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Enhancement of avian influenza virus infectivity by proteolytic bacteria

Byrum, Beverly Rieser, Ph.D.
The Ohio State University, 1994
ENHANCEMENT OF AVIAN INFLUENZA VIRUS INFECTIVITY
BY PROTEOLYTIC BACTERIA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

by

Beverly Rieser Byrum, D.V.M., M.S.

* * * * *

The Ohio State University

1994

Dissertation Committee: S. Bech-Nielsen

D.J. Jackwood

Y.M. Saif

R.D. Slemons (Advisor)

Approved by

R.D. Slemons
Advisor
Department of Veterinary Preventive Medicine
To Jim, Jamie and Eric.
VITA

1994. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Ph.D.
Department of Veterinary
Preventive Medicine
The Ohio State University

1989. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . D.V.M.
College of Veterinary Medicine
The Ohio State University

1977. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . M.S.
Department of Microbiology
The Ohio State University

1973 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . B.A.
The Ohio State University

FIELD OF STUDY

Major Field: Veterinary Preventive Medicine
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CHAPTER I
LITERATURE REVIEW

The Problem

One hinderance to the development of avian influenza (AI) control policy is the disparity in pathogenicity expressed by AI viruses under field conditions compared to in the laboratory. The vast majority of AI virus isolates associated with significant morbidity and mortality in commercial turkeys under field conditions are consistently characterized as low or nonpathogenic when evaluated under laboratory conditions (Newman, 1981). Co-factors including host age, management conditions and bacterial co-infections have been proposed to account for this dichotomy (Johnson, 1976, Newman, 1981). Of these co-factors, concurrent bacterial infection has received the widest acceptance; however, no specific mechanism has been identified, nor has one specific bacteria been consistently associated with increased pathogenicity observed during avian influenza outbreaks. Understanding the mechanisms by which co-infecting bacteria increase AI virus infectivity and
pathogenicity would present new opportunities for disease control and strategic intervention.

**Economic Significance of Influenza Viruses in Humans and Poultry in the United States.**

In humans, influenza is a pandemic respiratory disease which results in high morbidity and significant mortality. In 1985, a nonpandemic year, influenza alone accounted for 2054 human deaths in the United States while an additional 67,615 human deaths were attributed to influenza and bacterial pneumonia (National Center for Health Statistics, 1987). The economic cost of human influenza can also be high. In 1976 the economic costs of the human influenza epidemic in the United States were estimated at $6 billion including medical costs and loss of wages (Schoenbaum, 1976).

In poultry, avian influenza is associated with a wide spectrum of disease ranging from subclinical infections to severe systemic disease with high morbidity and mortality. The economic costs of influenza in birds can be high. The 1983 Pennsylvania avian influenza outbreak typifies the dramatic and significant losses which characterize the infrequent avian influenza epidemics among commercial chickens. This outbreak led to the loss of over 12 million chickens with direct costs of approximately $60 million
dollars (Stuart-Harris, 1985). Consumers paid an estimated additional $349 million over a four to six month period to cover losses passed on by producers (Lasley, 1987). The economic impact of avian influenza is not limited to chickens. Annual avian influenza outbreaks occur in commercial turkeys, and though these outbreaks are less dramatic, they have significant impact on that industry. Losses have been suffered by turkey producers for many years in Europe, Israel, Great Britain, Australia as well as the United States of America (Alexander, 1987; Meulemans, 1987; Weisman, 1987; Cross, 1987). Estimated costs of outbreaks in Minnesota alone between 1977 and 1987 totaled more than $10 million (Poss, 1987). The development of effective avian influenza prevention and control strategies would be welcomed among all poultry producers and consumers.

**Biology of Avian Influenza Virus**

Avian influenza virus is a type A orthomyxovirus consisting of an outer lipoprotein envelope and an internal helical ribonucleoprotein core, the nucleocapsid, which contains eight segments of RNA of the negative sense. Those eight segments are known to code for ten viral proteins, eight of which are structural constituents of the virions; HA, NA, NP, M₁, M₂, PB₁, PB₂, and PA. Two glycoproteins spikes project from the surface of the viral envelope: hemagglutinin (HA) and neuraminidase (NA). There are
approximately 500 hemagglutinin molecules and 100 neuraminidase molecules per virus particle (Murphy, 1990). Within the envelop is a shell comprised of the matrix protein (M) which encloses the viral RNA associated with the nucleoprotein (NP) and the polymerase proteins PA, PB1, and PB2, which are responsible for RNA replication and transcription (Murphy, 1990). The functions of the two nonstructural proteins, NS1 and NS2, are not yet defined (Easterday, 1991).

Strain taxonomy is based on the HA and NA subtype. In 1960 the World Health Organization classified 13 antigenically distinct hemagglutinin groups (H1-H13) and nine neuraminidase groups (N1-N9) of type A influenza viruses. Recently, an additional hemagglutinin, H14, has been isolated from mallard ducks from the USSR (Kawaoka, 1990).

Major antigenic changes in the HA and/or NA proteins resulting in the appearance of new influenza subtypes occur periodically. This is referred to as antigenic shift, and is believed to be the result of genetic reassortment between type A influenza viruses. Antigenic shift has been reported to occur between human viruses in nature, (Bean, 1980), between avian viruses in nature (Desselberger, 1978) and between human viruses and avian strains in vivo (Webster, 1971).
Evidence exists which suggests reassortment between human influenza viruses and avian influenza in nature may result in the production of new pandemic human influenza viruses. The A/Aichi/2/68 (H3N2) strain of type A influenza virus contains the NA and all other genes from an Asian human (H2N2) strain and an HA that is antigenically related to that of the A/duck/Ukraine/63 (H3N8) (Ward and Dopheide, 1981). The amino acid sequence homology between the HA s of the A/duck/Ukraine/63 and A/Aichi/2/68 (H3N2) viruses was 96% suggesting that the duck strain donated the HA gene to the new H3N2 virus.

Antigenic drift, the accumulation of sequential antigenic changes in HA and NA, is the result of point mutations in the genes which code for viral glycoproteins. Information on variation of the HA and NA genes has been generated using nucleotide sequencing analysis of the genes from viruses isolated over time. The resulting amino acid changes alter the antigenic sites of the virus such that antibodies generated against an initial viral infection may not be protective against infection with a later emerging virus. Antigenic drift is best described in viruses that effect humans, but also occurs within the HA and NA of avian strains (Hinshaw, 1984; Kida, 1987). Higher levels of genetic homology are observed in the NS, NP, M and P genes within and between influenza subtypes of human, avian, swine
and avian origin. Sequence analysis has suggested that point mutations within these genes leading to antigenic variation are limited (Smith, 1989). Avian influenza presents unique problems to the researcher attempting to develop disease prevention and control techniques. In nature, AI viruses express high variability and remarkable plasticity in their genome. Their propensity to undergo gene reassortment and their high mutation rate results in the development of progeny virus with new biological features such as altered antigenicity, host specificity and pathogenic properties (Rott, 1984). These characteristics make control of AI through routine flock vaccination programs difficult and impractical.

Immune Response to Type A Influenza

Two animal models are frequently used to study the immune response to type A influenza. The chicken model is most commonly used to study avian influenza, and the mouse model is most commonly used to study mouse-adapted human origin type A influenza viruses. In the following discussion of cellular, humoral and local immunity to influenza viruses, human and avian models are presented independently with species cited for clarity.
Cellular Immunity

Studies of the cell mediated immunity to AI in chickens suggest that immunity is directed against a broad range of antigenic characteristics with the hemagglutinin protein eliciting the primary cell mediated response (Easterday, 1981). One report of a virulent AI strain in poultry, A/turkey/Ont/7732/66 (H5N9), suggested that the virus had a marked effect on the immune system of chickens, infecting lymphocytes and macrophages and resulting in severe lymphopenia (Van Campen, 1989). Subsequent follow-up studies involving other virulent H5 AI strains, including A/chick/Penn/1370/32 and A/tern/South Africa/1961 indicated that these virulent viruses had little or no effect on the lymphoid tissues of infected chickens (Van Campen, 1989). Recent work with mallard ducks and the low-pathogenic AI strain A/mallard/Ohio/184/86 demonstrated an apparent suppression of T-cell activity and enhanced macrophage activity (Laudert, 1993). Just as there are great differences in the pathogenicity among AI viruses, there appears to be significant differences in the effect that various AI strains have upon the immune systems of birds.

In mice, it has been demonstrated that cellular immunity is necessary for recovery from influenza infection although cell-mediated immunity does not prevent viral infection (Ada, 1986). Influenza challenge in mice results
in cytotoxic T cells capable of recognizing influenza encoded proteins including HA, NA, NP, and M (Wysocka, 1990) which are proteins expressed on the surface of influenza infected cells (Els, 1989). Cytotoxic T cells appear to recognize mainly the NP antigen, although a minor population of cytotoxic T cells from influenza infected mice (10-15%) is capable of recognizing HA (Yewdell, 1989). Nude mice lacking a functional cellular immune system display prolonged shedding of virus from their noses and lungs following influenza infection (Kris, 1989), demonstrating the importance of cellular immunity in recovery from influenza infection.

Humoral Immunity

Agents that infect the respiratory tract of chickens induce specific humoral antibodies, including IgM, IgA and IgG (Darbyshire, 1987). With avian influenza, virus neutralizing antibodies in sera are directed against the hemagglutinin and neuraminidase antigens (Easterday, 1981). Serum IgG antibodies to NA have been shown to prevent systemic spread of virus and death of chickens, but are less effective than anti-HA IgG antibodies in mediating protection (Webster, 1988). Antibodies to NP are used in AI surveillance programs since this antigen is type specific and shared by all avian influenza viruses (Easterday, 1991).
Antibodies to M and NP proteins are not believed to be important in protection (Derbyshire, 1987).

In mice, as in chickens, antibodies to the HA neutralize viral infectivity, and resistance to lethal infection correlates with serum anti-HA IgG antibody levels (Potter, 1982). Antibody specific for viral neuraminidase reduced the severity of lung lesions and lowered viral titers in mice (Schulman, 1968) but did not prevent infection.

Local Immunity

Local immunoglobulin in respiratory tract secretions of infected or vaccinated chickens have been demonstrated with Newcastle disease virus, avian paramyxovirus type (PMV 1). The bulk of mucosal secretions initially consist of IgA, although IgA and IgG are found in the saliva of infected chickens (Darbyshire, 1987). It has been suggested that local immunity is more important than humoral immunity in preventing virus replication in the avian respiratory mucosa (McFerran, 1981), but studies with avian influenza which confirm this proposal have not been reported.

Protection studies in mice have demonstrated that serum antibodies are sufficient to prevent infection in the lung (Loosli, 1953), but protection of the upper respiratory tract from intranasal viral challenge is not prevented by
serum antibody (Kris, 1988). Recent studies have shown that intranasal administration of mice with a recombinant vaccinia virus containing the hemagglutinin gene of H1N1 influenza virus induce local protection against H1N1 influenza virus (Meitin, 1991). This protection correlated with an increase in IgA titer of nasal washes. Virus specific IgA has been found in the nasal washes of influenza convalescent humans (Brown, 1985). These studies suggest that the secretory immune system plays a role in protection against nasopharyngeal influenza challenge.

The importance of the role that IgA plays was further elucidated recently in two protection abrogation studies (Renegar, 1991a,b). First it was demonstrated that passive transfer of local immunity to influenza virus (H1N1) infected mice could be conferred by intravenous inoculation of mice with monoclonal anti H1N1 IgA (Renegar, 1991a). IgA was shown to be selectively transported to nasal washings relative to IgG. The transported IgA was demonstrated to be functional in that it bound to virus in ELISA assay and protected nonimmune mice against intranasal infection with H1N1 but not H3N2 influenza virus. The transported IgA was shown to protect 80% of the mice, as they did not shed virus following intranasal inoculation. The remaining 20% of the mice were conferred partial protection in that the virus titer shed was lower than nonimmune saline inoculated.
control mice. To confirm that IgA was the mediator of the local immunity, passively immune mice were given anti-IgA antiserum by intranasal administration. Their passive local immune protection was found to be abrogated in that they shed virus in the same quantities as mice that had not received passive immunity. Similar intravenous neutralizing doses of IgG antibody were not protective.

In a second study (Renegar, 1991b), convalescent mice were shown to be immune to influenza infection, in that they did not shed virus in their nasal secretions following intranasal challenge with influenza virus. Intranasal treatment with anti-IgA, but not anti-IgG or anti-IgM antiserum was shown to eliminate protection immunity in influenza convalescent mice. Convalescent mice treated with anti-IgA shed virus in their nasal secretions at the same rate as nonimmune mice. Anti-IgG and anti-IgM had no effect on convalescent immunity suggesting that IgA is the major, if not the sole mediator of mucosal immunity to influenza virus in the murine nasopharynx.

Pathogenicity of Avian Influenza

The pathogenicity of type A influenza virus infection in poultry varies from subclinical infections to highly pathogenic systemic disease with high morbidity and mortality (Klenk, 1988). The term "fowl plague" was
historically used to refer to the clinical disease or virus involved in outbreaks with high mortality. A recommendation was adopted in 1981 by the participants of the First International Symposium on Avian Influenza to discard the term "fowl plague" in favor of the term "highly pathogenic influenza viruses" (Lancaster, 1981). This recommendation was subsequently revised by the U.S. Animal Health Association Committee on Transmissible Diseases of Poultry and Other Avian species. They suggested that isolates be classified as highly pathogenic avian influenza if they result in the death of 6, 7 or 8 of eight four to six week old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1:10 dilution of bacteria-free infectious allantoic fluid (Pearson, 1987). Pathogenicity of an AI isolate cannot be predicted based upon host of origin or antigenic subtypes. Highly pathogenic strains of avian viruses belonging to the H5 and H7 subtypes have been associated with disease in chickens, turkeys, ducks, and terns (Alexander, 1987). However, there are many examples of H5 and H7 viruses that are not pathogenic.

Hemagglutinin as a Determinant of Virus Pathogenicity

The molecular basis for pathogenicity is not totally understood, however studies have indicated that the most
clearly defined determinant of avian influenza pathogenicity is hemagglutinin (Bosch, 1979, Rott, 1982). This glycoprotein is of considerable biological interest because, as previously discussed, it is the major target for the protective immune response of the host (Looslic, 1953; Burlington, 1983, Meitin, 1991), and because it plays a key role in the entry of the virus in the cell (Rott, 1986).

Hemagglutinin has two different functions in the initiation of the viral infection. First it is responsible for the attachment of the virus to neuramic acid-containing receptors at the host's cell surface (Rogers, 1986). The receptor binding site is at the globular region of the hemagglutinin spike. More recently it has become apparent that hemagglutinin is also involved in penetration by triggering fusion of the viral envelope with cellular membranes (Gething, 1986; Guy, 1992). For fusion to occur, the hemagglutinin must undergo posttranslational cleavage of the hemagglutinin molecule at an arginine residue by a "trypsin-like" endoprotease provided by the host. Recent studies have identified a mammalian cellular serine protease of the subtilisin family which cleaves HA proteins at multibasic (Stieneke-Grober, 1992) and dibasic (Barr, 1991), amino acid sites.

When a host cell does not contain an appropriate serine protease, non-infectious progeny virus with uncleaved
hemagglutinin is released (Webster, 1987). Under these in vitro conditions, trypsin and other trypsin-like proteases such as plasmin can substitute for the intracellular enzyme and cleave the HA which activates the virus (Lazarowitz, 1975).

The hemagglutinins of the mammalian influenza viruses and the nonpathogenic avian influenza viruses, both of which can cause a local infection, are susceptible to proteolytic cleavage only in a restricted number of cell types (Webster, 1987). The hemagglutinins of highly pathogenic avian viruses are cleaved by proteases present in a broad range of different host cells. Those highly pathogenic viruses, which include members of the H5 and H7 subtypes, cause systemic infections and are associated with high mortality. Sequence analysis have revealed that the HA1 and HA2 fragment of the HA molecules of the low pathogenic avian and of all mammalian influenza viruses are linked by a single basic amino acid arginine. This is in contrast to the highly pathogenic AI virus strains, which have a sequence of several basic amino acids at the cleavage site with the common denominator lysine-arginine or arginine-arginine. Examples of the cleavage sites for four avian influenza strains are as follows:
Though the amino acid sequence is important in determining the cleavability and associated pathogenicity of the virus, the tertiary structure of the HA has also been found to be important. This fact was demonstrated when single point mutations of the HA molecule were shown to be responsible for dramatic increases in pathogenicity that occurred within a series of outbreaks of avian influenza in 1983 (Kawaoka and Webster, 1985). Comparison of different isolates which belong to the H5N2 subtype revealed that virus in an early outbreak had low pathogenicity and contained a HA with restricted cleavability. Viruses in a late outbreak was highly pathogenic and had a HA that was easily cleaved. Sequence analysis revealed that the highly pathogenic variant had a cleavage site structure lysine-lysine-lysine-arginine which was susceptible to ubiquitous proteases. The apathogenic precursor strain had the same cleavage site, but it was masked by an adjacent oligosaccharide which was subsequently lost as the result of a point mutation.
Importance of Non-hemagglutinin Proteins to Pathogenicity

While the HA is clearly associated with virulence, reassortment studies involving highly pathogenic fowl plague viruses (FPV), have demonstrated that pathogenicity of AI virus is also polygenic in nature (Rott, 1976). Pathogenic FPV whose HA gene was replaced through reassortment were always less pathogenic in nature. However, when one or more of the other segments of a FPV were replaced by segments from a less pathogenic virus, the resulting new virus was also often less pathogenic. It has been suggested that the nature of the second virus glycoprotein, neuraminidase, determines whether cleavage of HA may occur. Reassortment studies with A/WSN/33 and several AI viruses including A/turkey/Ontario/7732/66 (H5N9) indicated that the NA from WSN strain was necessary for cleavage of HA molecule in the AI recombinant viruses. The mechanism by which a viral NA in some instances may facilitate cleavage of HA is not understood (Klenk, 1988), nor is the specific effect that each of the other genes has on reducing or enhancing pathogenicity.

Potential Mechanisms for Synergism Between Influenza and Bacteria

Many years ago workers studying human influenza noted that the pathology observed when both influenza and bacteria
are present is greater than the pathology observed with either agent alone (Loosli, 1968; 1973). It has also been reported that combined bacterial-viral pneumonia is three times more common than primary viral pneumonia among humans (Stuart-Harris, 1985). *Staphylococcus aureus* and *Streptococcus pneumonia* are the bacteria most commonly involved with combined human bacterial-influenza pneumonias. Increased pathogenicity of mammalian influenza in the presence of bacteria has been reported by several workers, yet the exact mechanisms by which this occurs is not well delineated.

There have been several mechanisms suggested in the literature by which human influenza viruses and bacteria could act synergistically. One mechanism suggests that bacteria may have a primary role in increasing pathogenicity of influenza infections. This mechanism proposes that the production of IgA protease by certain bacteria contributes to viral-bacterial synergism. As previously discussed, the presence of local mucosal IgA appears to be important in the prevention of influenza infections. Workers have found that *Hemophilus influenza* and *Streptococcus pneumonia* produce proteases which cause degradation of IgA (Kilian, 1979), thereby potentially enhancing the establishment of influenza infections.
A second mechanism which may account for synergism between bacteria and influenza virus suggests that bacteria may play a secondary role. Several workers have reported that bacteria adhere to influenza infected epithelial cells (Fainstein, 1980; Davison, 1981, Beachey, 1983). The mechanisms by which viruses alter bacterial adherence include alteration of host cell surface membrane receptors and alteration of the microenvironment in which bacterial attachment occurs (Babiuk, 1984). Viral infections decrease the levels of fibrinonectin production by the cell. Bacterial colonization has been correlated with low fibrinonectin levels (Woods, 1987), therefore decreased production of fibrinonectin in virus infected cells may increase bacterial adherence to virus infected cells.

**Synergism Via Cleavage Activation by Bacterial Proteases**

Another specific mechanism has recently been identified which suggests that bacterial protease may play a primary role in increasing the pathogenicity of influenza infections. Tashiro et. al., (1987) has demonstrated that some strains of *Staphylococcus aureus* can produce proteases capable of activating hemagglutinin by proteolytic cleavage under in vitro conditions. The presence of bacterial protease in cell culture media allowed mammalian influenza viruses to undergo multiple additional replicative cycles.
In in vivo studies, co-infection of mice with human-origin Staphylococcus aureus and mouse-adapted human origin type A influenza resulted in fatal disease and more extensive lesions in the lung tissues compared to mice infected with influenza alone. Akaike et al. (1989) demonstrated that the presence of a protease produced by Serratia marcesans increased replication of mouse adapted human influenza virus in cell culture, and resulted in enhancement of lung lesions and lethality in the murine model. Scheiblauer et al. (1992), subsequently demonstrated that a protease produced by Aerococcus viridans enhanced influenza infectivity and pathogenicity in mice by proteolytic activation of the hemagglutinin. These findings suggest that protease from co-infecting bacteria may enhance the infectivity of the influenza virus and play a primary role in increasing the severity of disease.

**Mechanisms for Cleavage of Hemagglutinin by Bacterial Proteases**

Proteolytic bacteria can enhance the infectivity of influenza via cleavage of the HA molecule by at least three mechanisms. First, some bacteria, including Staphylococcus aureus and Aerococcus viridans can produce a protease which directly cleaves the viral HA (Tashiro, 1987; Scheiblauer, 1992). Secondly, some bacteria, including Serratia
Staphylococcus aureus (Arvidson, 1983) produce a protease which converts host cellular plasminogen to plasmin. Plasmin can cleave the HA of some influenza particles, and thus activate the virus (Tashiro, 1987). Thirdly, it has been demonstrated that the combined effect of bacteria and virus in the respiratory tract increases the host cellular response, potentially resulting in increased production of cellular proteases capable of cleaving HA (Scheiblauer, 1992). Workers have reported that a protease produced by Pseudomonas, though unable to activate influenza in vitro, resulted in increased pathology and higher viral titers in the mice lungs. Examination of the bronchialveolar lavage fluids indicated the presence of increased trypsin-like activity in co-inoculated mice. Specific cellular enzymes responsible for this effect have not been determined, however, kallikrein, urokinase, plasmin and thrombin may be candidates. Kallikrein, urokinase and thrombin have been shown to activate some influenza virus subtypes (Scheiblauer, 1992). Further work is necessary to define the exact mechanisms by which proteolytic bacteria could enhance infectivity of influenza viruses.

Bacterial Activation of Low-Pathogenic Avian Influenza

Since it is known that HA1 and HA2 fragments of the hemagglutinin molecules of mammalian and low-pathogenic
avian influenza viruses have similar basic amino acid structures at their HA cleavage sites, and that protease secreting bacteria can exacerbate influenza infections in mice, we hypothesized that bacterial proteases may play an important part in outbreaks of avian influenza that occur annually among commercial turkeys. We believe that specific bacteria present as permanent or transient flora in the respiratory tract of poultry species secrete proteases capable of activating low-pathogenic influenza viruses and increasing the pathogenicity of the resulting infection.

The overall goal of this project was to investigate the potential for co-infecting proteolytic bacteria to produce proteases which enhance avian influenza virus infectivity resulting in increased pathogenicity. We propose that specific proteases produced by pathogenic or nonpathogenic bacteria from the upper respiratory tract flora of poultry may increase AI virus titers and severity of lesions in the lung. The demonstration of enhancement of specific strains of AI virus in the presence of specific proteolytic bacteria could explain the paradox of the variable pathogenicity observed in the field and laboratory and could potentially lead to the development of new avenues for strategic intervention and control of avian influenza.
The specific objectives of this project were:

**Phase 1:** Determine if proteolytic bacteria are present in the respiratory tract of poultry.

**Phase 2:** Isolate an avian origin bacterium whose protease enhances the infectivity of avian influenza virus under in vitro conditions.

**Phase 3:** Determine if concurrent inoculation of turkeys with the proteolytic bacteria isolated in phase one and the avian influenza enhanced by the avian-origin bacterial protease in phase two would result in increased AI virus titers and pathogenicity in the lungs compared to turkeys inoculated with the bacterium or virus alone.

The three phases of this study are addressed in separate chapters as follows.

**Chapter II.** Detection of proteolytic bacteria among the respiratory tract flora of poultry.

**Chapter III.** In vitro enhancement of avian influenza by a bacterial protease.

**Chapter IV.** Proteolytic bacteria enhance virulence and replication of avian influenza virus in turkeys.
List of References


CHAPTER II
Detection of Proteolytic Bacteria Among the Respiratory Tract Flora of Poultry

Summary

Highly proteolytic bacteria were recovered from tracheal swabs of 20-50% of the birds in four poultry flocks. The highly proteolytic bacteria recovered included five different species of staphylococcus as well as two gram negative species, Flavobacterium sp and Vibrio alginolyticus. All species of staphylococcus isolated have been previously reported to be associated with animal sources, with S. hyicus receiving recent recognition as a poultry pathogen. Flavobacterium sp and Vibrio alginolyticus isolates are considered to be opportunistic pathogens from soil and surface water origins respectively.

A method requiring only minimal supplies and equipment for isolating proteolytic bacteria from tracheal swabs of poultry is described. This method could potentially be used for isolating proteolytic and highly proteolytic bacteria associated with AI outbreaks.
Introduction

Activation of avian influenza (AI) virus by the mammalian serine protease trypsin (Lazarowitz, 1975) raised the question of whether bacterial proteases enhance the infectivity of influenza viruses in the natural host. Recent studies have shown that serine type bacterial proteases from Staphylococcus aureus (Tashiro, 1987), Serratia marsecans (Akaike, 1989) and Aerococcus viradans (Scheiblauer, 1992) increase the infectivity of type A influenza viruses in the respiratory tract of mice. Our work has shown that a protease secreted by Vibrio alginolyticus recovered from the upper respiratory tract of a chicken increases the infectivity of A/turkey/Wisconsin/68 and A/mallard/Ohio/184/86 under in vitro conditions (Chapter III). An initial obstacle to further examining the potential relationship between AI viruses and proteolytic bacteria was the need for a method which distinguished proteolytic bacteria among the flora of the respiratory tract of poultry. This chapter describes a rapid technique for screening the respiratory tract bacterial flora for the presence of nonproteolytic, proteolytic and highly proteolytic bacteria. The technique is suitable for screening large numbers of birds and will allow expedient recognition of proteolytic bacteria associated with outbreaks of avian influenza.
Materials and Methods

Flocks Tested

Four poultry flocks were surveyed for the presence of proteolytic bacteria (Table 1). These flocks included:
(1) a research flock of leghorn chickens housed in cages;
(2) a commercial flock of leghorn chickens housed in cages;
(3) specific pathogen free turkeys housed in isolators; and
(4) a research flock of turkeys on pasture. The age of birds and numbers of birds sampled in each flock is given in Table 1.

Collection of Proteolytic Bacterial Specimens for Culture

Tracheal samples were collected on sterile Dacron swabs and streaked onto selected agar plates within one hour to prevent drying and subsequent death of organisms present. If time lapses of greater than one hour were anticipated, samples were refrigerated and the time between collection and plate inoculation was extended by approximately one hour to two hours. Samples were refrigerated (not frozen) during transport.

Isolation of Proteolytic Bacteria

Each swab was streaked onto trypticase soy agar with 5% sheep blood (BAP), MacConkey's Agar (MAC), and Standard Methods Caseinate Agar (CAS). Plates were incubated aerobically at 37°C for 24-72 hours, with plates being
checked every 24 hours. Proteolytic bacteria were identified as colonies on CAS surrounded by a caseinate precipitate. Highly proteolytic bacteria were identified as colonies on CAS with a clear zone between the colonies and caseinate precipitation (Frank, 1985). Proteolytic and/or highly proteolytic bacteria were isolated by re-streaking onto CAS agar. Gram stains were completed on highly proteolytic isolates. Gram positive, catalase positive cocci were identified using the STAPH Trac\textsuperscript{R} system (bioMerieux Vitak, Inc., Hazelwood MO.). Gram negative rods were identified using the API 20E\textsuperscript{R} system (bioMerieux Vitek, Inc., Hazelwood, MO).

**Screening Bacteria for Proteolytic Activity**

The relative proteolytic activity of each isolate was determined via the agar gel diffusion test (Bio-Rad Laboratories, Richmond, CA). Each highly proteolytic colony was inoculated into a Brain Heart Infusion Broth tube and incubated at 37°C for 18 hours. The culture was then centrifuged for 10 min. at 10,000 x g. Agar gel diffusion (AGD) plates were prepared and contained 1% bovine casein in Tris buffered PBS, pH 7.2 (Bio-Rad Laboratories, Richmond, CA.). Aliquots (15 ul) of each bacterial supernatant and of each trypsin control concentration (5, 10, 15 and 20 ug/ml purified bovine pancreatic trypsin in 0.05 M Tris-HCl, pH
7.5, 0.05M CaCl\textsubscript{2}) were placed into 4 mm diameter wells on the AGD plate. AGD plates were incubated 24 hours at 22\textdegree C. After incubation plates were overlaid with 3% acetic acid (v/v in distilled H\textsubscript{2}O). A plot of ring diameter of each bacterial supernatant vs. trypsin concentration was made. A qualitative assessment of proteolytic activity was completed by comparing the ring diameters of the bacterial supernatants with those of the trypsin controls.

**Results**

Highly proteolytic bacteria (Plate I) were recovered from tracheal swabs of one or more birds in all four flocks tested. The percentage of birds with proteolytic, highly proteolytic or non-proteolytic bacteria within each of the four flocks tested is presented in Table 2. Birds were classified by the highest level of proteolytic bacteria recovered. Highly proteolytic bacteria were recovered from 20-50% of the birds depending on the flock.

The identification of the highly proteolytic bacteria isolated and the flocks in which they were found is given in Table 3. Five different species of staphylococcus bacteria were found. *S. aureus*, *S. hyicus*, and *S. xylosus* were isolated from 3 of the 4 flocks tested. *S. epidermis* was present in 2 of the flocks tested, while *S. sciuri* was present in one flock. Highly proteolytic gram negative bacteria were found in two flocks tested. *Flavobacterium sp*
and *Vibrio alginolyticus* were the two highly proteolytic gram negative bacteria isolated.

**Agar Gel Diffusion Test**

The agar gel diffusion test results demonstrated proteolytic activity in all 18 hour BHIB supernatants of highly proteolytic bacteria. Proteolytic activity ranged from barely detectable zones, which characterized *Staphylococcus epidermis* strains, to very wide zones, which characterized *Staphylococcus hyicus* strains. Most of the bacterial supernatants had clear zones which appeared similar to zones of the 10 to 20 ug/ml trypsin controls (Plate II).

**Discussion**

Proteolytic and highly proteolytic bacteria were frequently recovered from tracheal swabs of the upper respiratory tract of poultry. To the best of our knowledge, no studies on the prevalence or identification of proteolytic bacteria from tracheal swabs of poultry have been reported. Proper collection and handling of bacterial specimens are essential for valid results (Needham, 1987). Prompt processing of samples was considered essential to ensure recovery of bacterial isolates. If a delay of more than one or two hours is anticipated before samples can be
inoculated onto an agar media, an appropriate transport media should be used.

Plating media was selected in effort to recover the maximal numbers of proteolytic organisms. Blood agar supports the growth of most aerobic organisms. MacConkey's agar selectively supports the growth of gram-negative organisms, which may be overgrown on the blood agar plates. Standard Methods Caseinate Agar supports the growth of most aerobic bacteria, while differentiating between proteolytic and nonproteolytic bacteria. Similar numbers and types of bacteria were recovered from the blood agar plates and the Standard Methods Caseinate Plates. Bacterial growth from tracheal swab samples did not overcrowd the BAP or CAS plates, and few bacteria grew on the MacConkey's agar plates. Primary isolation of proteolytic bacteria should be made on CAS plates with BAP streaked as a secondary plate.

Agar gel diffusion results did not quantitatively assess proteolytic activity among the bacterial supernatants because the edges of the clear zones were difficult to identify. This screening test however gave a simple preliminary indication of the protease activity in each supernatant by comparing the size of clear zone to trypsin standards. Plate II demonstrates the relative assessment of protease activity in the agar gel diffusion test for four concentrations of trypsin in control wells and the Vibrio
algino|olyticus protease recovered from a broth supernatant. Quantitative assessment of specific activity of purified protease from each bacterial isolate is described in Chapter III.

The highly proteolytic bacteria were found to be members of seven different bacterial species. A brief description of the natural habitat of each highly proteolytic poultry tracheal swab isolate follows.

**Staphylococcus aureus**

*Staphylococcus aureus* was isolated from three of the four flocks tested. It is considered part of the normal flora of the nasal membrane and nasopharynx of warm blooded animals and is present in 10-40% of non-hospitalized normal humans (Linton, 1990). *Staphylococcus aureus* infections in poultry are common and may or may not be associated with disease (Skeeles, 1991; Jensen, 1987). *Staphylococcus aureus* in poultry is associated with omphalitis, pododermatitis, cellulitis, discospondylitis, osteomyelitis, pericarditis, bursitis, arthritis as well as granulomatous lesions of the liver and lung (Whiteman, 1989; Munger, L.L., 1973). *Staphylococcus aureus* infections in poultry are also associated with septicemia and death. As in man, most staphylococci species are considered normal flora, which help suppress other possible pathogens by their presence.
through interference or competitive exclusion. (Skeeles, J.K., 1991). Strains of *Staphylococcus aureus* associated with poultry have been divided for many years into specific biotypes based on biochemical testing (Devriese, L.A., 1973). Phage typing studies have suggested that *Staphylococcus aureus* in poultry are differentiated from strains associated with other animals and humans (Linton, A.H., 1990). The potential for proteases produced by *Staphylococcus aureus* poultry isolates to enhance avian influenza is supported by the finding that several strains of human origin *Staphylococcus aureus* produce protease that enhance mouse adapted human origin strains of type A influenza and one avian influenza strain, A/duck/Ukr/1/63 (H3N8) under in vitro conditions (Tashiro, 1987).

**Staphylococcus epidermis**

Highly proteolytic strains of *Staphylococcus epidermis* were also found in three of the four flocks tested. *Staphylococcus epidermis* is the major inhabitant of man's skin (Linton, 1990) and is frequently isolated from poultry (Skeeles, 1991). The biotypes of *Staphylococcus epidermis* isolated from poultry is different than those most frequently present on man, but may be similar to strains associated with porcine and bovine strains (Devriese, L.A., 1975). It has been suggested that exposing turkeys to a
specific strain of **Staphylococcus epidermis** (strain 115) by aerosol at 10 days and 4 to 5 weeks substantially reduces losses from arthritis and synovitis (Meyer and Jensen, 1987).

**Staphylococcus hyicus**

This bacterium was originally described as a gram positive coccus isolated from pigs with exudative epidermatitis. It is now associated with bovine mastitis and is frequently isolated from healthy poultry skin. Historically, poultry strains of *S. hyicus* have not been reported to be pathogenic for poultry nor pigs (Kloos, 1980). There is growing evidence however, that *S. hyicus* might play a significant role in osteomyelitis/synovitis in turkeys (Tate, 1993). *S. hyicus* is similar to *S. aureus* with respect to appearance on gram stain as well as catalase and coagulase tests. The suggestion has been made that a complete battery of biochemical tests is required and should be employed on poultry isolates to definitively differentiate between these two species (Tate, 1993).

**Staphylococcus xylosus**

This highly proteolytic bacterium is found occasionally on the skin of a variety of lower mammals including cattle, sheep and horses (Kloos, 1980; Kloos 1981). It is capable
of free living existence and is associated with environmental sources including soil, beaches and natural waters. This species has not been associated with specific disease conditions.

*Staphylococcus sciuri*

*Staphylococcus sciuri* is a highly proteolytic bacterium commonly found in large populations of squirrels as well as rodents. This bacterium is capable of a free living existence in that it does not require an organic source of nitrogen and has been isolated from natural waters (Kloos, 1981). It is not considered to be a pathogen (Kloos, 1980).

*Flavobacterium sp*

These gram negative yellow pigmented non-motile highly proteolytic bacteria are free-living and can be frequently isolated from fresh water and soil. They are not associated with disease in birds, with the exception of one report where *Flavobacterium sp* was implicated with arthritis in ducks (Bissgard, M., 1981).

*Vibrio alginolyticus*

The gram negative rod shaped bacteria of the genus *Vibrio* are divided into twenty species. *V. cholera* and *parahaemolyticus* have long been identified as human
Vibrio anguillarum is pathogenic for fish. Vibrio vulnificus, V. cholerae, V. parahaemolyticus, and V. alginolyticus have all been isolated from superficial lesions on humans where they may be simply be colonizes or opportunistic pathogens (Bauman, P., 1984). Vibrio metschnikovi is the only species in this genus which has been associated with disease in poultry, and it has been associated with a choleraic diarrhea in birds (Lee, J.V., 1978). Vibrio alginolyticus is capable of free living existence and is associated with surface water.

Conclusion

Highly proteolytic bacteria were frequently recovered from tracheal swabs of four poultry flocks. Staphylococcus bacteria were the highly proteolytic bacteria most frequently recovered. All of the Staphylococcus species isolated have been previously associated with animal sources. Flavobacterium sp and Vibrio alginolyticus were two highly proteolytic gram negative bacteria recovered. These bacteria are considered nonpathogenic to opportunistic and are associated with soil and surface water, suggesting that they may have entered the flocks through the water supply.


CHAPTER III

In Vitro Enhancement of Avian Influenza Virus Infectivity by a Bacterial Protease

Summary

Extra-cellular proteases secreted by *Staphylococcus aureus* Woods 46 (ATCC 10832), *Staphylococcus gallinarum* (ATCC 35539), and poultry respiratory tract isolates *Staphylococcus aureus*, *Staphylococcus epidermis*, *Staphylococcus hyicus*, *Flavobacterium sp* and *Vibrio alginolyticus* were purified from culture filtrates. Mean tissue culture dose (TCID₅₀) for human influenza A/PR/8/34 (H1N1), and avian influenza isolates A/turkey/Wisconsin/68 (H5N9), A/turkey/Ohio/88 (H1N1), A/chicken/Alabama/75 (H4N8), A/mallard/Ohio/184/86 (H5N1) and A/mallard/Ohio/338/86 (H4N8), were then determined in chicken embryo fibroblast (CEF) tissue culture in the presence and absence of each of the purified proteases.

The TCID₅₀ of A/turkey/Wisconsin/68 and A/mallard/Ohio/184/86 viruses were increased in the presence of chicken-origin *Vibrio alginolyticus* protease by 10¹·¹ and 10²·¹, respectively. The infectivity of the human-origin
A/PR/8/34 virus was increased in the presence of the protease from human origin S. aureus Woods 46 as previously reported (Tashiro, 1987a), but not by the six avian-origin bacterial proteases.

These findings raise the possibility that the severity of avian influenza outbreaks in poultry may be due in part to bacteriological co-factors and that the relationship between a bacterium and virus enhanced is very specific.

Introduction

It is well established that cleavage of the avian influenza (AI) virus hemagglutinin (HA) molecule is necessary to maintain viral infectivity and that the HA of more pathogenic virus is cleaved by proteases for a wider variety of cell types (Bosch, 1979; Lazarowitz, 1975; Webster, 1987). The mammalian-source serine protease trypsin has classically been used to demonstrate that cleavage of the influenza A virus HA will increase infectivity under in vitro conditions (Lazarowitz, 1975; Garten, 1981). Recent studies have shown that proteases secreted by specific strains of Staphylococcus aureus will also increase infectivity of human-origin influenza viruses in cell culture media (Tashiro 1987a,b). The potential importance of virus cleavage by bacterial protease was demonstrated by co-inoculation of mice with specific
proteolytic strains of *Staphylococcus aureus*, *Serratia marcesans* or *Aerococcus viridans* or their proteases and mouse-adapted strains of human influenza virus. These co-inoculation studies resulted in recovery of higher influenza viral titers in lung tissues and more severe lesions in the mice compared to mice inoculated with influenza alone. (Tashiro, 1987a; Akaike, 1989; Scheiblauer, 1992).

Our previous work has demonstrated that proteolytic bacteria are frequently present in tracheal swabs of poultry. This raises the possibility that bacteria present in the respiratory tract of poultry may increase the infectivity of some avian influenza viruses and exacerbate the severity of the disease. The objective of this study was to determine if proteases secreted by bacteria recovered from tracheal swabs of poultry could enhance the infectivity of AI virus under in vitro conditions.

**Materials and Methods**

**Bacteria.** One human origin and six avian-origin strains of highly proteolytic bacteria were selected for this study. Highly proteolytic bacteria include those that produce colonies surrounded by caseinate precipitates and a clear zone between the colony and the caseinate precipitate when grown on Standard Methods Caseinate Agar (Chapter II). Organisms obtained from the American Type Culture Collection
include *Staphylococcus aureus* Woods 46 (ATCC 10832), a human-origin bacteria, and avian-origin *Staphylococcus gallinarum* (ATCC 35530). A strain of *Staphylococcus aureus* and of *Staphylococcus epidermis* were isolated from tracheal swabs of turkeys on pasture. *Staphylococcus hyicus*, *Flavobacterium sp* and *Vibrio alginolyticus* were isolated from tracheal swabs of caged leghorn chickens (Chapter II).

**Viruses.** Six strains of type A influenza virus were included in this study. Human origin A/PR/8/34 (H1N1) (ATCC VR-95) was obtained from the American Type Culture Collection. The other five isolates included: two waterfowl-origin isolates A/mallard/Ohio/338/86 (H4N8) [A/ma/Oh/338/86] and A/mallard/Ohio/184/86 (H5N1) [A/ma/Oh/184/86] which were both recovered in this laboratory and were in second passage (Slemons, 1991); fifth passage A/chicken/Alabama/75 (H4N8) [A/ck/AL/75], obtained from Dr. Max Brugh (Southeast Poultry Research Laboratory, U.S. Department of Agriculture, Athens, Ga.); A/turkey/Wisconsin/68 (H5N9) [A/ty/WI/68] obtained from Dr. V. Hinshaw (University of Wisconsin, unknown passage history); and second passage A/turkey/Ohio/1/88 (H1N1) [A/ty/Oh/88] obtained from Dr. Y.M. Saif (Ohio Agricultural Research and Development Center, Wooster, Ohio).
Trypsin.  TPCK treated Type XIII trypsin (Sigma, Cat. No. T8642, St. Louis, MO.) having 12,000 Nε-Benzoyl-L-arginine Ethyl Ester (BAEE) units/mg activity as reported by the manufacturer was used throughout the study. One BAEE unit will produce a \( \Delta A_{25} \) of 0.001 per minute at pH 7.6, 25°C using Nε-Benzoyl-L-arginine Ethyl Ester as substrate (Sigma, St. Louis MO.).

Purification of bacterial proteases. Proteases secreted by each strain of bacteria were purified using a slight modification of the procedure described by Drapeau (1972). Basically, the bacteria were inoculated into a defined broth medium (Drapeau, 1972) and incubated for 18 hours at 37°C. The culture was centrifuged at 10,000 x g for 15 minutes to remove the cells. The protein in the supernatant was precipitated by adding ammonium sulfate to 60% (w/v) and then was collected by centrifugation (10,000 x g). The pellet was dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 1mM CaCl₂ and was precipitated again with 1.35 volume of cold acetone. The pellet was resuspended in a small volume of 10 mM Tris buffer with mM CaCl₂ and was dialyzed against a weak Tris buffer for four hours at 4°C. The protease was then purified via KCl ion exchange through a DEAE cellulose exchange column. Qualitative protease
activity of each fraction was measured using an agar gel digestion technique (Chapter II). Fractions found to have proteolytic activity were pooled. Molecular weight of the bacterial protease was determined on 12% SDS-PAGE gels (Sambrook, 1989).

Assay of Proteolytic Enzyme Activity. Proteolytic activity was assayed using sodium caseinate as a substrate (Tashiro, 1987a). Enzyme solution in 200 ul was added to 4 ml of 1 % w/v sodium caseinate in the same buffer and incubated for 30 minutes at 22°C. The reaction was stopped by adding 3 ml of 10% trichloracetic acid. After removal of the precipitated proteins by filtration through a No. 1 Whatman filter, the filtrate was assayed for absorbance at 280 nm. One unit of proteolytic activity was defined as the amount of enzyme which increased the absorbance by 1.0 OD under these conditions. Protein concentration of each protease preparation was determined by the Bradford technique (Bradford, 1976). Specific activity/ug is equal to proteolytic activity divided by the protein concentration.

Assay for Enhancement of Influenza Virus Infectivity. The mean tissue culture infective dose (TCID₅₀) of each stock virus was determined in monolayered primary chicken embryo fibroblasts in 96 well plates. Ten-fold serial dilutions of
each stock virus was inoculated onto CEF monolayers (in replicates of 4 wells/dilution). After a virus absorption period of 60 minutes at 37°C, the cells in each well were fed with one of the following:

(1) serum free M199 (Sigma, St. Louis, MO.) containing 0.3μg/ml = 3.9 BAEE units/ml, purified bovine pancreatic trypsin (Sigma, St. Louis, MO.) (trypsin control) (4 wells/virus dilution), or

(2) serum free M199 containing no trypsin (negative control) (4 wells/virus dilution), or

(3) serum free M199 containing one of five concentrations of the bacterial protease being tested (4 wells/virus dilution/each concentration of bacterial protease).

Each bacterial protease was tested at activity levels which bracketed the activity of the positive control (3.9 BAEE units of trypsin/ml). All cell cultures were incubated in 5% CO₂ at 37°C. At 72 hours the culture medium from each well was harvested and assayed for the presence of virus production by the microtiter hemagglutination test (Beard, 1989). The TCID₅₀ was calculated by the Reed-Muench method (Reed, 1938).

For initial screening of virus infectivity enhancement by a protease, a one log₁₀ increase in the TCID₅₀ compared to the negative control was considered a positive increase in infectivity. Assays were repeated for a total of five
repetitions for protease-virus combinations which were positive on initial screening. The Wilcoxon Sign Rank procedure was used to test the hypothesis that there was no difference in the mean TCID$_{50}$ of the negative controls compared to each protease treatment group. Results were considered statistically significant at p values of <0.05.

**Results**

The specific activity (amount of enzyme which increased the absorbance of 1% sodium caseinate suspension in 30 min. at 22°C by 1.0 OD$_{280}$) per mg of protein for each bacterial protease preparation is given in Table 4. *Staphylococcus hyicus* was found to have the highest specific activity (88.0 units/mg) and *Staphylococcus epidermis* was found to have the lowest specific activity (6.7 units/mg). These results are consistent with the qualitative proteolytic results obtained with the agar gel diffusion test (Chapter II) where *S. hyicus* supernatants produced the widest zones and *S. epidermis* produced barely detectable zones. The protein concentrations of the bacterial protease tested in cell culture are also given in Table 4. These concentrations were selected because their activity was similar to the BAEE activity of the 0.3 ug/ml trypsin control as determined in proteolytic enzyme activity assays.
Results from the screening of each bacterial protease for the ability to enhance influenza virus infectivity are reported in Table 5. As expected, the trypsin control (0.3 ug/ml) increased the TCID₅₀ of all viruses (values ranged from $10^{1.5}$ to $10^{3.3}$). Also *Staphylococcus aureus* Woods 46 protease (292 ug/ml) increased the TCID₅₀ titer of human origin A/PR/8/34 which is consistent with previous reports (Tashiro, 1987a). *Vibrio alginolyticus* protease (250 ug/ml) increased the TCID₅₀ of avian influenza viruses A/turkey/Wisconsin and A/mallard/184/86 on initial screening. The proteases of the *Staphylococcus gallinarum* (ATCC 35530), chicken-origin *Staphylococcus hyicus*, chicken-origin *Flavobacterium* sp, turkey-origin *Staphylococcus aureus* and turkey-origin *Staphylococcus epidermis* all failed to increase the TCID₅₀ for the five avian influenza strains in this study at the protease concentrations tested (Table 5).

The differences in the TCID₅₀ titers for multiple repetitions of type A influenza viruses with and without bacterial proteases added to the cell culture media is given in Table 6. The presence of *Staphylococcus aureus* Woods 46 protease (292 ug/ml) in cell culture media increased infectivity of A/PR/34/8 by an average of $10^{2.0}$. The human-origin *Staphylococcus aureus* Woods 46 protease, did not increase viral titers of A/ty/WI/68 and A/ma/OH/184/86, also
reported in Table 5. The addition of the protease from chicken-origin *Vibrio alginolyticus* (250 ug/ml) to the cell culture media increased the TCID₅₀ of A/ty/WI/68 and the waterfowl isolate A/ma/OH/184/86 by a mean titer (in 5 TCID₅₀ repetitions) of 10¹·¹ and 10²·¹, respectively. Wilcoxon Sign Rank analysis indicates that the replicate TCID₅₀ increases associated with the *Vibrio alginolyticus* protease are statistically significant for both the A/ty/WI/68 (p=0·02) and A/ma/OH/184/86 (p=0·02).

The magnitude of virus enhancement observed with *V. alginolyticus* protease in cell culture medium was found to be concentration dependent (Figure 1). The *V. alginolyticus* protease concentration which resulted in the greatest virus titer increase was 250 ug/ml. Protease concentrations that were greater or less than 250 ug/ml resulted in smaller magnitudes of virus infectivity enhancement for both A/ma/OH/184/86 and A/ty/WI/68.

SDS-PAGE analysis of the *Vibrio alginolyticus* protease indicates that the molecular weight of the purified virus-activating protease is 12,000 daltons.

Discussion

A strain of *Vibrio alginolyticus* recovered from a tracheal swab of a chicken secreted protease which, when added to cell culture medium, enhanced the infectivity of
A/ty/Wi/68 and A/ma/Oh/184/86 AI viruses in primary chicken embryo cell culture. The Vibrio protease did not enhance the infectivity of three other avian influenza viruses. Human-origin Staphylococcus aureus Woods 46 enhanced the infectivity of human-origin A/PR/8/34 influenza virus in this study, but was not effective in enhancing the infectivity of any of the avian-origin influenza viruses. The S. aureus protease results confirm work reported by Tashiro et al. 1987a, where the addition of staphylococcal proteases resulted in in vitro enhancement of infectivity for some human influenza strains tested, but was not observed for other human influenza strains. Most interesting was the infectivity of none of the AI viruses was increased by proteases of staphylococcus isolates or Flavobacterium sp at concentrations tested. Since no bacterial protease tested to date has been effective in enhancing the infectivity of all influenza viruses and the infectivity of no one virus is enhanced by all bacterial proteases, the interaction between bacterial protease and influenza virus seems to be very specific.

Enhancement of virus infectivity by Vibrio alginolyticus appeared to be concentration dependent with 250 ug/ml resulting in the greatest activation of both A/ty/WI/68 and A/ma/OH/184/86. This is consistent with Tashiro (1989a) studies where influenza virus enhancement
observed with Staphylococcus proteases was also concentration dependent. It is well known that enhancement of influenza viruses by trypsin is concentration dependent (Lazarowitz, 1975).

Trypsin concentrations of 0.3 ug/ml were used throughout this study after preliminary studies showed that lower concentrations activated fewer viruses and greater concentrations resulted in cell toxicity. However, unlike trypsin, it is not clear why higher Vibrio protease concentrations did not result in increased viral replication. No visible cell cytopathic effects were observed at higher bacterial protease concentrations used in the study. Lower Vibrio protease concentrations may have resulted in smaller increases in the viral titers due to the fact that there was insufficient protease available to activate all of the virus.

Our work focused on a bacterium recovered from poultry that probably came from an environmental source. *Vibrio alginolyticus* is most commonly associated with water, and was probably recovered from the tracheal swab after being ingested with water ingested by the birds. The potential for this or any other organism to be present in the trachea and respiratory epithelium in significant numbers during an influenza infection may be increased due to adherence of bacteria to influenza infected cells (Davison, 1981) and/or
due to impaired tracheal clearance function during influenza infections (Nuggent, 1983). This issue will be addressed more thoroughly in Chapter IV. Increased bacterial numbers in the respiratory tract of influenza infected birds may provide the opportunity for normal flora or transient bacterial to secrete protease which activate progeny influenza virus. If this association occurs it represents a truly synergistic relationship which could have clinical significance.

Conclusion

A strain of Vibrio alginolyticus recovered from a tracheal swab of a chicken produced protease which enhanced the infectivity of A/ty/Wi/68 and A/ma/Oh/184/86 in primary chicken embryo cell culture. The study also demonstrated that the relationship between bacterial protease and influenza viruses is very specific and complex. Co-infection leading to increases in influenza viral infectivity under in vivo conditions would appear to require a specific proteolytic bacteria and a specific strain of avian influenza virus. Also, the bacteria responsible for the enhancement of AI infectivity would not necessarily need to be a pathogen, but could be transient or part of the birds normal flora.
LIST OF REFERENCES


CHAPTER IV
Proteolytic Bacteria Enhances Virulence and Replication of Avian Influenza Virus in Turkeys

Summary

Compared to inoculation with virus or bacteria alone, intratracheal co-inoculation of specific pathogen free (SPF) turkeys with *Vibrio alginolyticus* and A/turkey/WI/68 (H5N9) resulted in enhancement of virus replication and increased severity of disease. Co-infection resulted in recovery of significantly higher virus titers in the lung (p=0.002) as well a significant increase in tracheal lesion scores (p=0.01). Prior experiments following a similar protocol but using another bacterium, *Staphylococcus aureus* Woods 46, and either A/chicken/Alabama/75 (H4N8) or A/mallard/OH/184/86 (H5N1) did not demonstrate an increase in virus titers in lung tissues of SPF chickens. These data provide preliminary evidence for a role by which select proteolytic bacteria may increase the severity of avian influenza (AI) infections in poultry, and also suggest that the interaction between influenza virus and proteolytic bacteria is specific to the virus-bacteria combination. The increased
replication and virulence of AI in the presence of a specific bacterium may help explain why poultry AI isolates associated with significant morbidity and mortality in the field are consistently classified as nonpathogenic or low pathogenic under laboratory conditions.

Introduction

Activation of type A influenza viruses by the proteolytic enzyme trypsin (Lazarowitz, 1975) led to many subsequent reports on the requirements of posttranslational cleavage of type A influenza virus for initiating the next viral replication cycle (Klenk, 1988). Tashiro (1987a,b) was the first to examine the potential effect of bacterial proteases on increasing influenza virus infectivity under in vitro conditions. Protease secreted by Staphylococcus aureus Woods 46 was shown to activate 7 of 12 mammalian influenza viruses in cell culture studies. In vivo studies by Tashiro (1987a,b) and others showed that co-inoculation of mice with type A influenza and proteolytic bacteria or bacterial exoproteases could increase the severity of influenza infections in mice (Akaike, 1989; Scheiblauer, 1992). These data raised the question of whether a synergistic relationship exists between avian-origin proteolytic bacteria and avian influenza viruses.

Recent studies in this laboratory demonstrated proteolytic bacteria are frequently present in the
respiratory tract of poultry (Chapter II). Additional studies demonstrated that protease produced by a chicken-origin *Vibrio alginolyticus* increased the average TCID₅₀ titers of A/mallard/Ohio/186/84 and A/turkey/Wisconsin/68 stock viruses by 10⁷⁻¹ and 10¹⁻¹, respectively (Chapter III), while not increasing the TCID₅₀ titers of three other avian influenza viruses. These data justify further inquiry into the potential role of proteolytic bacteria in increasing the severity of avian influenza outbreaks in poultry.

The objective of this study was to determine if co-infection of SPF poultry with a proteolytic bacteria, *Staphylococcus aureus* or *Vibrio alginolyticus* and AI viruses, A/chicken/Alabama/75, A/mallard/Ohio/184/86 or A/turkey/Wisconsin/68 would increase the severity of disease and elevate viral titers in lung tissues.

**Materials and Methods**

**Hosts and Housing:**

One-day-old specific pathogen free (SPF) chickens and SPF turkeys were obtained from the Ohio Agriculture Research and Development Center, Wooster, Ohio. Upon arrival, birds were divided into 4 equal groups and housed in rigid negative pressure Horsfall-Bauer isolator cabinets fitted with HEPA biologic filters on both air inlet and exhaust. Birds were provided with feed and water ad libitum.
Bacteria selected for this study include:

**Staphylococcus aureus Woods 46:** This proteolytic bacterium was obtained from the American Type Culture Collection (ATCC 10832). Protease secreted by this bacterium was shown to enhance infectivity of seven of twelve mammalian type A influenza viruses, [including mouse-adapted human influenza A/PR/8/34 (H1N1)], (Tashiro, 1987a; Chapter III). The increase in infectivity reported by Tashiro (1987a) was associated with cleavage of type A influenza virus hemagglutinin. Intranasal co-infection of mice with this bacterium and A/swine/1976/31 (H1N1) resulted in recovery of increased virus titers from the lung as well as increased severity of disease (Tashiro, 1987a,b).

**Vibrio alginolyticus:** This proteolytic bacterium was recovered on Standard Methods Caseinate Agar from a tracheal swab of a leghorn chicken (Chapter I). In primary chicken embryo fibroblast cell culture, protease secreted by this bacterium enhanced the infectivity of A/turkey/WI/68 (H5N9) and A/mallard/OH/184/86 (H5N1) but not three other avian influenza isolates tested, (Chapter III).

Viruses selected for this study include:

**A/chicken/AL/75 (H4N8):** This avian influenza isolate was identified in 1975 as the cause of a self-limiting influenza
outbreak in chickens, with mortality rates ranging from 8.6 to 69.1% (Johnson, 1977). While early experimental efforts to reproduce disease with this virus were unsuccessful (Pearson, 1981), subsequent studies at the United States Department of Agriculture Southeast Poultry Research Laboratory and in this laboratory have shown that this virus is pathogenic and has an IVPI score of 0.49 (Slemons, 1991). This virus is activated in cell culture by trypsin (Chapter, III).

**A/mallard/OH/184/86 (H5N1):** This isolate was obtained from a cloacal swab of an asymptomatic mallard duck during an influenza surveillance program on Lake Erie (Slemons, 1990). It is activated by trypsin (Jeffery, 1991) and *Vibrio alginolyticus* protease (Chapter III). This isolate is reported to have a low pathogenicity index (IVPI score 0.30) in chickens (Slemons, 1991).

**A/turkey/WI/68 (H5N9):** This isolate was recovered from a clinical outbreak of AI in turkeys associated with severe drops in egg production and mild respiratory illness (Smithies, 1969). It is activated by trypsin and *Vibrio alginolyticus* protease and, based on the IVPI test, is nonpathogenic in chickens (IVPI 0.00) (Slemons, 1991).
Treatment Groups

The four groups of birds established upon arrival were used as separate treatment groups. Treatment groups were challenged as follows:

(1) Virus only challenge (V): avian influenza virus in allantoic fluid (AF) + sterile brain heart infusion broth (BHIB) (sham bacteria inoculation).

(2) Bacteria only challenge (B): bacteria in BHIB + sterile AF (sham virus inoculation).

(3) Virus and Bacteria challenge (V+B): avian influenza virus in AF + bacteria in BHIB.

(4) Control (CNTRL): sterile BHIB + sterile AF.

Preparation of Viral and Bacterial Challenge Doses

Preparation of virus challenge dose: Viruses were propagated in the allantoic sac of ten-day-old embryonated eggs. After a 48 hour incubation at 37°C followed by 24 hours at 4°C, the AF was harvested, virus was aliquoted into vials, then stored at -70°C. On challenge day, stock virus was thawed, then diluted 1/10 (v/v) in 50% phosphate buffered saline and 50% allantoic fluid. Titration of challenge virus was accomplished in ten-day-old embryonated eggs using standard techniques (Beard, 1980).

Preparation of bacterial challenge dose: Bacteria were propagated in brain heart infusion broth (BHIB), washed in
BHIB, suspended in BHIB + 10% glycerol, then aliquoted into vials and stored at -70°C until used. Frozen vials of bacteria were thawed then inoculated into 100 ml of BHIB. After incubation at 37°C for 24 hour, bacteria were centrifuged at 12,000 rpm for 12 minutes, washed three time in BHIB, then resuspended in 50 ml of BHIB. Titration of challenge bacteria was accomplished following a ten-fold serial dilution of the bacterial challenge suspension and subsequent plating onto Standard Method Plate Count agar using the pour plate technique.

Experimental Protocol

Four experiments were completed following the same basic design. Variations in design of individual experiments are described below and are summarized in Table 7.

1. Hosts:
Expts. 1 and 2: Six-week-old specific pathogen free chickens
Expts. 3 and 4: Seven-day-old specific pathogen free turkeys

2. Days of challenge:
Expts. 1 and 2: day 0 and day 1 post inoculation (PI).
Expts. 3 and 4: day 0 only
3. Challenge Virus and Bacteria

Expt. 1: A/chicken/AL/75 (H4N8) and *S. aureus* Woods 46
Expt. 2: A/mallard/Oh/184/86 (H5N1) and *S. aureus* Woods 46
Expt. 3: A/turkey/WI/68 (H5N9) and *Vibrio alginolyticus*
Expt. 4: A/turkey/WI/68 (H5N9) and *Vibrio alginolyticus*

4. Route of Challenge:

All birds were challenged with 50 ul quantities of virus and bacteria (or sham substitutes) via intratracheal inoculation.

5. Euthanasia:

Expts. 1 & 2: Challenged birds were euthanized with sodium pentobarbital (50mg/kg body weight, IV) on appropriate sample days.

Expts. 3 & 4: Challenged birds were anesthetized with ketamine (50 mg/kg body weight) and xylazine (10 mg/kg body weight) given together, IM, then euthanized with sodium pentobarbital (50 mg/kg body weight, IV) on appropriate sample days. This technique modification was made to prevent aspiration of bacteria into the trachea, which may have occurred with the former method.

6. Samples collected at necropsy:

Virology: Lung tissues, tracheal and cloacal swabs were collected from all birds on day of necropsy. Cloacal and tracheal swabs were placed in 2-dram screw cap tubes
containing 2 ml of brain heart infusion broth plus penicillin (10,000 units/ml) and streptomycin (10 mg/ml) (BHIB+P/S). Lung tissues were placed in individual sterile whirl-pak bags. Swabs and tissues were stored at -70°C until tested.

**Bacteriology:**
Lung tissues were collected from all birds on day of necropsy. Tracheal washes were collected in Experiment 4 only. Ventral thoracic approach was used to obtain lung tissues in Expts. 1, 2 and 3. A dorsolateral approach was used for Expt. 4 to reduce bacterial contamination during collection of lung tissues at necropsy. Lungs were collected in sterile whirl-pak bags, held at 4°C for less than 2 hours, then immediately transported to the laboratory for processing. Tracheal wash samples were taken in attempts to recover challenge bacteria. Following aseptic isolation of the trachea, sterile forceps were used to close the trachea just below the glottis. The trachea was then transected between the glottis and the forceps and just anterior to the thoracic inlet. A syringe and needle was used to rinse the interior of the trachea section with 0.5 ml of BHIB. Tracheal washings were placed into a 2 dram screw cap vial and held at 4°C for less than two hour before processing.
**Histopathology:**

In Expts. 3 & 4 lung, trachea, pancreas and kidney were collected for histopathological evaluation. All tissues were collected and placed in 10% neutral buffered formalin solution within 10 minutes of euthanasia. All formalin fixed tissues were trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H+E).

7. **Processing of samples collected:**

**Virology samples:**

Re-isolation of challenge virus was accomplished in ten-day-old embryonated eggs using standard techniques (Beard, 1980). Basically, 0.1 ml of either BHIB+P/S from swabs or tissue homogenate (1:10 w/v BHIB+PS) was inoculated into the allantoic cavity of ten-day-old embryonated commercial chicken eggs. After 48 hour incubation at 37°C, followed by 24 hour at 4°C, the allantoic fluids were checked for hemagglutinating activity. Hemagglutinating agents recovered were identified as challenge virus using the hemagglutinin inhibition test (Palmer, 1975). Inoculation of ten fold serial dilutions of tissue supernatants from virus isolation positive tissue homogenates eggs was used to determine the mean Egg Infective Dose (EID<sub>50</sub>) titers. Titer were calculated by the method of Reed and Mensch (Reed, 1938).
**Bacteriology samples:**

Tracheal wash samples were processed and lung tissue homogenates (10% w/v) were prepared in BHIB within two hours of necropsy. Serial dilutions of lung tissue homogenates and tracheal wash samples were plated onto duplicate Standard Methods Caseinate Agar using the pour plate technique. Agar plates were incubated at 37°C for 48 hours. Bacterial titers as colony forming units (cfu) per gram of tissue were recorded. Highly proteolytic colonies were evaluated via gram stain and biochemical testing to identify challenge bacterium.

**Histology samples:**

H&E stained slides were evaluated for presence and severity of lesions by Dr. David Swayne, Ohio State University Department of Veterinary Pathobiology. Lesions were assigned a score from 1 to 4 with 1 indicating no lesions, 2 indicating focal lesions, 3 indicating multifocal lesions and 4 indicating diffuse lesions.

**8. Statistical Analysis**

The Wilcoxon Rank Sum tests were used to determine if significant differences existed in viral titers, bacterial titers and histopathological lesion scores among treatment groups.
Results

EXPERIMENTS 1 and 2

Virology

There was no statistical difference in the viral concentrations in lung tissues of co-inoculated birds (virus & bacteria challenge) compared to virus only inoculated birds within Expts. 1 or 2 (Wilcoxon Rank Sum $>0.05$). However, virus was recovered from lung tissues of birds in the virus only challenge group, but not the virus-bacteria challenge group in Expt. 1. Patterns for virus recovery from lung tissues are reported in Table 8 (Expt. 1) and Table 9 (Expt. 2). There also was no significant difference in the frequency of virus isolation from tracheal or cloacal swabs among treatment groups within Expts. 1 and 2 (Table 10). The frequency of virus recovery was higher in Expt. 1 (A/ch/AL/75) than in Expt. 2 (A/ma/OH/184/86), which is consistent with previous reports of recovery of these viruses from intratracheal challenge of chickens birds (Slemons, 1992).

Bacteriology

The bacterial titers recovered from the lungs of bird in Expts. 1 and 2 are reported in Tables 8 and 9 respectively. Proteolytic bacteria recovered from these experiments were characterized as coagulase positive gram
positive cocci. No attempt was made to definitively identify the recovered bacteria. Bacterial titers in lung tissues ranged from $10^1$ to $10^{5.2}$. There were no statistical differences in bacteria concentration recovered from lung tissues between treatment groups within these two experiments (Wilcoxon Rank Sum $p > 0.05$).

**EXPERIMENTS 3 and 4**

Experiments 3 and 4 were duplicate trials involving the same virus/bacteria combination (A/ty/WI/68 and *Vibrio alginolyticus*). The results obtained with each of these experiments were very similar (Table 11 and Table 12, respectively).

**Virology**

*Lung Tissues*: In Expts. 3 & 4 combined, challenge virus was recovered from lung tissues of 2 of 23 birds ($EID_{50}$ $10^{2.3}$ & $10^{7.3}$/g lung tissue) in the virus only treatment groups, and 12 of 23 lungs from birds inoculated with bacteria and virus ($EID_{50}$ $10^{2.3}$ to $10^{5.8}$/g lung tissue). The virus only inoculated bird which had a virus lung titer of $10^{7.3}$, was found dead in the cage on day 2 PI.

Statistical analysis of the virus isolation data from lung tissues recovered from Experiments 3 and 4 indicated that a significantly higher titer of challenge virus was recovered from the lungs of birds inoculated with both *Vibrio alginolyticus* and A/ty/WI/68 than birds inoculated
with influenza alone (Wilcoxon Rank Sum p=.002).

Tracheal Swabs: In Expts. 3 & 4 combined, challenge virus was recovered from tracheal swabs of 1/23 birds in the virus only inoculated group and 6/23 birds in the bacteria and virus inoculated groups (Table 10).

Cloacal Swab: Virus was not recovered from cloacal swabs in Expts. 3 and 4 (Table 10).

Bacteriology

The bacteria titers in the lungs of birds in Expts. 3 and 4 are reported in Tables 11 and 12 respectively. Bacterial titers recovered ranged from \(<10^1\) to \(10^{5.0}\) cfu/g tissue. Challenge bacterium *Vibrio alginolyticus* was not recovered from lungs tissues or tracheal wash samples of any birds in Experiment 3 or 4. There were no statistical differences in bacterial titers recovered among treatment groups within each of these two experiments (Wilcoxon Rank Sum p >0.05). The bacterial counts are markedly lower in Expt. 4 than in Expt. 3 which most likely due to a change in dissection technique. The dorsolateral approach to the lung appears to have reduced inadvertent bacterial contamination, and resulted in a higher quality sample for bacterial analysis.

Histopathology

Tracheal Lesions: Results of tracheal lesion scores on day 3 & 5 PI are reported for Expt. 3 in Tables 13 and 14 and
for Expt. 4 on day 1, 3, & 5 in Tables 15, 16 and 17. No lesions (Plate III) were present in the tracheas of control birds on any day (lesion index 1). Tracheal lesions present in birds challenged with *Vibrio alginolyticus* only, A/ty/WI/68 only, or *Vibrio alginolyticus* and A/ty/WI/68 included severe tracheitis characterized by numerous lumenal heterophils, epithelial hyperplasia with loss of orientation of cilia, submucosal infiltration of heterophils, lymphocytes and macrophages (Plate IV).

The dynamics of tracheal lesions over time is presented in Figure 2, where the average tracheal lesion scores for Expts. 3 & 4 are reported by treatment group and by day. On day 1 the bacteria only treatment group and the virus and bacteria treatment group had significantly higher tracheal lesion scores than the control or virus only inoculated groups. On day 3 no tracheal lesions were present in any of the birds in the bacteria only inoculated groups. The birds in the virus and bacteria inoculated groups had the highest tracheal lesion scores on day 3. On day 5, the tracheal lesions of the virus only and the virus and bacteria groups were less severe than on day 3, with the virus and bacteria inoculated treatment groups still having the highest average tracheal lesion score.

Statistical analysis of tracheal lesions from all groups suggests that the lesions present in the virus and
bacteria inoculated groups were more severe than the lesions present in the virus only (p=.0002) or bacteria alone (p=.0002) groups. Wilcoxon Rank Sum analysis of day 3 data only indicates the tracheal lesions in the virus and bacteria treatment group were significantly greater than the lesions in the virus only treatment group (p value of .01).

**Lung Lesions:** Lesions present in the lungs of the *Vibrio alginolyticus* only, A/ty/WI/68 only and *Vibrio alginolyticus* and A/ty/WI/68 inoculated treatment groups consisted of inflammation of the primary bronchus with peribronchial pneumonia (Plate V) characterized by lymphocytes, fibrin, heterophils and necrotic cells (Plates VI and VII). These lesions were most consistently present in the area where the tertiary bronchi comes off the secondary bronchi, in the ventral most aspect of the lungs. The average lung lesion scores for Expts. 3 & 4 combined are presented by treatment group and by day PI in Figure 3. There were no statistical differences in the average lung lesion scores between birds in the virus only or virus and bacteria treatment groups. Severe lesions were present only in the bird from the virus only inoculated group, which had died on day 2 (Table 13).
Discussion

These data provide preliminary evidence which suggests co-infection of turkeys with a specific bacterium can increase the infectivity and pathogenicity of a specific avian influenza virus. This conclusion is supported by data which measures synergism by three different methods. First, co-inoculation of turkeys with *Vibrio alginolyticus* and A/ty/WI/68 resulted in significantly higher viral titers in lungs (Wilcoxon Rank Sum p=0.002), compared to birds inoculated with A/ty/WI/68 alone (Table 11). Secondly, AI virus was recovered more frequently from tracheal swabs of birds inoculated with A/ty/WI/68 and *Vibrio alginolyticus* (6/23 birds) than from birds inoculated with A/ty/WI/68 alone (1/23 birds)(Table 10). Thirdly, the tracheal lesion scores in the A/ty/WI/68 and *Vibrio alginolyticus* inoculated treatment group are significantly higher (Wilcoxon Rank Sum p=0.01) than the birds in the treatment group inoculated with virus alone (Figure 2).

These in vivo results are consistent with previous reports where co-infection of mice with mouse-adapted human-origin type A influenza and the bacteria or bacterial proteases of *Staphylococcus aureus* Woods 46 (Tashiro, 1987a), *Serratia marcesans* (Akaike, 1989) or *Aerococcus viridans* (Scheiblauer, 1992) resulted in increased viral titers. However, in these experiments, increased severity
mouse studies.

Based on our theory, the failure of *Staphylococcus aureus* Woods 46 to enhance the infectivity and pathogenicity of A/ch/AL/75 or A/ma/OH/184/86 in vivo was not unexpected. Results obtained in tissue culture studies (Chapter III) indicated that the titers of the five AI influenza viruses tested, including A/ch/AL/75 and A/ma/OH/184/86, were not increased in the presence of *S. aureus* Woods 46 protease. This suggests that the interaction between bacteria and virus is very specific. The specificity observed between virus and bacteria in these in vivo studies was also reported by Tashiro (1987a). Co-inoculation of *S. aureus* Woods 46 increased the severity of pulmonary lesion of mice co-infected with A/swine/1976/31 (H1N1) but not A/chicken/Germany/49 (H10N1).

One unexpected result obtained in the in Expt. 1 was the recovery of challenge virus from the tracheal swab of a bacteria only inoculated bird (Table 10). This result is attributed to inappropriate use of the ventilation equipment during this experiment, which allowed cross ventilation between isolators. This occurrence was not believed to have effected our conclusions, since Expt. 1 did not show significant viral enhancement.

A second unexpected result in study was that the most severe pulmonary lesions observed were from a bird which was
severe pulmonary lesions observed were from a bird which was inoculated with A/ty/WI/68 only in Expt. 3 (Table 13). This bird died in day 2 PI from apparent primary viral pneumonia. This finding suggests that the direct role of low pathogenic influenza virus in producing severe primary pulmonary damage and death in an individual animal cannot be ignored.

The hypothesis that proteolytic bacteria may increase the severity of influenza in turkeys was recently given credence by field studies of a natural outbreak (Ficken, 1989). The demonstration of influenza A virus in pulmonary macrophages and the simultaneous recovery of bacteria, including Staphylococcus aureus and Streptococcus spp. from the lungs (Ficken, 1989) presents a scenario where synergism may occur. As expected, experimental attempts to reproduce the disease via re-inoculation of the virus isolated did not result in the mortality rate observed in the original outbreak (Swayne, 1992). The pulmonary lesions described in that natural outbreak, however, appear similar in distribution and character to the lesions reported in this study (Personal communication, Dr. David Swayne).

The specific mechanism responsible for the increased influenza titers and pathology observed in this study has not been identified. There appear to be at least two possible mechanisms which could be responsible for the increase in pathogenicity and influenza virus titers in
birds co-infected with *Vibrio alginolyticus* and A/ty/WI/68. First, the bacterial protease may have cleaved the HA directly, which resulted in the activation of the virus. This possibility is supported by the fact that the presence of the *Vibrio alginolyticus* protease in tissue culture medium resulted in increased A/ty/WI/68 titers recovered (Chapter III). However, the finding that challenge bacteria were not isolated from lung tissues or tracheal swabs on day 1, 3 or 5 PI, having been cleared from the trachea via the mucociliary apparatus, makes the direct effect mechanism more difficult to support. A local direct effect of low numbers of proteolytic bacteria which may be present in the upper trachea cannot be discounted, but other possible mechanisms should also be considered. While cleavage of HA by bacterial proteases has been proposed as a mechanism for activation of influenza virus in the mouse host, (Tashiro, 1987a), researchers have not reported attempts to recover challenge bacteria.

A second possible mechanism for viral-bacterial synergism in this study is that the presence of bacteria and/or bacterial protease increased the release of the host's cellular enzymes and these enzymes cleave the viral HA. This mechanism was suggested by Scheiblauer (1992). The fact that tracheal and pulmonary lesions were present in the bacteria only treatment group on day 1 supports the
suggestion that increased amounts of the host's cellular enzymes may be released. The presence of increased host cellular enzymes on day 1 could result in cleavage of the HA of progeny virus, which facilitated virus replication in the early stage of infection which led to increased tracheal viral shedding, increased tracheal lesions, and increased viral lung titers on day 3 PI.

Two other mechanisms have been reported in the literature which may be responsible for increased severity of avian influenza infection in the presence of proteolytic bacteria. First it has been suggested that bacterial proteases may transform host-origin plasminogen to plasmin, which has been shown to cleave the HA of some influenza viruses (Akaike, 1989). The use of plasmin-resistant influenza viruses has eliminated this variable in some in vivo studies (Scheiblauer, 1992) but cannot be ruled out in this study. Another proposed mechanism by which bacterial protease could enhance AI pathogenicity is that the presence of the protease could result in degeneration of host IgA, which would impede the hosts immune response (Kilian, 1979). However a significant specific IgA response by day 3 PI seems unlikely.

The results presented in this study are consistent with a sequence of events in which bacteria facilitate the early replication of influenza viruses producing tracheitis and
becomes effective. In most flocks, as well as laboratory studies of field isolates, influenza virus alone cannot accomplish this because the majority of the virus particles produced possess uncleaved HA and are therefore noninfectious. The presence of a co-infecting organism such as *Vibrio alginolyticus*, though harmless itself, during the initial exposure may allow multiple replication cycles early in the infection.

**Conclusions**

Co-infection of turkeys with an avian origin proteolytic bacteria resulted in significantly higher AI virus titers in the lung and significantly higher tracheal lesion scores on days 3 and 5 post inoculation. The early disappearance of the bacteria from the respiratory tract indicates that proteolytic bacteria not recovered in the later stages of disease may be responsible for facilitating initiation of AI virus infections in turkeys. Further work is necessary to identify the specific mechanisms by which co-infecting bacteria contribute to higher influenza titers.
List of References


APPENDIX A

TABLES
Table 1

Poultry flocks tested for the presence of proteolytic bacteria from tracheal swabs

<table>
<thead>
<tr>
<th>Flocks</th>
<th>Age (weeks)</th>
<th>Housing</th>
<th>Number Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>56</td>
<td>Caged</td>
<td>30</td>
</tr>
<tr>
<td>F2</td>
<td>52</td>
<td>Caged</td>
<td>30</td>
</tr>
<tr>
<td>F3</td>
<td>6</td>
<td>Isolators</td>
<td>10</td>
</tr>
<tr>
<td>F4</td>
<td>12</td>
<td>Pasture</td>
<td>30</td>
</tr>
</tbody>
</table>

F1 = Leghorn chickens, research flock
F2 = Leghorn chickens, commercial flock
F3 = Specific pathogen free turkeys
F4 = Turkeys on pasture
Table 2

Percent of birds with proteolytic, highly proteolytic, or non-proteolytic bacteria recovered from tracheal swabs of poultry. Birds classified by highest level of proteolytic bacteria recovered.

<table>
<thead>
<tr>
<th>Flock</th>
<th>Highly Pro&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Proteolytic&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Non-Pro&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>37</td>
<td>20</td>
<td>43</td>
</tr>
<tr>
<td>F2</td>
<td>20</td>
<td>n/d*</td>
<td>n/d</td>
</tr>
<tr>
<td>F3</td>
<td>50</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>F4</td>
<td>27</td>
<td>3</td>
<td>70</td>
</tr>
</tbody>
</table>

F1 = leghorn, research flock
F2 = leghorn, commercial flock
F3 = specific pathogen free turkeys
F4 = turkeys on pasture

<sup>1</sup> Highly Pro = % highly proteolytic bacteria
<sup>2</sup> Proteolytic = % proteolytic bacteria
<sup>3</sup> Non-Pro = % non-proteolytic bacteria
<sup>4</sup> n/d = not differentiated
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus sciuri</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Staphylococcus xylosus</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Staphylococcus hyicus</em></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Flavobacterium sp</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

F1 = Leghorn, Research Flock

F2 = Leghorn Chickens, Commercial Flock

F3 = Specific Pathogen Free Turkeys

F4 = Turkeys on Pasture
Table 4

Specific activity\(^1\) of bacterial proteases and total protein concentration of protease preparations evaluated for influenza virus enhancing effect in primary cell culture.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Specific Activity</th>
<th>Total Protein Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg protein</td>
<td>Tested(^2) - μg/ml</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Woods 46</td>
<td>13.3</td>
<td>146, 219, 292, 365, 438</td>
</tr>
<tr>
<td><em>Staphylococcus gallinarum</em></td>
<td>15.0</td>
<td>131, 196, 262, 327, 393</td>
</tr>
<tr>
<td><em>Staphylococcus hyicus</em></td>
<td>88.0</td>
<td>22, 66, 88, 110, 132</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>30.0</td>
<td>65, 97, 130, 162, 195</td>
</tr>
<tr>
<td><em>Staphylococcus epidermis</em></td>
<td>6.7</td>
<td>290, 435, 580, 725, 1000</td>
</tr>
<tr>
<td><em>Flavobacterium sp.</em></td>
<td>10.9</td>
<td>175, 262, 350, 437, 525</td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em></td>
<td>15.6</td>
<td>125, 187, 250, 312, 375</td>
</tr>
</tbody>
</table>

\(^1\) 1 unit = amount of protease which increased absorbance of sodium caseinate suspension by 1.0 OD\(_{280}\) after 30 minutes at 22°C.

\(^2\) Protein concentrations tested are those which bracketed 3.9 BAEE units/mg = 0.3 μg/ml trypsin.
Table 5

Screening for the ability of trypsin and bacterial proteases to enhance the infectivity of type A influenza virus in primary chicken embryo fibroblasts cell culture

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Trypsin</th>
<th>SAW</th>
<th>VA</th>
<th>SG</th>
<th>SH</th>
<th>SA</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34 (H1N1)</td>
<td>+2</td>
<td>+</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>A/ch/AL/75 (H4N8)</td>
<td>+</td>
<td>.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/ty/WI/68 (H5N9)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>A/ty/OH/88 (H1N1)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A/ma/OH/184/86 (H4N8)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>A/ma/OH/338/86 (H5N1)</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

1 TCID$_{50}$ of viruses determined in PCEF cell culture at 37°C with M199 containing no protease, purified bovine pancreatic trypsin (trypsin; 0.3 μg/ml), Staphylococcus aureus protease (SA), Vibrio alginolyticus protease (VA) Staphylococcus aureus Woods protease (SAW), Staphylococcus gallinarum ATCC 35530 protease (SG), Staphylococcus hyicus protease (SH), Staphylococcus epidermis (SE) protease.

2 + - > 1 log$_{10}$ increase in TCID$_{50}$ after 72 hours at 37°C.

3 - < 1 log$_{10}$ increase in TCID$_{50}$ after 72 hours at 37°C, at any of the five protein concentrations tested.

4 - Not done
### Table 6

Results from repeat infectivity assays measuring TCID<sub>50</sub> of type A influenza virus concentrations with and without the addition of bacterial proteases to cell culture media.

<table>
<thead>
<tr>
<th></th>
<th>S. aureus Woods 46 protease (292 µg/ml)</th>
<th>V. alginolyticus protease (250 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With</td>
<td>Without</td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>10.3</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>10.2</td>
<td>8.0</td>
</tr>
<tr>
<td>A/ty/WI/68</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.2</td>
<td>5.5</td>
</tr>
<tr>
<td>A/ma/OH/184/86</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>5.8</td>
<td>4.0</td>
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<td></td>
<td>7.2</td>
<td>4.6</td>
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<tr>
<td></td>
<td>6.5</td>
<td>4.3</td>
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</tbody>
</table>
Table 7

Design of Individual Experiments: Co-inoculation studies of specific pathogen free chickens and turkeys with proteolytic bacteria and avian influenza viruses.

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Host</th>
<th>Age</th>
<th>Chal Days</th>
<th>Virus Chal</th>
<th>Bact Chal</th>
<th>Smpl Days</th>
<th>Viral Smpl</th>
<th>Bacterial Smpl</th>
<th>Histological Smpl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>chks</td>
<td>6 wk</td>
<td>0, 1</td>
<td>ck/A</td>
<td>SAW</td>
<td>1, 3</td>
<td>lung, t, cl swab</td>
<td>lung</td>
<td>none</td>
</tr>
<tr>
<td>2</td>
<td>chks</td>
<td>6 wk</td>
<td>0, 1</td>
<td>ma/O</td>
<td>SAW</td>
<td>1, 3</td>
<td>lung, t, cl swab</td>
<td>lung</td>
<td>none</td>
</tr>
<tr>
<td>3</td>
<td>tkys</td>
<td>7 dy</td>
<td>0</td>
<td>ty/W</td>
<td>VA</td>
<td>3, 5</td>
<td>lung, t, cl swab</td>
<td>lung</td>
<td>lung, t, kd, panc</td>
</tr>
<tr>
<td>4</td>
<td>tkys</td>
<td>7 dy</td>
<td>0</td>
<td>ty/W</td>
<td>VA</td>
<td>1, 3, 5</td>
<td>lung, t, cl swab</td>
<td>lung</td>
<td>lung, t, kd, panc</td>
</tr>
</tbody>
</table>

Chal Days - Days birds challenged with virus and/or bacteria  
Virus Chal - Challenge Virus  
Bact Chal - Challenge Bacterium  
Smpl Days - Sample Days  
Viral Smpl - Virology Samples collected/bird  
Bacterial Smpl - Bacteriology Samples collected/bird  
Histological Smpl - Histology Samples collected/bird  

chks - specific pathogen free chickens  
tkys - specific pathogen free turkeys  
ck/A - A/chicken/AL/75  
ma/O - A/mallard/OH/184/86  
ty/W - A/turkey/WI/68  
SAW - S. aureus Woods 46  
VA - Vibrio alginolyticus  
t - trachea  
cl - cloaca  
k - kidney  
t/wh - tracheal wash  
panc - pancreas
Table 8

Experiment 1: Viral and bacterial titers recovered from LUNGS of specific pathogen free chickens post intratracheal inoculation with A/chicken/AL/75 and/or Staphylococcus aureus Woods 46.

<table>
<thead>
<tr>
<th>Trt Grp</th>
<th>Chal</th>
<th>Repl</th>
<th>Viral D1</th>
<th>Viral D3</th>
<th>Bacterial D1</th>
<th>Bacterial D3</th>
</tr>
</thead>
<tbody>
<tr>
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<td>ck/A</td>
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<td>2.1</td>
<td>&lt; 1.0</td>
<td>&lt; 1.0</td>
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<td>ck/A</td>
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<td>&lt; 1.5</td>
<td>3.5</td>
<td>0.7</td>
<td>&lt; 1.0</td>
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<tr>
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<td>ck/A</td>
<td>3</td>
<td>&lt; 1.5</td>
<td>0.9</td>
<td>1.4</td>
<td>&lt; 1.0</td>
</tr>
<tr>
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<td>ck/A</td>
<td>4</td>
<td>---</td>
<td>&lt; 1.5</td>
<td>---</td>
<td>2.6</td>
</tr>
<tr>
<td>1</td>
<td>ck/A</td>
<td>5</td>
<td>---</td>
<td>&lt; 1.5</td>
<td>---</td>
<td>&lt; 1.0</td>
</tr>
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<td>SAW</td>
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<td>&lt; 1.5</td>
<td>&lt; 2.0</td>
<td>2.3</td>
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<tr>
<td>2</td>
<td>SAW</td>
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<td>&lt; 1.5</td>
<td>&lt; 2.0</td>
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<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>&lt; 2.0</td>
<td>&lt; 2.0</td>
</tr>
<tr>
<td>2</td>
<td>SAW</td>
<td>4</td>
<td>---</td>
<td>&lt; 1.5</td>
<td>---</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>SAW</td>
<td>5</td>
<td>---</td>
<td>&lt; 1.5</td>
<td>---</td>
<td>&lt; 2.0</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>1</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>5.2</td>
<td>&lt; 2.0</td>
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<td>v+b</td>
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<td>&lt; 1.5</td>
<td>4.1</td>
<td>&lt; 2.0</td>
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<tr>
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<td>v+b</td>
<td>3</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.4</td>
<td>&lt; 2.0</td>
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<tr>
<td>3</td>
<td>v+b</td>
<td>4</td>
<td>---</td>
<td>&lt; 1.5</td>
<td>---</td>
<td>&lt; 2.0</td>
</tr>
<tr>
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<td>v+b</td>
<td>5</td>
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<td>&lt; 1.5</td>
<td>---</td>
<td>&lt; 2.0</td>
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<td>&lt; 1.0</td>
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<td>&lt; 1.5</td>
<td>1.5</td>
<td>&lt; 1.0</td>
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<td>&lt; 1.0</td>
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<tr>
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<td>5</td>
<td>---</td>
<td>&lt; 1.5</td>
<td>---</td>
<td>&lt; 1.0</td>
</tr>
</tbody>
</table>

Trt Grp = Treatment Group  
Chal = virus and/or bacterial challenge  
Repl = Replication  
Viral D1/D3 = challenge Virus recovered, Day 1, Day 3 (Log10)  
Bacterial D1/D3 = aerobic Bacteria recovered, Day 1, Day 3 (Log10)  
ck/A = challenged with A/chicken/AL/75 (10^7.6 day 0, 10^7.9 day 1  
PI-Egg Infective Dose50/bird)  
SAW = challenged with S. aureus Woods 46 (10^7.3 day 0, 10^7.4 day 1  
PI-colony forming units/bird)  
v+b = challenge with A/chk/AL/75 and S. aureus Woods 46  
ctrl = sham inoculated with allantoic fluid and brain heart infusion broth
Table 9

Experiment 2: Viral and bacterial titers recovered from LUNGS of specific pathogen free chickens post intratracheal inoculation with A/mallard/OH/184/84/ and Staphylococcus aureus Woods 46

<table>
<thead>
<tr>
<th>Trt Grp</th>
<th>Chal</th>
<th>Repl</th>
<th>Vlrs D1</th>
<th>Vlrs D3</th>
<th>Bact D1</th>
<th>Bact D3</th>
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<tr>
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<td>&lt;1.5</td>
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<td>1.8</td>
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<td>ma/OH</td>
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<td>&lt;1.5</td>
<td>2.5</td>
<td>3.2</td>
</tr>
<tr>
<td>1</td>
<td>ma/OH</td>
<td>3</td>
<td>---</td>
<td>&lt;1.5</td>
<td>---</td>
<td>3.1</td>
</tr>
<tr>
<td>1</td>
<td>ma/OH</td>
<td>4</td>
<td>---</td>
<td>&lt;1.5</td>
<td>---</td>
<td>2.5</td>
</tr>
<tr>
<td>1</td>
<td>ma/OH</td>
<td>5</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>SAW</td>
<td>1</td>
<td>&lt; 1.5</td>
<td>&lt;1.5</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
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<td>SAW</td>
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<td>&lt; 1.5</td>
<td>&lt;1.5</td>
<td>1.3</td>
<td>1.7</td>
</tr>
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<td>2</td>
<td>SAW</td>
<td>3</td>
<td>---</td>
<td>&lt;1.5</td>
<td>---</td>
<td>3.1</td>
</tr>
<tr>
<td>2</td>
<td>SAW</td>
<td>4</td>
<td>---</td>
<td>&lt;1.5</td>
<td>---</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>SAW</td>
<td>5</td>
<td>---</td>
<td>&lt;1.5</td>
<td>---</td>
<td>5.1</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>1</td>
<td>&lt; 1.5</td>
<td>&lt;1.5</td>
<td>&lt;1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>2</td>
<td>&lt; 1.5</td>
<td>&lt;1.5</td>
<td>&lt;1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>3</td>
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<td>&lt;1.5</td>
<td>---</td>
<td>1.9</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>4</td>
<td>---</td>
<td>&lt;1.5</td>
<td>---</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>5</td>
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<td>&lt;1.5</td>
<td>---</td>
<td>5.2</td>
</tr>
<tr>
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<td>ctrl</td>
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<td>&lt;1.5</td>
<td>&lt;1.0</td>
<td>2.2</td>
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<tr>
<td>4</td>
<td>ctrl</td>
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<td>&lt;1.5</td>
<td>&lt;1.0</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>ctrl</td>
<td>3</td>
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<td>&lt;1.5</td>
<td>---</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>4</td>
<td>ctrl</td>
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<td>&lt;1.5</td>
<td>---</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>ctrl</td>
<td>5</td>
<td>---</td>
<td>&lt;1.5</td>
<td>---</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Trt Grp = Treatment Group  
Chal = virus and/or bacterial challenge  
Repl = Replication  
Vlrs D1/D3 = challenge Virus recovered, Day 1, Day 3 (Log_{10})  
Bact D1/D3 = aerobic Bacteria recovered, Day 1, Day 3 (Log_{10})  
ma/OH = challenged with A/mallard/OH/184/84/ (10^8.7 day 0, 10^9.0 day 1 PI-Egg Infective Dose_{50}/bird)  
SAW = challenged with S. aureus Woods 46 (10^{7.1} day 0, 10^{7.0} day 1 PI colony forming units/bird)  
v+b = challenge with A/ck/AL/75 and S. aureus Woods 46  
ctrl = sham inoculated with allantoic fluid and brain heart infusion broth
Table 10

Frequency of virus recovery from tracheal and cloacal swabs of specific pathogen free turkeys and chickens post intratracheal inoculation. Data from sample days pooled.

<table>
<thead>
<tr>
<th>Trt Group</th>
<th>Exp 1 tr</th>
<th>Exp 1 cl</th>
<th>Exp 2 tr</th>
<th>Exp 2 cl</th>
<th>Exp 3 tr</th>
<th>Exp 3 cl</th>
<th>Exp 4 tr</th>
<th>Exp 4 cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>virus</td>
<td>5/8(^1)</td>
<td>4/8</td>
<td>1/8</td>
<td>0/8</td>
<td>1/10</td>
<td>0/10</td>
<td>0/13</td>
<td>0/13</td>
</tr>
<tr>
<td>bact</td>
<td>1/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/10</td>
<td>0/10</td>
<td>0/13</td>
<td>0/13</td>
</tr>
<tr>
<td>v &amp; b</td>
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<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>3/10</td>
<td>0/10</td>
<td>3/13</td>
<td>0/13</td>
</tr>
<tr>
<td>cntrl</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/10</td>
<td>0/10</td>
<td>0/11</td>
<td>0/11</td>
</tr>
</tbody>
</table>

Experiment 1: A/chicken/AL/75 and *S. aureus* Woods 46
Experiment 2: A/mallard/OH/184/86 & *S. aureus* Woods 46
Experiment 3: A/turkey/WI/68 and *Vibrio algalyticus*
Experiment 4: A/turkey/WI/68 and *Vibrio algalyticus*

\(5/8^1\) = 5 positive swabs/8 birds tested
Table 11

Experiment 3: Viral and bacterial titers recovered from LUNGS of specific pathogen free turkeys post intratracheal inoculation with A/turkey/VI/68 and *Vibrio alginolyticus*.

<table>
<thead>
<tr>
<th>Trt Grp</th>
<th>Chal</th>
<th>Repl</th>
<th>Vlrs D3</th>
<th>Vlrs D5</th>
<th>Bact D3</th>
<th>Bact D5</th>
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</thead>
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<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.4</td>
<td>2.6</td>
</tr>
<tr>
<td>1</td>
<td>tv/WI</td>
<td>2</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>1</td>
<td>tv/WI</td>
<td>3</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>1</td>
<td>tv/WI</td>
<td>4</td>
<td>2.3</td>
<td>&lt; 1.5</td>
<td>---</td>
<td>2.9</td>
</tr>
<tr>
<td>1</td>
<td>tv/WI</td>
<td>5</td>
<td>7.3</td>
<td>&lt; 1.5</td>
<td>2.7</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>VA</td>
<td>1</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.1</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>VA</td>
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<td>&lt; 1.5</td>
<td>3.4</td>
<td>3.3</td>
</tr>
<tr>
<td>2</td>
<td>VA</td>
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<td>&lt; 1.5</td>
<td>3.8</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>VA</td>
<td>4</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.2</td>
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<tr>
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<td>5</td>
<td>&lt; 1.5</td>
<td>&lt; 1.5</td>
<td>3.1</td>
<td>4.6</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>1</td>
<td>&lt; 1.5</td>
<td>4.5</td>
<td>3.7</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>v+b</td>
<td>2</td>
<td>5.5</td>
<td>&lt; 1.5</td>
<td>3.5</td>
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Trt Grp = Treatment Group  
Chal = virus and/or bacterial challenge  
Repl = Replication  
Vlrs D3/D5 = challenge Virus recovered, Day 3, Day 5 (Log<sub>10</sub>)  
Bact D3/D5 = aerobic Bacteria recovered, Day 3, Day 5 (Log<sub>10</sub>)  
ty/WI = challenged with A/turkey/WI/68 (10<sup>7.9</sup> Egg Infective Dose<sub>50</sub>/bird)  
VA = challenged with *V. alginolyticus* (10<sup>7.8</sup> colony forming units/bird)  
v+b = challenge with A/turkey/WI/68 and *V. alginolyticus*  
ctrl = sham inoculated with allantoic fluid and brain heart infusion broth
Table 12

Experiment 4: Viral and bacterial titers recovered from LUNGS of specific pathogen free turkeys post intratracheal inoculation with A/turkey/WI/68 and Vibrio alginolyticus.

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<td>&lt;1.0</td>
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<td>&lt;1.5</td>
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<td>ctrl 5</td>
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<td>&lt;1.5</td>
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</tr>
</tbody>
</table>

Trt Grp = Treatment Group
Chal = virus and/or bacterial challenge
Repl = Replication
Virs D1,3,5 = challenge Virus recovered, Day 1,3,5 (Log10)
Bact D1,3,5 = aerobic Bacteria recovered, Day 1,3,5 (Log10)
tv/WI = challenged with A/turkey/WI/68 (10^4.2 Egg Infective Dose50/bird)
VA = challenged with V. alginolyticus (10^1.4 colony forming units/bird)
v+b = challenge with A/tv/WI/68 and V. alginolyticus
ctrl = sham inoculated with allantoic fluid and brain heart infusion broth
Table 13

Experiment 3

Tracheal and lung lesion scoring of SPF turkeys day 3 post intratracheal challenge with A/ty/WI/68 and/or *Vibrio alginolyticus*.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Tracheal Lesion</th>
<th>Tracheal Score</th>
<th>Lung Lesion</th>
<th>Lung Score</th>
</tr>
</thead>
<tbody>
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<td>Negative controls-sterile</td>
<td>No</td>
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<td>BHIB and AF</td>
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<td>4</td>
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<td>1</td>
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<td>Vibrio alginolyticus</td>
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<td>No</td>
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<td>A/ty/WI/68 and Vibrio alginolyticus</td>
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<td>3</td>
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</table>

BHIB = Brain Heart Infusion Broth
AF = Allantoic Fluid
1 = no lesions
2 = focal lesions
3 = multifocal lesions
4 = diffuse lesions
<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Tracheal Score</th>
<th>Tracheal Lesion</th>
<th>Lung Score</th>
<th>Lung Lesion</th>
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</thead>
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</table>

BHIB = Brain Heart Infusion Broth
AF = Allantoic Fluid
1 = no lesions
2 = focal lesions
3 = multifocal lesions
4 = diffuse lesions
Table 15

Experiment 4
Tracheal and lung lesion scoring of SPF turkeys day 1 post intratracheal challenge with A/ty/WI/68 only, *Vibrio alginolyticus* only or A/ty/WI/68 and *Vibrio alginolyticus*.

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<th>Tracheal Score</th>
<th>Lung Lesion</th>
<th>Lung Score</th>
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</table>

BHIB = Brain Heart Infusion Broth
AF = Allantoic Fluid
1 = no lesions
2 = focal lesions
3 = multifocal lesions
4 = diffuse lesions
## Table 16

**Experiment 4**

Tracheal and lung lesion scoring of SPF turkeys day 3 post intratracheal challenge with A/ty/WI/68 only, *Vibrio alginolyticus* only or A/ty/WI/68 and *Vibrio alginolyticus*.

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</table>

**BHIB** = Brain Heart Infusion Broth  
**AF** = Allantoic Fluid  
1 = no lesions  
2 = focal lesions  
3 = multifocal lesions  
4 = diffuse lesions
Table 17

Experiment 4
Tracheal and lung lesions of SPF turkeys day 5 post intratracheal challenge with A/ty/WI/68 only, *Vibrio alginolyticus* only or A/ty/WI/68 and *Vibrio alginolyticus*.

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<th>Tracheal Lesion</th>
<th>Tracheal Score</th>
<th>Lung Lesion</th>
<th>Lung Score</th>
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</table>

BHIB = Brain Heart Infusion Broth
AF = Allantoic Fluid
1 = no lesions
2 = focal lesions
3 = multifocal lesions
4 = diffuse lesions
APPENDIX B

FIGURES
Log increase in AI virus TCID vs Vibrio alginolyticus protease concentration after 72 hours incubation

Figure 1

Mean tissue culture infective dose of A/mallard/OH/184/86 and A/ty/WI/68 vs. V. alginolyticus protease concentration in cell culture media.
Figure 2

EXPERIMENTS 3 and 4:
Average tracheal lesion scores of SPF turkeys on days 1, 3 and 5 post intratracheal inoculation with:

- **C** = Control - sterile brain heart infusion broth and sterile chorioallantoic fluid.
- **V** = Virus. *A/ty/WI/68*
- **B** = Bacteria. *Vibrio alginolyticus*
- **V+B** = *A/ty/WI/68* and *Vibrio alginolyticus*

Lesion score scale
- 1 = No lesion
- 2 = Mild, focal lesions
- 3 = Multifocal lesions
- 4 = Severe diffuse lesions
EXPERIMENTS 3 and 4:
Average lung lesion scores of SPF turkeys on days 1, 3 and 5 post intratracheal inoculation with:

- **C** = Control - sterile brain heart infusion broth and sterile chorioallantoic fluid.
- **V** = Virus. A/ty/WI/68
- **B** = Bacteria. *Vibrio alginolyticus*
- **V+B** = A/ty/WI/68 and *Vibrio alginolyticus*

Lesion score scale
- 1 = No lesion
- 2 = Mild, focal lesions
- 3 = Multifocal lesions
- 4 = Severe diffuse lesions
PLATE I

Standard Methods Caseinate agar plate streaked with a tracheal swab of a 56 week old leghorn hen from a research flock 48 hours after incubation at 37°C. Majority of isolates are highly proteolytic bacteria.
PLATE II
Agar gel diffusion plate. Control wells loaded with 15 ul of 5, 10, 15 and 20 μg/ml purified bovine pancreatic trypsin in 0.05 M Tris-HCl, 0.05 M CaCl₂, pH 7.5. Well 1 is a positive well containing 15 μl of an 18 hour Brain Heart Infusion Broth culture of Vibrio alginolyticus.
PLATE III

Normal ciliated tracheal epithelium with mucosal mucous gland.
Specific pathogen free (SPF) turkey control. (H&E stain, 750X).
PLATE IV
Severe tracheitis characterized by numerous luminal heterophils (arrows), epithelial hyperplasia with loss of orientation of cilia. Submucosal infiltration of heterophils, lymphocytes and macrophages. Specific pathogen free turkey three days post intratracheal challenge with A/ty/WI/68 and Vibrio alginolyticus. (H+E stain, 750X).
PLATE V

Inflammation of primary bronchus with peribronchial pneumonia. Specific pathogen free turkey three days post intratracheal challenge with A/ty/VI/68 and Vibrio alginolyticus. (H+E stain, 45X).
PLATE VI

Necrosis in air capillary with fibrin and necrotic inflammatory cells. Specific pathogen free turkey three days post intratracheal challenge with A/ty/WI/68 and *Vibrio alginolyticus*. (H+E stain, 750X).
PLATE VII
Fibrinocellular bronchitis within tertiary bronchus. Specific pathogen free turkey three days post intratracheal challenge with A/ty/WI/68 and *Vibrio alginolyticus*. (H+E stain, 750X).
APPENDIX D

ISOLATION OF PROTEOLYTIC AND HIGHLY PROTEOLYTIC BACTERIA
ISOLATION OF PROTEOLYTIC AND HIGHLY PROTEOLYTIC BACTERIA

Proteolytic bacteria can be isolated from tracheal swabs using standard methods caseinate agar plates as described below.

MATERIALS

STANDARD METHODS CASEINATE AGAR

1. Add 1.1025 g Sodium Citrate to 250 ml of ddH₂O.

2. Dissolve 5.9 g Standard Methods agar in 125 ml of citrate solution. Place in boiling water bath for 10 minutes to aid dispersion.

3. Dissolve 2.5 g of sodium caseinate in the other 125 ml of citrate solution and also place in the boiling water bath.

4. Combine the two solutions and autoclave at 121°C for 15 minutes.

5. Also prepare CaCl₂ solution by adding 7.35 g CaCl₂·2H₂O to 50 ml of ddH₂O and autoclave at 121°C for 15 minutes.

6. Before pouring plates, add 5 ml of CaCl₂ solution to molten Caseinate Agar.

PROTOCOL

1. Prepare Standard Methods Caseinate Agar (SMCA).

2. Streak the swab being tested onto a SMCA plate so that single colonies will be isolated.

OR

3. Plate 0.1 ml quantities of sample (i.e. swab in transparent medium) and spread evenly with a sterile bent-glass rod. To insure absorption of the sample, allow incubated plates to dry for 15 minutes and incubate plates for 48-72 hours at 37°C.
4. Colonies surrounded by a white or off-white zone of casein precipitate are proteolytic. Highly proteolytic bacteria will also produce a clear zone (Frank, 1985).
APPENDIX E

PURIFICATION OF BACTERIAL EXOPROTEASES
PURIFICATION OF BACTERIAL EXOPROTEASES

The following technique is used to purify exoproteases produced by proteolytic bacteria (Tashiro, 1987a; Drapeau, 1972).

**MATERIALS**

Bacterial Protease Broth

Add the following to 1000 ml of ddH$_2$O:
- 2.4g K$_2$HPO$_4$
- 0.4g NaH$_2$PO$_4$
- 0.2g MgSO$_4$·7H$_2$O
- 5.0g Yeast Extract
- 7.5g B-glycerophosphate
- 15g Casein
- 10mg MnCl$_2$
- 6.3mg FeSO$_4$
- 1 stir bar

CaCl$_2$ solution. In a separate small bottle, add 0.74g CaCl$_2$ and 10 ml of ddH$_2$O, mix until dissolved. Autoclave both suspensions at 121°C for 30 minutes.

Add the sterile CaCl$_2$ solution to the Bacterial Protease Broth just before use.

**ADDITIONAL MATERIALS**

- Frozen stock vial of bacteria
- .1 g Centrimide
- 4-250 ml polypropylene centrifuge tubes
- 600 g Ammonium Sulfate
- 10 mM Tris HCl buffer
- 175 ml Cold Acetone (-15°C)
- 25 cm DEAE Cellulose Column
- 1 M KCl solution

**PROTOCOL**

1. Thaw frozen stock vial of bacteria by submerging in lukewarm water.
2. Resuspend 1.0 ml of thawed bacterial suspension in 15 ml of Bacterial Protease Broth and incubate at 37°C for 6-7 hours.

3. Inoculate 1000 ml of Bacterial Protease Broth with the 15 ml pre-incubated suspension. Incubate the inoculated bacterial protease broth with slow stirring at 37°C for 18 hours.

4. At the end of this incubation period, add centrimide to achieve a 1:10,000 concentration (.1 g/1000 ml). Continue incubation with stirring for one hour, then chill suspension in an ice bath to approximately 4°C.

   NOTE: All remaining steps must be carried out at 4°C.

5. Centrifuge the suspension (pre-chill Beckman) at 9000 g (7000 rpm Beckman J2 21 with J5 7.5 rotor) for 30 minutes to remove bacterial cells. Discard pellet.

6. Precipitate the proteins in the supernatant by slowly adding pulverized solid ammonium sulfate while stirring, to achieve a 60% w/v concentration (600 g ammonium sulfate/1000 ml supernatant). After last amount of ammonium sulfate is added, continue stirring for 1 hour.

   NOTE: Ammonium sulfate precipitate either before centrifugation or as a pellet is a good form to keep sample in, i.e. it can be left overnight.

7. Centrifuge ammonium sulfate suspension for 30 min. at 9000 g. Pour off supernatant (discard). Resuspend remaining pellet in 100 ml of 10 mM Tris HCl buffer, pH 7.5 with 2 mM CaCl₂. Protease will be in supernatant. Centrifuge at 9000 g for 30 min., discard pellet and save supernatant.

8. Slowly add 125 ml of cold (-15°C) acetone to Tris Buffer supernatant while stirring. Centrifuge immediately at 9000 g for 20 min.

9. Pour off supernatant. Resuspend the pellet in < 50 ml of 50 mM Tris HCl pH 7.5. Centrifuge 10 min at 16,000 rpm. Discard pellet.

10. Dialyze supernatant for 4 hours against weak Tris HCl Buffer (1:20 dilution of 10 mM Tris HCl 2 mM CaCl₂ buffer). Dialysis conditions = ≥ 500 ml buffer, 4°C with stirring, change buffer after 2 hours.
11. Apply sample (25 ml maximum) to prepared 25 cm DEAE Cellulose ion exchange column (flow 2.5 ml/min paper speed 3.0 cm/min 2V) which was equilibrated with 50 mM Tris-HCl buffer.

12. Use fractionator to monitor protein concentration and collect all fractions.

13. Initiate elution of sample buffer (10 mM Tris HCl, 2 mM CaCl₂, pH 7.5). Continue elution until first peak comes back down to zero.

14. Begin gradient 0.6 M KCl. Continue elution with gradient 0.6 M KCl until second peak comes back down. (Total gradient volume = 80 ml 0.6 M KCl, 80 ml ddH₂O).

15. After gradient volume has been applied to the column, continue elution with straight 0.6 M KCl until any residual peaks come down. Place eluted fractions on ice. (Clean DEAE column with 1 M KCl after each use).

16. Determine activity (APPENDIX F) and protein content (Bradford, 1976) of eluted fractions. Combine and freeze (-70°C) fractions with the greatest activity.
APPENDIX F

EVALUATION OF ACTIVITY OF PURIFIED PROTEASES
EVALUATION OF ACTIVITY OF PURIFIED PROTEASES

MATERIALS

1% CASEIN SUBSTRATE IN 0.5M TRIS HCl BUFFER

1. Add 39.4 g of Tris HCl to 500 ml of distilled H2O. Stir until dissolved.

2. Add 11 NaOH pellets to the Tris HCl suspension and stir until dissolved. Adjust pH of the mixture to 7.5 with 1N NaOH or 1N HCl.

3. Add 5 g of sodium caseinate to the buffer. Mix. Heat the mixture in a boiling waterbath for 15 min.

4. Remove the suspension from the boiling waterbath and cool on counter top. Store at 4°C. This substrate has a shelf life of 2 weeks.

ADDITIONAL MATERIALS

10% (w/v) Trichloroacetic Acid in ddH2O
10 x 100 ml Test Tubes (clean)
Sigma Bovine Pancreatic Trypsin--Stock Dilution
10 mm Tris HCl 2mM CaCl2 Buffer (pH 7.5 at 4°C).

PROTOCOL

1. Prepare 500 ml of 1% Sodium Caseinate substrate and 500 ml of 10% Trichloroacetic Acid.

2. Prepare trypsin standards as follows:

Reference trypsin is crystallized Bovine Pancreatic Trypsin, TPCK treated, Sigma T-8642.

Stock Trypsin = Sigma 100 ug/ml in 10 mM Tris HCl + 2 mM CaCl2 buffer, pH 7.5 at 4°C. (This concentration of stock trypsin is stored in -70°C freezer).
Prepare 500 ul of each trypsin standard concentration as follows:

**PREPARATION OF TRYPSIN STANDARD CONCENTRATIONS**

<table>
<thead>
<tr>
<th>ul of trypsin stock in .2ml</th>
<th>ul of buffer</th>
<th>ul buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>470</td>
<td>12</td>
</tr>
<tr>
<td>60</td>
<td>440</td>
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<tr>
<td>90</td>
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<td>150</td>
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<td>180</td>
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<td>108</td>
</tr>
<tr>
<td>300</td>
<td>200</td>
<td>120</td>
</tr>
</tbody>
</table>

3. Add 4 ml of 1% sodium caseinate to 22 tubes (for blank control and trypsin standards) and 2 times the number of unknown protease (e.g. if testing 2 proteases, prepare 22 + 4 = 26 4 ml sodium caseinate tubes). Place in 37°C incubator to pre-warm the tubes— at least 5 minutes.

4. Add 0.2 ml of each standard trypsin concentration to pre-warmed 4 ml tube of 1% sodium caseinate. Do this in duplicate.

5. Add 0.2 ml of 10 mM Tris HCl 2 mM CaCl₂ buffer to each of two 1% sodium caseinate tubes—these are blank controls.

6. Add 0.2 ml of each Protease to be tested to each of two 1% sodium caseinate tubes.

7. Continue incubation of all tubes at 37 degrees celsius for 30 minutes.

8. At the end of 30 minutes, stop reaction immediately by adding 3 ml 10% Trichloroacetic acid to all tubes.

9. Let sit for 15 minutes.

10. Filter all precipitated caseinate suspensions through Whatman No. 1 filters. Save filtrate.

11. Calibrate the spectrophotometer against the buffer blank filtrates at a wavelength of 280 nm. Read and record O.D.₂₈₀.
for all standards and protease filtrates.

12. Prepare a standard graph of O.D.\textsubscript{280} vs. [Trypsin]. Compare the activity of the unknown protease against the prepared standard graph.
APPENDIX G

SDS-PAGE ELECROPHORESIS
Materials

ELECTROPHORESIS RUNNING BUFFER

Tris Base 13.62 g
Glysine 64.86 g
SDS 4.5 g
ddH₂O 4.5 L

The pH of the buffer should be around pH 8.3. If the pH is much lower, the buffer should be discarded and a new buffer should be made. Do not adjust the pH using acids or bases. Store at 4°C. This buffer may be used 3-4 time before discarding.

SAMPLE BUFFER or LYSIS BUFFER STOCK SOLUTION

ddH₂O 4.0 ml
0.5 M Tris-HCl, pH 6.8 1.0 ml
Glycerol 0.8 ml
10% (w/v) SDS 0.4 ml
0.05% (w/v) bromophenol blue 0.2 ml

Add 2-mercaptoethanol 4.0 ul to every 76 ul stock solution prior to use

Store solution at 4°C. Before use, remove from refrigerator and warm to 37°C. Mix by inverting until SDS is dissolved. Add 2-mercaptoethanol to the aliquot to be used immediately.

ACRYLAMIDE STOCK SOLUTION BIS (30% T, 2.6% C)

Acrylamide 29.2 g
N’N’-BIS methylene acrylamide 0.8 g
ddH₂O <100 ml

Mix the ingredients using a stir bar. q.s. to 100 ml with ddH₂O. Filter through a Whatman No. 1. paper. Cover bottle with aluminum foil. NOTE: Wear gloves when handing acrylamide.
SEPARATION GEL TRIS SOLUTION, 1.5 M Tris-HCl, pH 8.8

Tris base

ddH₂O

Conc. HCl

18.15 g

<100 ml

to adjust pH

Dissolve Tris in distilled H₂O, adjust the pH with concentrated HCl. q.s. to 100 ml using ddH₂O. Store at 4°C.

STACKING GEL TRIS SOLUTION, 0.5 M Tris-HCl, pH 6.8

Tris base

ddH₂O

Conc. HCl

6.05 g

<100 ml

to adjust pH

Dissolve Tris in H₂O, adjust the pH with concentrated HCl. q.s. to 100 ml using ddH₂O. Store at 4°C.

10% (W/V) SDS

SDS

ddH₂O

2.0 g

20.0 ml

Dissolve SDS in ddH₂O, store at 4°C. Warm to 37°C to re-dissolve SDS before using.

COOMASSIE BLUE GEL STAIN

Commassie Brilliant Blue R-250

Methanol (40%)

Acetic Acid, glacial

ddH₂O

0.45 g

240 ml

36 ml

324 ml

Add Commassie Blue to ddH₂O. Add acetic acid and methanol. Stir to dissolve the dye.

DESTAINING SOLUTION, 10% acetic acid

Acetic Acid, glacial

ddH₂O

60 ml

540 ml

Add acetic acid to premeasured ddH₂O.
**SDS-PAGE PROTOCOL**

The preparation of a discontinuous gel consisting of a 4% acrylamide stacking gel and a 12% acrylamide separating gel is described. Preparations for two gels are given. Divide by 2 if only 1 gel is desired (Sambrook, 1989).

1. Wash 16x18 cm glass plates with tap water, then 10% acetic acid. Rinse with ddH₂O. Wash all parts of the gel electrophoresis apparatus with tap water.

2. Assemble casting stand as described by manufacturer.

3. Prepare separating gel. (14 cm x 14 cm x 0.75 mm gels)
   a. Preliminary steps: Make fresh solution of ammonium persulfate solution by adding 0.1 g ammonium persulfate to 1.0 ml ddH₂O, dissolve. Redissolve 10% SDS by warming to 37°C.
   b. Pour the following ingredients into a clean vacuum flask, in the order in which they are listed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>13.5 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>Acrylamide: BIS (30% T, 2.6% C)</td>
<td>16.0 ml</td>
</tr>
</tbody>
</table>

   c. Degas mixture for 30 minutes by hooking to vacuum source. Then add:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEMED</td>
<td>40 ul</td>
</tr>
<tr>
<td>10% ammonium persulfate-fresh</td>
<td>200 ul</td>
</tr>
</tbody>
</table>

   d. Swirl gently. Immediately (< 5 minutes) fill each gel sandwich with 14-16 ml of this mixture. Gently stream 2 ml of ddH₂O to solution surface and rock apparatus stand to aid in giving gel even surface. Allow gel to polymerize 1 hour.

4. Prepare Stacking Gel (4% acrylamide)
a. Pipet the following ingredients into a clean vacuum flask.

\[
\begin{align*}
\text{ddH}_2\text{O} & \quad 6.1 \text{ ml} \\
0.5 \text{ Tris-Cl, pH 6.8} & \quad 2.5 \text{ ml} \\
10 \% (w/v) \text{ SDS} & \quad 0.1 \text{ ml} \\
\text{Acrylamide: BIS (30\% T, 2.6\% C)} & \quad 1.3 \text{ ml}
\end{align*}
\]

b. Degas the gel solution for 15 minutes. Pour the overlaying H\textsubscript{2}O of the separating gel. Place selected comb above glass plates in a tilted position so air bubbles can escape as the gel is being poured. Add the following to the degassed solution.

\[
\begin{align*}
\text{TEMED} & \quad 15 \text{ ul} \\
10\% \text{ ammonium persulfate} & \quad 100 \text{ ul}
\end{align*}
\]

c. Swirl gently after adding each solution. Lower the comb as the stacking gel solution is added to the separating gel. Allow gel to polymerize for 2.5-3 hours.

d. Remove comb and add electrophoresis running buffer. Store at 4°C.

5. Sample Preparation

a. Dilute sample 1/4 or 1/5 with Sample (Lysis) Buffer in a microtube (desired final volume=35-40ul).

b. Add 4 ul of Low and High molecular weight markers to 30 ul sample buffer.

c. Cap tightly and place all samples and MW markers in boiling H\textsubscript{2}O.

d. Centrifuge tubes in microfuge for 1 minute.

e. Carefully apply sample to bottom of wells. Record sample lanes.

6. Assemble the gel electrophoresis according to manufacturers directions.

a. Display should read "3000 V, 300mA 400 W". Current "15 mA" should be used for one gel and "30 mA" should be used for two gels. The dye should take 45 to 1 hour to pass through the stacking gel. The mA setting should be increased to "20 mA" when the dye is in the separating gel. It should take 6-8 hours to for the dye to run through the separating gel.

b. Remove acrylamide gel from the gel sandwich into a glass pan using a squirt bottle of distilled water. Drain the water from the gel.

8. Staining the Acrylamide Gel

a. Pour enough coomassie blue R 250 staining solution to cover the gel. Cover glass pan with foil and stain for 30 to 60 minutes.

b. Discard the staining solution. Rinse gel with 10% acetic acid. Change rinse solution several times until gel has destained sufficiently.

c. Gel can be photographed for permanent records and/or dried using the BIO Design, Inc. Bio Gel Wrap Drying System.
List of References


41. Jeffery, J. Plausing properties of Waterfowl Origin Type A Influenza Viruses of Chicken, Duck, and Turkey Primary Cell Cultures. The Ohio State University, Department of Poultry Science, Master of Science, Thesis. 1990.


