemolytic ORT isolates. However, embryo lethality assays performed in our laboratory (data not shown) and the live-bird trial described herein do not support this observation. In fact, our study revealed that the non-hemolytic isolate had a negative effect on weight gain, caused higher antibody responses suggesting greater invasiveness, and produced more severe lung lesions. The non-hemolytic isolate also had the ability to survive longer in the host. The clinical effects seen in this trial were milder, but the trends exhibited were comparable to those observed by other investigators evaluating non-hemolytic isolates (10, 12). Therefore, it would appear that the increased mortality and morbidity thought associated with the hemolytic isolate in the field was more likely due to prior or concomitant exposure to other disease agents and/or the presence of additional management issues.

From a production perspective, it appears that non-hemolytic isolates may be of greater economic significance. In this study, an 11% loss of gain was seen over a 1 week period following infection with this phenotype. Compensatory gain was subsequently noted, but the true financial impact would depend on age at time of infection, especially if feed conversion was dramatically altered. Unfortunately, this indicator could not be accurately determined in the present trial due to excessive feed wastage.

Further live trials are necessary to determine whether prior or concomitant infection with other respiratory pathogens will influence morbidity and mortality associated with hemolytic versus non-hemolytic ORT challenge. In addition, a higher density of poult placement may enhance the experimental effect.

Finally, if the hemolytic isolate is truly less pathogenic and phenotypically stable, it may have value as vaccine candidate if serologic cross-reactivity on ELISA is reflective of cross-protectivity.

References:

1. Banani, M., S.A. Pourbakhsh, and P. Khaki. Characterization of Ornithobacterium rhinotracheale Isolates from Commercial Chickens. Arch. Razi Ins. 52:27-36. 2001.
2. Charlton, B.R., S.E. Channing-Santiago, A.A. Bickford, C.J. Cardona, R.P. Chin, G.L. Cooper, R. Droual, J.S. Jeffrey, C.U. Meteyer, H.L. Shivaprasad, and R. Walker. Preliminary characterization of a pleomorphic gram-negative rod associated with avian respiratory disease. J Vet Diagn Invest 5:47-51. 1993.
3. Chin, R.P., C.P. van Empel, and H.M. Hafez. Ornithobacterium rhinotracheale infection. In: Diseases of poultry, 12th ed. Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan, and D.E. Swayne, eds. Wiley-Blackwell, Ames, Iowa. pp. 765-774. 2008.
4. Hafez, H.M. Current Status on the role of Ornithobacterium rhinotracheale (ORT) in respiratory disease complexes in poultry. Arch Geflùgelkd 60:208-211. 1996.
5. Hinz, K.H., C. Blome, and M. Ryll. Acute exudative pneumonia and airsacculitis associated with Ornithobacterium rhinotracheale in turkeys. Vet Rec 135:233-4. 1994.
6. Hinz, K.H. and H.M. Hafez. The early history of Ornithobacterium rhinotracheale (ORT). Arch Geflùgelkd 61:95-96. 1997.
7. Minshew, B.H., J. Jorgensen, G.W. Counts, and S. Falkow. Association of hemolysin production, hemagglutination of human erythrocytes, and virulence for chicken embryos of extraintestinal Escherichia coli isolates. Infect Immun 20:50-4. 1978.
8. Roepke, D.C. Field observations of ORT infection in an integrated turkey operation. In: Proceedings of The Turkey ORT Scientific Symposium organized by Roche Animal Nutrition and Health. Minneapolis, Minnesota. September 4-6, 1996.

9. Ryll, M., K.H. Hinz, H. Salisch, and W. Kruse. Pathogenicity of Ornithobacterium rhinotracheale for turkey poults under experimental conditions. Vet Rec 139:19. 1996.

10. Sprenger, S.J., A. Back, D.P. Shaw, K.V. Nagaraja, D.C. Roepke, and D.A. Halvorson. Ornithobacterium rhinotracheale infection in turkeys: experimental reproduction of the disease. Avian Dis 42:154-61. 1998.

11. Tabatabai, L.B., M.K. Zimmerli, E.S. Zehr, R.E. Briggs, and F.M. Tatum. Ornithobacterium rhinotracheale North American field isolates express a hemolysin-like protein. Avian Dis 54:994-1001. 2010.

12. van Empel, P., H. van den Bosch, D. Goovaerts, and P. Storm. Experimental infection in turkeys and chickens with Ornithobacterium rhinotracheale. Avian Dis 40:858-64. 1996.

13. van Empel, P., H. van den Bosch, P. Loeffen, and P. Storm. Identification and serotyping of Ornithobacterium rhinotracheale. J Clin Microbiol 35:418-21. 1997.

14. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, L.A. Devriese, M. Bisgaard, K.H. Hinz, and W. Mannheim. Ornithobacterium rhinotracheale gen. nov., sp. nov., isolated from the avian respiratory tract. Int J Syst Bacteriol 44:24-37. 1994.

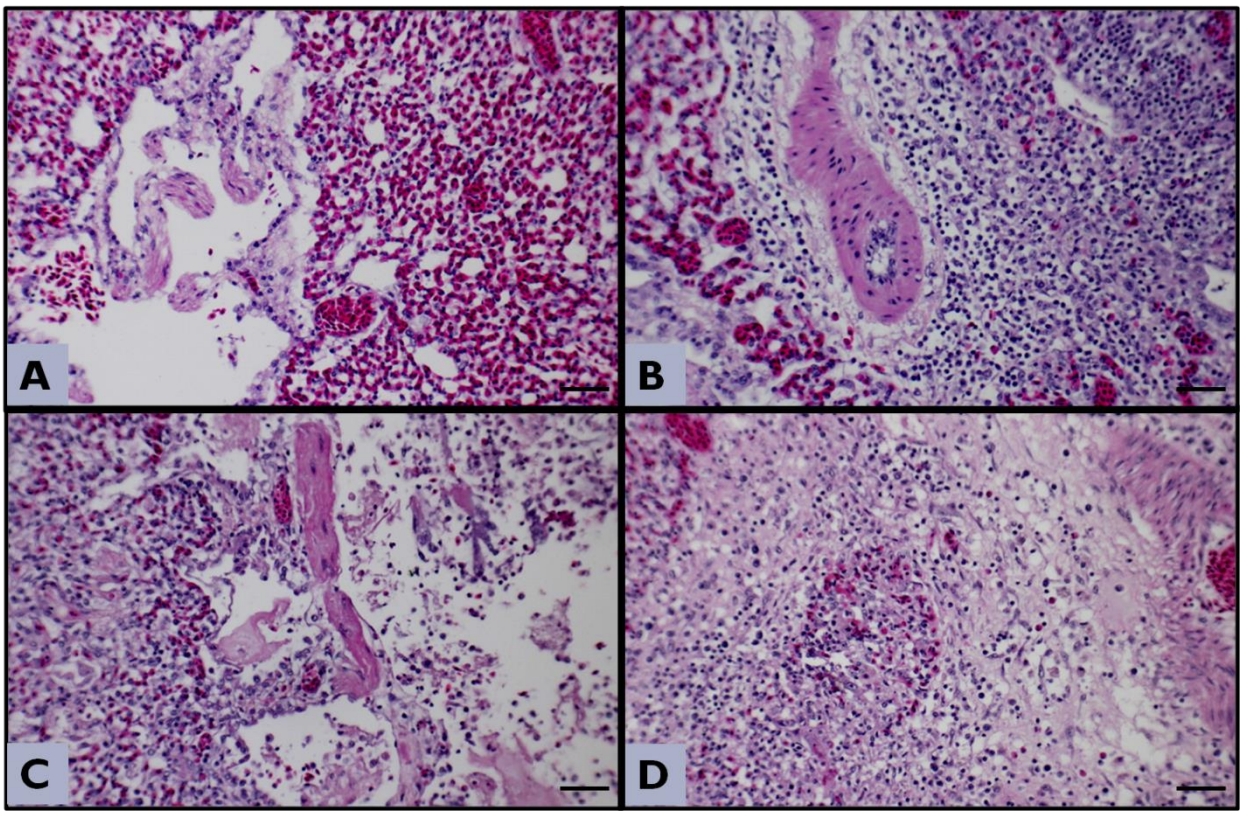

Figure 1. Photomicrographs one week post-inoculation showing varying degrees of inflammation following ORT inoculation. A. Score of 0, within normal limits. B. Score of 1, minimal inflammation. C. Score of 3, moderate inflammation. D. Score of 4, severe inflammation. Bars are approximately 100 microns.

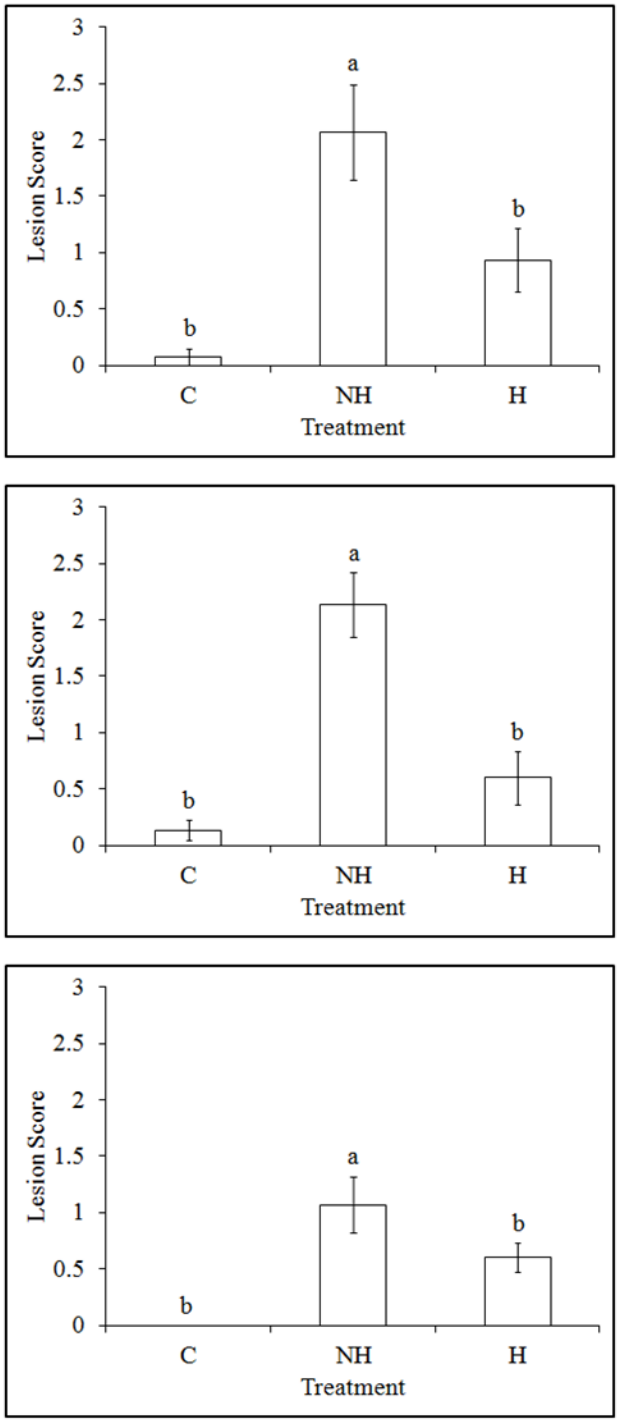

Figure 2. Histopathologic lesions scores top: 7 dpi ($p=0.0002$), middle: 14 dpi ($p<0.0001$), and bottom: 21 dpi ($p=0.0002$) showing a significant increase in lesion score associated with NH compared to H and C treatments.

Treatment[A]	n[B]	Mean Body Weight 0 dpi (kg)	Mean Body Weight 7 dpi (kg)	Mean Weekly Weight Gain 0-7 dpi (kg)[C]	Mean Body Weight 14 dpi (kg)[D]	Mean Weekly Weight Gain 7-14 dpi (kg)	Mean Body Weight 21 dpi (kg)	Mean Weekly Weight Gain 14-21 dpi (kg)
C	3	2.05 (+/- 0.09)	2.78 (+/- 0.09)	0.74 (+/- 0.01)[a]	3.67 (+/- 0.15)	0.88 (+/- 0.06)	4.68 (+/- 0.21)	1.01 (+/- 0.09)
NH	3	2.17 (+/- 0.04)	2.83 (+/- 0.02)	0.66 (+/- 0.04)[b]	3.79 (+/- 0.06)	0.96 (+/- 0.07)	4.89 (+/- 0.09)	1.10 (+/- 0.07)
H	3	2.14 (+/- 0.04)	2.93 (+/- 0.04)	0.78 (+/- 0.01)[a]	3.93 (+/- 0.05)	1.02 (+/- 0.01)	4.88 (+/- 0.29)	0.94 (+/- 0.31)

[A] C = sham treatment controls, NH = poults inoculated with the non-hemolytic isolate, H = poults inoculated with the hemolytic isolate.

[B] All birds each replicate (room) were weighed and replicate averages were used calculate treatment means ($n = 3$ replicates / treatment).

[C] Within column, means having different superscripts are significantly different ($p<0.05$).

[D] Due to the removal of 5 poults for tissue harvesting at 7 and 14 dpi, the number of poults available for weighing at 14 and 21 dpi was proportionately reduced.

Table 1. Average weekly body weights and weight gains by treatment. Infection with non-hemolytic ORT caused a depression in weight gain between 0 and 7 dpi (days post-inoculation) (p=0.0032)

	Mean Antibody Response (OD at λ 450 nm)[A,B]			
Treatment[C]	Day 0 Post-Inoculation	Day 7 Post-Inoculation	Day 14 Post-Inoculation[D]	Day 21 Post-Inoculation
C	0.105 +/- 0.009[b]	0.092 +/- 0.005[c]	0.099 +/- 0.004[c]	0.122 +/- 0.029[c]
NH	0.166 +/- 0.026[a]	0.556 +/- 0.052[a]	0.605 +/- 0.120[a]	0.592 +/- 0.081[a]
H	0.105 +/- 0.014[b]	0.291 +/- 0.029[b]	0.377 +/- 0.010[b]	0.256 +/- 0.020[b]

[A] Within column, means having different superscripts are significantly different ($p<0.05$)

[B] All birds in each replicate (room) were sampled, values averaged and treatment means ($n = 3$ replicates / treatment) calculated. By comparison, the average OD for positive control wells was 0.569 and that for negative control wells was 0.127.

[C] C = sham treatment controls, NH = poults inoculated with the non-hemolytic isolate, H = poults inoculated with the hemolytic isolate

[D] Due to the removal of 5 poults for tissue harvesting at 7 and 14 dpi, the number of poults available for serological testing at 14 and 21 dpi was proportionately reduced.

Table 2. Average antibody response (OD) by treatment. Responses were seen in both NH and H inoculated groups 7-21 dpi, but a significantly higher antibody response was seen in the birds inoculated with the NH isolate (7 dpi-$p<0.0001$, 14 dpi $p=0.0003$, 21 dpi $p<0.0001$) (Table 2). Antibodies to ORT were not detected in the controls

	ORT Re-isolation[A,B]		
Treatment[C]	Day 7 Post-Inoculation	Day 14 Post-Inoculation	Day 21 Post-Inoculation
C	0/15	0/15	0/15
NH	4/15	5/15	3/15
H	9/15	2/15	0/15

[A] Total number of birds by treatment (5 birds per replicate, 3 replicates / treatment)

[B] The isolate phenotype recovered corresponded to isolate phenotype used for inoculation.

[C] C = sham treatment controls, NH = poults inoculated with the non-hemolytic isolate, H = poults inoculated with the hemolytic isolate.

Table 3. Ability to recover respective ORT isolate by treatment. Isolation was able to be completed through day 21 post-inoculation in the non-hemolytic treatment group while only through day 14 post-inoculation in the hemolytic treatment group. Number of positive birds is out of total number of birds sampled per treatment group.

Chapter Five: Conclusions and Future Work

Ornithobacterium rhinotracheale (ORT) is a highly contagious respiratory pathogen in poultry. While its modes of transmission, pathophysiology, and virulence mechanisms are not fully elucidated, ORT is known to be a cause of significant economic loss (23). Commonly, ORT infection can cause morbidity, mortality, a decrease in weight gain, higher feed conversion, and reduction in egg production. Disease is normally more severe in older birds (20), but younger birds can be affected as well (14). There are no vaccines commercially available and antibiotic resistance proves to be an obstacle to effective treatment (6, 10).

The research described in this dissertation was designed to evaluate the pathogenicity of and identify any unique characteristics of newly-isolated, atypical hemolytic ORT strains found in Virginia's Shenandoah Valley. Further evaluation and studies are necessary, but a framework has been laid for future research.

Serological surveillance using ELISA methodology showed the widespread presence of antibodies (70.9% prevalence) to ORT on commercial turkey farms in Virginia. While not all farms had increased mortality or morbidity, decreased final body weight and increased feed conversion seemed to be associated with ORT seroconversion. Sera received and tested were all pre-slaughter, so whether the corresponding isolates found on the farm were hemolytic (H) or not (NH) was unknown. Additionally, based on serologic data, a significant relationship between the presence of antibodies to ORT and those against other agents such as PMV-1 and *B. avium* was not found, which suggests that ORT might play a role as a primary disease-causing pathogen. A sero-survey that correlates isolation of a specific phenotype with the development of antibodies could be useful, but all isolates cultured from flocks with ORT-related respiratory disease since 2009 by the Virginia Department of Agriculture and Consumer Services (Harrisonburg, Virginia

Laboratory) have shown hemolytic characteristics on Columbia Blood Agar. This consistency would suggest that the H phenotype is the dominant phenotype and may have an increased virulence and greater ability to cause respiratory disease, regardless of results from *in vivo* trials (25). Additionally, the H phenotype dominance could be a result of antibiotic selection pressure associated with resistance. Of potential benefit would be a survey of all flocks at a given age to determine if ORT can be cultured from the lungs and tracheas regardless of the presence of clinical respiratory signs. This may help establish whether the H phenotype can be directly correlated with increased disease rates. Additionally, isolation of other agents like *E. coli* or *B. avium* at the time that clinical respiratory disease first appears could help further define ORT's role as a primary or secondary agent.

Determining external reservoirs that may be contributing to the ability of ORT to survive and become endemic would be of value. Early in this study, the discovery of antibodies to the hemolytic phenotype in lambs on an endemic farm using a serum plate agglutination test suggested the presence of a potential reservoir in a mammalian species. In comparison, lambs from a farm where there was no exposure to commercial turkeys were seronegative (unpublished data). We were unable to isolate ORT from the nasal passages of lambs, but this may have been due to heavy contamination with other bacteria. To overcome the contamination issue, it may be useful to obtain samples from the lungs, tracheas and other internal organs of slaughtered lambs or those that have respiratory disease. Additionally, selective media that would hinder other organisms like blood agar with 5.2 ug/ml of gentamicin and 5.0 ug/ml of polymyxin B (4) could enhance isolation of ORT. A case-control study evaluating multiple farms with both species and those with either turkeys or sheep alone would be helpful answering the question of an alternate reservoir. In order to estimate an appropriate sample size for this study, we have to consider an

expected proportion of controls exposed, an assumed odds ratio, confidence level, and power. Using an expected proportion of controls exposed as 0.05, an assumed odds ratio of 10 (farms with lambs are ten times more likely to have birds positive for ORT), a confidence level of 0.95 and a power of 0.8, an appropriate sample size for this study would be 25 farms in each category (Epitools, AusVet Animal Health Services, 2014). During the study, both lambs and turkeys would be tested for the presence of antibodies via serum samples and live bacteria via culture, and any evidence of clinical signs associated with respiratory disease would be recorded and evaluated. While serology comparisons were originally determined via a SPAT, the ELISA developed as part of this dissertation (Chapter Two) would likely be more sensitive. This could be accomplished by modifying the methodology to include conjugated rabbit or goat anti-sheep antibody. In addition to the work with lambs, analysis of the reservoir potential of insects and rodents would also be useful. Particularly on poultry farms, issues with pest management contribute to large numbers of rodents and darkling beetles. Both of these species are able to live in the poultry house and have access to food, water, litter, and the birds themselves. If found to be disease-carrying vectors, both could contribute to disease spread on farm, between houses, and between neighboring farms.

In addition to hemolysis characteristics, differences between the two ORT phenotypes were identified. Differences in 16S rRNA and SDS-PAGE protein analysis were not found, but further work involving Western Blot techniques determining cross reactivity and immunogenicity as well as serotyping both isolates should be completed. Identical 16S rRNA sequences found in the hemolytic, non-hemolytic, and the ATCC Type strains of ORT showed that the hemolytic strain does contain the conserved 784 bp fragment identifying it as ORT (1). Differences in plasmid-like bands, growth conditions (Appendix A), and antimicrobial

susceptibility were present. Plasmid analysis between the two phenotypes identified a suspected super-coiled plasmid in the hemolytic phenotype that was not present in the non-hemolytic isolate studied. Further evaluation matching sequence data to specific enzymes or transcriptomes may be useful to determine more precisely the purpose of the plasmid. However, sequence and BLAST analysis suggest that portions of the plasmid may have evolved from *Reimerella anatipestifer* and/or *Flavobacterium*, both of which can cause disease in poultry. This suggests that ORT may have picked up extra-chromosomal DNA from these bacterial species during a co-evolutionary event, presumably host co-infection. When ORT was first identified, it was initially thought to be a variant of *R. anatipestifer* (24). This suggests the close taxonomic relationship of the two organisms and may propose a mechanism of plasmid transfer if *R. anatipestifer* is a definitive source of genetic material in the H ORT plasmid. Specifically, the 4.0 kb plasmid seen in the H isolate appears to be similar to a 3.9 kb plasmid found in *R. anatipestifer*. This *Riemerella* insert was found to carry four open reading frames (ORFs) corresponding to virulence-associated proteins from external DNA sources. Two of these ORFs contain chromosomal DNA homologous to virulence proteins found in *Dichelobacter nodosus* (a pathogen associated with footrot in sheep), *Haemophilus influenza, Actinobacillus actinomycetemcomitans*, and *Niesseria gonnorhoeae* (3). This association is of particular interest when considering the finding of antibodies to H ORT in sheep. While ORT has never been cultured from a mammalian species, the presence of antibodies could suggest inter-species transmission between poultry and other livestock species. Additionally, the organism being harbored in both species could contribute to an endemic status of ORT and persistent respiratory disease on farms.

The H phenotype was shown to be less virulent *in vivo* (Chapter Four) than the non-hemolytic organism, which is unusual because hemolysis has been associated with virulence in other bacterial species (8, 11, 12). Additionally, the extra-chromosomal material was stable in the organism. Efforts to cure the suspected plasmid from the hemolytic phenotype using Ethidium Bromide were unsuccessful (Appendix A), therefore, it was impossible to determine if the gene(s) coding for a hemolysin or hemolysin-like protein were located in the plasmid. Alternate methodologies to remove the plasmid must be employed if its role in hemolysis is to be elucidated. The use of Congo Red medium has been used to distinguish between pathogenic *E. coli* species in chickens, and could potentially be of value due to ORT's Gram-negative characteristics (2). Antimicrobial resistance patterns shown here suggest the H isolate has acquired resistance to some antimicrobials, particularly the tetracyclines, which are commonly used to treat poultry respiratory infections. Data from other experiments suggested that the plasmid found in this hemolytic isolate did not correlate with pathogenicity due to its apparent avirulent nature in turkey poults (25). This finding is consistent with plasmids found in other respiratory-disease causing bacteria such as *Bordetella avium* that contain genes associated with antimicrobial resistance (9) but not increased pathogenicity (18). Resistance to tetracyclines through the efflux mechanism (preventing intracellular buildup) has been found on plasmids in bacterial species including *Staphylococccus aureus, Enterococcus, Enterobacteriacae, Vibrio, Streptococcus* and *Bacillus* (13, 19). Implications of this finding include the inability to treat ORT infections with certain antimicrobial classes and the necessity of management changes for control.

Consistent with the findings of other investigators (7, 20, 22), the non-hemolytic phenotype caused a decrease in weight gain one week post-inoculation. van Empel and others

have suggested a substantial economic impact of ornithobacteriosis, but have not quantified the cost of disease (15). Current market values and production statistics of turkeys in the U.S. provide a basis for estimating a cost scenario. The average livability of light hens on a commercial farm at the time of marketing is 91.15% (AgriStats, Accessed 9/2/2014); if the number of poults placed on the farm at one day of age is 10,000, the average total number of birds going to slaughter is 9,115 birds. Farmers paid a per bird base price of $0.96 equates to a total market value of $8750.50 for an average flock. Data demonstrating an average mortality of ~2.4% in acutely affected ORT hen flocks (5), suggests livability drops to 88.75% and decreases the market value of the flock to $8,520. In an experimental aerosol challenge of birds at 31 days of age, van Empel showed that average daily weight gain of turkeys decreased by 47% in birds challenged with both ORT and turkey rhinotracheitis virus (TRT) and 44% in those challenged with ORT alone (22). Studies completed during this research period showed that the non-hemolytic isolate had a much bigger impact on weight gain than the hemolytic isolate. In the trial, a loss of gain of approximately 11% was seen in turkeys challenged with the non-hemolytic isolates, while there was no loss of gain in the control and hemolytic groups. With average gain in a commercial light hen flock being 1750 (0.1750 lbs/day) in 2014 (AgriStats, Accessed 9/2/2014), a decrease of 11% results in a gain of only 1558 (0.1558). With a seven day period of decreased gain on an average age of 86 days, average final bird weight would be decreased to 14.916 lbs from 15.225 lbs. When companies were paid $1.08/lb for product (21), a loss of $0.33 per bird and $3,015 per flock could be expected. While not as substantial as the 44% decrease seen in van Empel's study (22), this loss in pay could be amplified with the addition of concomitant diseases or management issues. With an ORT prevalence of ~70% in Virginia, and a complex slaughtering 300,000 head per week, 15,600,000 per year, flocks with exposure to

ORT total 10,920,000 birds. Field evidence showing diseased flocks experiencing mortality averaging 2.4% due to ORT infection alone (5) equates to a loss of 262,080 birds. At 15.00 lbs per bird, this mortality causes a production loss of 3,931,200 lbs of meat and $4,245,696 ($1.08/lb (21)) in lost income. When looking at loss of gain on a large scale, having a loss of even 0.3 lbs per bird could cause a production loss of 3,276,000 lbs of meat and $3,538,080 in lost income. Cost analysis here limits itself to the value of a bird at slaughter. This financial simulation assumes only one week of decreased ADWG resulting from ORT infection; no prior or concomitant infections, management issues, price fluctuations, or bird type other than light hen. As stated in Chapter One, any changes in these factors could cause increases in the cost of an ORT infection to the producer and the company. Other factors that would also affect the value of losses, but for which estimates are not available, would include such things as feed conversion ratio, medications costs and a decrease in line speeds at the processing plant due to lack of bird size uniformity.

The finding that nearly all field isolates currently being cultured are of the hemolytic phenotype could suggest a benefit in being less likely to cause disease (a greater ability to survive in the host), or it may be related to antimicrobial resistance to enhance survival capability. Originally, the hemolytic isolate was speculated to be associated with severe outbreaks of disease, but trials in both embryo and live poult models indicate a lack of virulence associated with the H pathotype. The plasmid found in the H phenotype is extremely stable, as evidenced by the inability to cure it from the bacterium (Appendix 1). Plasmids, by definition, are extrachromosomal genetic elements capable of stable autonomous replication in a cell (17). Maintenance of these extrachromosomal genetic elements requires not only resources but places a metabolic burden on their host bacteria (16). The purpose for conservation of this plasmid by H

ORT is unknown, but because the organism died rather than relinquish the plasmid we have reason to believe the plasmid is important for survival of its host (Appendix 1).

Yet to be fully elucidated is the role of a concomitant or prior exposure to other disease-causing agent in the production of ornithobacteriosis. If the H phenotype has a greater ability to survive in the host, it may have a greater ability to compromise the bird for a secondary infection by bacterial species such as *E. coli* or *B. avium* - both prominent organisms in the region. A trial using live birds following the methods previously described in Chapter Four could be useful in determining the role of concurrent infections for respiratory disease involving ORT. Inoculation with both ORT and BA could be used to determine an opportunistic relationship between the two that could be related to increased morbidity and mortality seen in the field. Mortality and morbidity would be recorded daily using the Likert scale previously described (25). In addition, weights, serum samples, and lung/tracheal samples for histopathology and bacterial cultures would be taken at 7, 14, and 21 days post-challenge to determine pathogenicity differences between a single ORT infection and a concomitant infection with *B. avium*.

The H phenotype may be a suitable live vaccine candidate in birds as evidenced by the production of an antibody response but very mild lesions and the absence of any effect on weight gain in poults. Current industry standard is vaccination of breeder hens to protect both the hens and progeny. This presents a problem in that if ORT is found to affect older birds (8-10 weeks) in the grower house and cause more severe disease, the presence of maternal antibodies would be ineffective due to the fact that vertically passed immunoglobulins only persist the first three weeks of life. While vaccinating breeder hens is likely effective in preventing disease in those birds and therefore preventing loss of eggs and subsequently progeny, the presence of maternal antibodies in the brooder end could potentially inhibit early, mild infection that could induce a

natural immune response in poults. If the H strain could be used at hatch or after wane of maternal antibodies to protect against field challenge, it would have great economic implications to protect against financial loss in the industry. Use of this strain would allow a live infection to stimulate the immune response in young birds to produce their own antibodies to ORT, hopefully protecting them from severe disease in the later grow-out stages.

In conclusion, research completed in this dissertation has shown that ORT is an important pathogen in the Virginia poultry industry, and that the hemolytic phenotype of ORT differs from the non-hemolytic phenotype in both *in vitro* and *in vivo* studies. Specifically, molecular findings showed that this phenotype contains a plasmid with sequence similarity to other plasmids found in related poultry disease-causing agents like *R. anatipestifer*- suggesting that its origin may be external and not chromosomal. This plasmid is stable in the organism, but there is a lack of evidence that the plasmid is responsible for the hemolytic phenotype. In addition to evidence that this pathotype has differing molecular properties, increased antimicrobial resistance, and the presence of alternate growth patterns, it appears to be avirulent in a live turkey model as a primary agent. This is unusual in that hemolysin production is considered a virulence factor in other bacterial species like *E. coli* (12). The discovery of this avirulent phenotype could have implications in live vaccine development against ORT, which could prove to be crucial in preventing disease and loss of profits in the commercial poultry industry. Research still needs to be completed to further evaluate this organism and particularly its plasmid, but this dissertation has formed a baseline of knowledge of which to build upon.

References:

1. Amonsin, A., J. Wellehan, L. Li, P. Vandamme, C. Lindeman, M. Edman, R. Robinson, and V. Kapur. Molecular epidemiology of Ornithobacterium rhinotracheale. J. Clin. Microbiol. 35:2894-2898. 1997.
2. Berkhoff, H. A., and A. C. Vinal. Congo red medium to distinguish between invasive and non-invasive Escherichia coli pathogenic for poultry. Avian Dis 30:117-121. 1986.
3. Chang, C. F., P. E. Hung, and Y. F. Chang. Molecular characterization of a plasmid isolated from Riemerella anatipestifer. Avian Pathol 27:339-345. 1998.
4. Chin, R. P., and B. R. Charlton. Ornithobacteriosis A Laboratory Manual for the Isolation and Identification of Avian Pathogens 4:89-91. 1998.
5. De Rosa, M., R. Droual, R. P. Chin, H. L. Shivaprasad, and R. L. Walker. Ornithobacterium rhinotracheale infection in turkey breeders. Avian Dis 40:865-874. 1996.
6. Devriese, L. A., P. De Herdt, and F. Haesebrouck. Antibiotic sensitivity and resistance in Ornithobacterium rhinotracheale strains from Belgian broiler chickens. Avian Pathol 30:197-200. 2001.
7. El-Sukhon, S. N., A. Musa, and M. Al-Attar. Studies on the bacterial etiology of airsacculitis of broilers in northern and middle Jordan with special reference to Escherichia coli, Ornithobacterium rhinotracheale, and Bordetella avium. Avian Dis 46:605-612. 2002.
8. Goebel, W., T. Chakraborty, and J. Kreft. Bacterial hemolysins as virulence factors. Antonie Van Leeuwenhoek 54:453-463. 1988.

9. Jackwood, M. W., Y. M. Saif, and D. L. Coplin. Isolation and characterization of Bordetella avium plasmids. Avian Dis 31:782-786. 1987.

10. Malik, Y. S., K. Olsen, K. Kumar, and S. M. Goyal. In vitro antibiotic resistance profiles of Ornithobacterium rhinotracheale strains from Minnesota turkeys during 1996-2002. Avian Dis 47:588-593. 2003.

11. May, A. K., T. G. Gleason, R. G. Sawyer, and T. L. Pruett. Contribution of Escherichia coli alpha-hemolysin to bacterial virulence and to intraperitoneal alterations in peritonitis. Infect Immun 68:176-183. 2000.

12. Minshew, B. H., J. Jorgensen, G. W. Counts, and S. Falkow. Association of hemolysin production, hemagglutination of human erythrocytes, and virulence for chicken embryos of extraintestinal Escherichia coli isolates. Infect Immun 20:50-54. 1978.

13. Mojumdar, M., and S. A. Khan. Characterization of the tetracycline resistance gene of plasmid pT181 of Staphylococcus aureus. J Bacteriol. 170:5522-5528. 1988.

14. Ryll, M., K. H. Hinz, H. Salisch, and W. Kruse. Pathogenicity of Ornithobacterium rhinotracheale for turkey poults under experimental conditions. Vet Rec 139:19. 1996.

15. Saif, Y. M., J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Diseases of Poultry, 12 ed. Blackwell Publishing, Ames, Iowa. 2008.

16. Sengupta, M., and S. Austin. Prevalence and significance of plasmid maintenance functions in the virulence plasmids of pathogenic bacteria. Infect Immun 79:2502-2509. 2011.

17. Sherratt, D. J. Bacterial plasmids. Cell 3:189-195. 1974.

18. Simmons, D. G., L. P. Rose, F. J. Fuller, L. C. Maurer, and G. H. Luginbuhl. Turkey coryza: lack of correlation between plasmids and pathogenicity of Bordetella avium. Avian Dis 30:593-597. 1986.

19. Speer, B. S., N. B. Shoemaker, and A. A. Salyers. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clinical microbiology reviews 5:387-399. 1992.

20. Sprenger, S. J., A. Back, D. P. Shaw, K. V. Nagaraja, D. C. Roepke, and D. A. Halvorson. Ornithobacterium rhinotracheale infection in turkeys: experimental reproduction of the disease. Avian Dis 42:154-161. 1998.

21. USDA. USDA AMS Livestock, Poultry & Grain Market News In., Des Moines, Iowa. 2014.

22. van Empel, P., H. van den Bosch, D. Goovaerts, and P. Storm. Experimental infection in turkeys and chickens with Ornithobacterium rhinotracheale. Avian Dis 40:858-864. 1996.

23. van Empel, P. C., and H. M. Hafez. Ornithobacterium rhinotracheale: a review. Avian Pathol 28:217-227. 1999.

24. Vandamme, P., P. Segers, M. Vancanneyt, K. van Hove, R. Mutters, J. Hommez, F. Dewhirst, B. Paster, K. Kersters, E. Falsen, L. A. Devriese, M. Bisgaard, K. H. Hinz, and W. Mannheim. Ornithobacterium rhinotracheale gen. nov., sp. nov., isolated from the avian respiratory tract. Int J Syst Bacteriol 44:24-37. 1994.

25. Walters, J., R. Evans, T. LeRoith, N. Sriranganathan, A. McElroy, and F. W. Pierson. Experimental comparison of hemolytic and nonhemolytic Ornithobacterium rhinotracheale field isolates in vivo. Avian Dis 58:78-82. 2014.

Appendix A: Further *in vitro* Characterization

Plasmid-Curing Attempts.

Materials and Methods

An *O. rhinotracheale* isolate obtained from a 2008 field case submitted to the Virginia Department of Agriculture and Consumer Services (VDACS) was used in this study: ORT 22470-08 lg (designated hemolytic, H, due to presence of consistent alpha-hemolysis after initial 48 hours incubation). This isolate contained a 4 kb plasmid that was not present in non-hemolytic pathotypes. To attempt to remove the plasmid, Ethidium Bromide (EtBr) in increasing concentrations (0 uM, 1.0 uM, 1.25 uM, 1.5 uM, 1.75 uM, 2.0 uM, 2.25 uM, and 2.5 uM) were added to Columbia agar plates supplemented with 5% turkey's blood. The isolate was grown in 37°C in a restricted-oxygen environment.

Results

Increasing concentration of EtBr prohibited growth of H ORT beyond 2 uM, and killed bacterial cells. Plasmid band remained stable and present up to 2 uM EtBr (Figure 1).

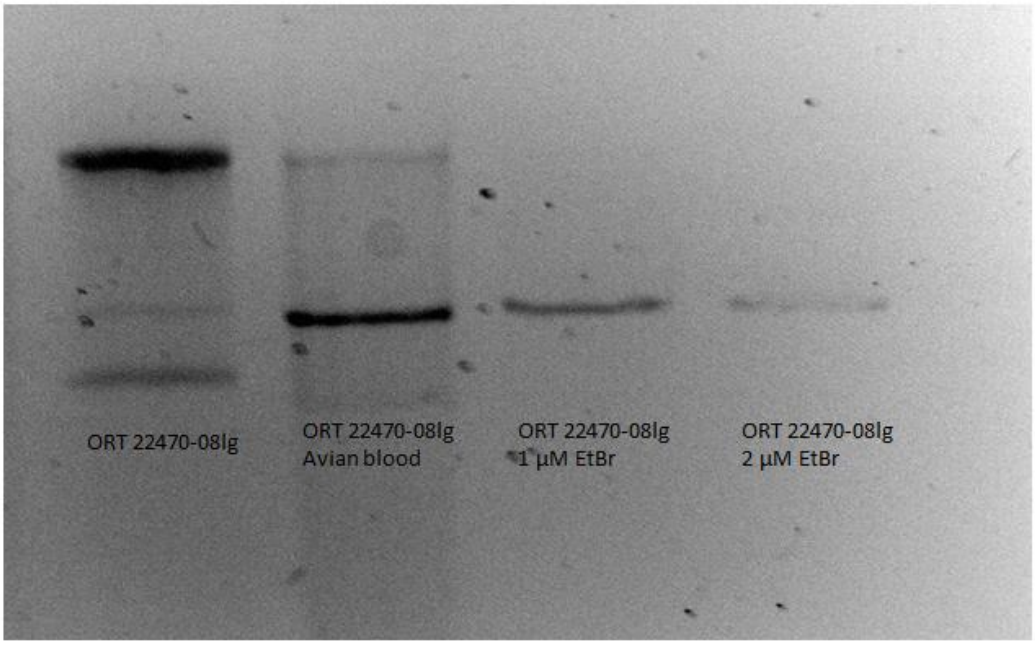

Figure 1: Presence of plasmid through concentration of 2 uM EtBr in blood agar.

Growth in Varying Media

Materials and Methods

O. rhinotracheale isolates obtained from 2008-2009 field cases submitted to the Virginia Department of Agriculture and Consumer Services (VDACS) were used in this study: ORT 22470-08 lg (designated hemolytic, H, due to presence of consistent alpha-hemolysis after initial 48 hours incubation), and ORT 725-09 (designated as non-hemolytic, NH, based on the consistent absence of alpha hemolysis after 48 hours of incubation). Original classification of isolates as H or NH was done by growth characteristics on solid Columbia Agar supplemented with 5% Sheep's Blood (CBA). In addition to growth on CBA, isolates were cultured and proliferation was attempted using the following media: Brain Heart Infusion (BHI) Broth, Tryptic Soy Broth (TSB), TSB + 5% Fetal Bovine Serum (FBS), BHI + 5% whole sheep's blood (SB), BHI + 5% whole turkey blood (TB), and BHI + turkey lung homogenate.

Results:

Using the NH isolate, positive growth was seen in BHI, TSB, TSB+5% FBS, BHI+5% SB, and BHI+ 5% TB. Additionally, mild growth was present in BHI+lung homogenate. With the H isolate, no growth was seen in BHI, TSB, TSB+5% FBS, or BHI+lung homogenate. Some growth was seen in BHI+5% sheep's blood, and best growth of the H isolate occurred with BHI+ 5% TB. Another difference noted between the two was that H colonies were much smaller at 24 hours on CBA.

Medium	NH	H
CBA	+, larger @ 24h	+, smaller @ 24h
BHI broth	++	-
TSB broth	++	-
TSB + 5% FBS	++	-
BHI+5% SB	++	+
BHI+5%TB	++	++
BHI+LH	+	-

Table 1. Growth capabilities of H vs. NH isolates in varying culture media.