Comparison of *in vivo* and *in vitro* methods for determining the pathogenicity of H5N2 avian influenza viruses isolated from chickens during the 1983/1984 epizootic in Pennsylvania

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Avian influenza (AI) is a viral disease which can affect the respiratory, enteric and reproductive tracts of many species of domestic poultry and wild birds (Easterday and Beard, 1984). The clinical disease can vary from inapparent, to an acute disease, characterized by high morbidity and mortality. The seriousness of AI infections in poultry is reflected in the fact that many countries have adopted regulations which require depopulation of birds infected with the highly pathogenic form of the disease. One critical element in developing an effective control program is the availability of rapid and accurate laboratory procedures for the isolation, identification and assessment of the pathogenic potential of the etiological agent.

In 1983/1984 a serious outbreak of AI occurred in poultry in Pennsylvania, New Jersey, Virginia, and Maryland. Attempts to control and eradicate the disease resulted in expenditures in excess of $63 million; one of the most expensive animal disease control efforts in the history of the United States. A total of 448 flocks were infected with the virus and 17 million birds died or were destroyed as a result of the eradication campaign (Animal and Plant Health Inspection Service, 1984). This outbreak was unique in that the nonpathogenic H5N2 AI virus which first appeared in April

became highly pathogenic in October, 1983. Viruses which were isolated from affected flocks were inoculated into chickens to distinguish between viruses of high and low pathogenicity. The pathogenicity testing of isolates did not always correlate well with the clinical signs observed in the field (Utterback, 1984; Fichtner, 1984), and repeated inoculations of chickens frequently produced inconsistent results (Senne et al., 1987).

Recent advances in the understanding of the molecular basis of pathogenicity of influenza viruses have indicated that *in vitro* correlates of pathogenicity may be important in evaluating the pathogenic potential of these viruses (Bosch et al., 1979; Rott, 1981; Kawaoka et al., 1984, 1987). These findings, as well as the problems and inconsistencies relating to pathogenicity testing of AI viruses isolated during the 1983/1984 AI outbreak in Pennsylvania, provided the incentive to evaluate *in vitro* methods to determine the pathogenicity of these viruses. This study was therefore initiated to compare various *in vivo* and *in vitro* assays with the idea that one or more of these methods might provide a rapid and reliable test for the evaluation of the pathogenic potential of isolates of influenza A virus from poultry.

LITERATURE REVIEW

History

Influenza is caused by any one of several type A influenza viruses belonging to the family Orthomyxoviridae. Countless isolations of influenza A viruses, representing several subtypes, have been obtained from many species of mammals and birds. Reports of influenza-like disease in humans date back as far as 412 B.C. when Hippocrates, the father of medicine, recorded such an occurrence (cited by Kaplan and Webster, 1977). The first well-recorded pandemic of human influenza, however, did not occur until 1580. Numerous other pandemics in humans have also been recorded throughout history, although none so severe as the one which occurred in 1918/1919, where it has been estimated that 20 million people died worldwide from the disease (Kaplan and Webster, 1977).

Influenza virus infections in poultry have been recognized, worldwide, for over a century. The highly pathogenic form of the disease, known as "fowl plague" (fowl pest), was first described by Perroncito in a chicken flock in Italy in 1878 (cited by Stubbs, 1965). The disease reportedly raged severely in northern Italy in 1894 and from there spread into Germany, and later into Belgium and France (Mohler, 1926). In Germany, in 1901, the disease was quickly

and widely disseminated by the Brunswick Fowl Exposition and has often been referred to as the Brunswick fowl disease (Stubbs, 1926). Outbreaks of fowl plague (FP) were reportedly widespread throughout the world during the first third of this century, with reports of the disease in Austria, Hungary, Switzerland, France, Belgium, Holland, England, USA, Egypt, China, Japan, Argentina and Brazil. The disease was enzootic in Italy and Germany until the 1930s, after which it became sporadic and occurred primarily in the Middle East.

The first report of FP in North America was in 1924 when severe losses among poultry in the New York City market occurred in the fall of that year (Krohn, 1925). The 1924/1925 outbreak was presumed to have been caused by virus which had escaped from a large eastern institute where research on filterable viruses was being conducted (Mohler, 1926). Several vials of the virus were reportedly brought into the United States (U.S.) in September of 1923, from France. Clinical cases of the disease were reported from 9 northeastern states including Pennsylvania (Stubbs, 1925), New York (Brunett, 1925), New Jersey (Beaudette, 1925), Connecticut, Indiana (Julien, 1925), Michigan (Johnson, 1925), West Virginia, Missouri (Mohler, 1926), and Illinois (Boughton and Tunnicliff, 1925). Eradication was

accomplished using improved sanitation procedures and a strict prohibition on movement of all poultry. The disease reappeared again in 1929, in New Jersey, but was quickly eradicated before the disease had a chance to spread significantly (Beaudette et al., 1934).

Influenza in Chickens

Since 1955, there have been very few reports of influenza infections in chickens when compared to infections in turkeys. Only four occurrences of AI in chickens in the United States have been reported since the presence of fowl plague-like disease in 1929. Chickens were infected in Alabama in 1975 with subtype H4N8 (Johnson and Maxfield, 1976; Johnson et al., 1977); in Minnesota in 1978 with subtype H6N1 (Halvorson et al., 1980); in Pennsylvania in 1983/1984 with subtype H5N2 (Buisch et al., 1984); and in 1986/1987 in New York, New Jersey, Massachusetts, and Ohio with subtype H5N2 (Garnett, 1987). Two outbreaks were associated with significant mortality in the field; Alabama and Pennsylvania (1983/1984). Attempts to experimentally reproduce the clinical disease with the AI virus isolated from the Alabama outbreak were unsuccessful. Isolates from the 1983/1984 Pennsylvania outbreak were highly variable in their ability to cause clinical disease, in that both highly pathogenic and nonpathogenic viruses were isolated (Pearson

et al., 1987). Isolates obtained from the 1986/1987 outbreak have been characterized as being nonpathogenic or low pathogenic in nature.

During the last 30 years, there have also been a few scattered reports on isolations of AI viruses from chickens in other countries: Scotland in 1959 (Pereira et al., 1965); Italy in 1966 and 1980 (Petek, 1981); Australia in 1975 and 1985 (Turner, 1976; Cross, 1987); Hong Kong (Shortridge et al., 1979; Shortridge, 1980); Belgium (Meulemans et al., 1980); France (Bennejean, 1981); and Israel in 1980 (Lipkind et al., 1981). Only the isolates from Scotland and Australia were demonstrated to be highly pathogenic in nature.

Influenza in Turkeys

Influenza in turkeys, in contrast to influenza in chickens, has recently become a major poultry disease problem, especially in countries where there is a turkey industry, namely, in the U.S. and Canada. Outbreaks of influenza in turkeys in the U.S. have been reported from at least 20 states since the first isolation from this species in 1964 (Bankowski, 1986). Avian influenza (AI) has been a serious problem in Minnesota where outbreaks in turkeys have occurred every year since 1966 (Bahl et al., 1979; Halvorson, et al., 1987). Extensive outbreaks occurred in 1978 and 1979, when more than 140 flocks and 2,000,000 birds were

affected (Bahl et al., 1979). The economic loss to the Minnesota turkey industry from AI was estimated to have been 4.2, 1.2, and 1.3 million dollars, in 1978, 1979, and 1980, respectively (Poss, 1981). Influenza virus infections of turkeys in Canada were prevalent during the years from 1960 to 1970 (Lang et al., 1965, 1968a, 1968b; Rouse et al., 1971; Pereira et al., 1966; Lang, 1981), but since 1970 there has been a considerable reduction in the incidence of AI in Canada, primarily as a result of avoiding contact between domestic turkeys and wild birds (Lang and Ferguson, 1981; Lang, 1981). Outbreaks have also been reported in Great Britain since 1963 (Allan et al., 1970; Madeley et al., 1971; Kendal et al., 1971; Alexander et al., 1979; Alexander, 1980, 1981, 1982; Alexander and Spackman, 1981; Alexander and Allan, 1982), in Italy since 1966 (Pereira et al., 1967; Franciosi et al., 1981; Petek, 1981), and in Israel (Weisman et al., 1987).

Epizootiology of Influenza A Viruses

Avian influenza, along with foot and mouth disease and African horse sickness, was one of the first diseases present at the turn of the century which was demonstrated to be caused by a filterable agent (Centanni, cited by Stubbs, 1965). Although at the time it was suspected the disease was caused by a virus, it was not until 1930 that the specific

viral etiology of influenza was identified, when Shope made the first virus isolation from swine (Shope, 1931). Three years later, influenza virus was finally isolated from man (Smith et al., 1933), despite earlier desperate attempts to find the etiological agent of the 1918/1919 pandemic of influenza-like disease in humans. In 1955, FP virus was first shown to share a common antigen with other influenza A viruses (Schäfer, 1955). In 1956, type A influenza viruses were found to be causing respiratory disease in ducks in England and Czechoslovakia (Pereira et al., 1965; Koppel 1956, cited by Easterday and Beard, 1984; Roberts, 1964; Blaškovič et al., 1959), and another type A virus was found to be responsible for acute respiratory disease in horses in Czechoslovakia (Sovinová et al., 1958). Within a few years, several avirulent viruses were isolated from avian species that were clinically different from FP, but shared common antigens with FP and other influenza A viruses. These and other reports served to set the stage for future work on what appeared to be a very complex ecological relationship involving the influenza viruses; and so the era of avian influenza had begun.

Much of the present knowledge of the ecology of AI viruses has been the result of extensive surveillance studies conducted during the 1970s. From these studies, waterfowl

have emerged as a significant reservoir of influenza viruses in nature, since representatives of all subtypes have been isolated from these species (Hinshaw et al., 1981). Reports of isolations of influenza viruses have also been made from pelagic birds (Becker, 1966; Downie and Laver, 1973); pet birds (Slemons et al., 1973a, 1973b; Alexander, 1982; Senne et al., 1983); seals (Webster et al., 1981); and from whales (Lvov et al., 1978). The presence of these vast pools of viruses in nature have stimulated a great deal of interest to determine the potential threat these viruses might pose to other species, including man.

Etiology

Classification

Identification and classification of influenza viruses isolated from avian and mammalian species were greatly enhanced by the discovery of the hemagglutination phenomenon by Hirst (1941), and the publication of the basic hemagglutination and hemagglutination-inhibition (HI) procedures by Salk (1944). During the next twenty-seven years, a series of discoveries and experiments pertaining to the identification of internal and surface antigens, culminated in the establishment of standard laboratory methods in 1971 when a World Health Organization Expert Committee published "A Revised System of Nomenclature for

Influenza Viruses" (WHO Report, 1971). The classification system was revised and simplified in 1980 (WHO Report, 1980) to reflect work which demonstrated antigenic and genetic similarities between viruses isolated from different species. This system divided influenza viruses into types A, B, or C based on the antigenic character of the internal ribonucleoprotein (RNP) antigen or the matrix (M) protein antigen. Both these antigens are regarded as type specific, i.e., they are common to all strains of influenza viruses of the same type (Schild, 1972; Dowdle et al., 1974). Only type A viruses have been isolated from avian species. Types B and C influenza have been recognized only in humans.

Influenza A viruses are further divided into subtypes on the basis of two surface antigens; the hemagglutinin (HA) and neuraminidase (N). At present, 13 HA subtypes, designated H1 through H13, and 9 N subtypes, designated N1 through N9, have been recognized. The heterogeneity of influenza viruses is exemplified by the fact that each virus possesses one HA and one N subtype which apparently can occur in any combination.

The WHO Expert Committee has also established rules for the nomenclature of influenza virus strains, in addition to the antigenic subtype nomenclature. Each new strain of influenza should contain the following information: 1) the antigenic type (A, B, or C); 2) host of origin (this is

omitted for man); 3) geographical location; 4) individual strain reference number; 5) year of isolation; and 6) for influenza A viruses, the H and N subtype. An example of this system for the prototype strain of highly pathogenic H5N2 virus isolated from chickens in Pennsylvania, in 1983, would be: A/Chicken/Pennsylvania/1370/83 (H5N2).

Properties of the virus

The influenza virus belongs to the family Orthomyxoviridae and are commonly referred to as myxoviruses. The term myxovirus was coined by Andrewes to denote the affinity of the virus for mucus in the form of mucopolysaccharides and glycoproteins (Andrewes et al., 1955). The influenza virus is highly pleomorphic and can vary in size from 80 to 120 nm and exhibit both spherical and filamentous forms. The helical nucleocapsid is enclosed within a protein matrix which is surrounded by a host derived lipid membrane covered with HA and N glycoprotein spikes (Figure 1). The virus genome consists of eight single-stranded segments (negative sense) which are transcribed into messenger RNA under the direction of a virus-associated transcriptase.

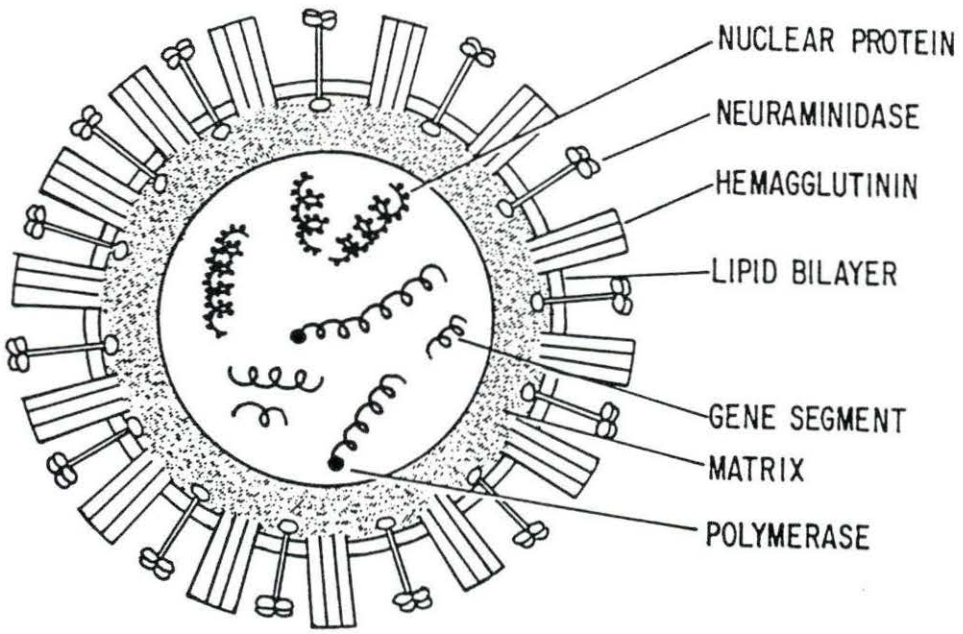


Figure 1. A diagram of the structure of influenza A virus. The hemagglutinin (HA) and neuraminidase (N) spikes are embedded through the lipid bilayer which is lined on the underside by the matrix protein. The matrix protein surrounds the core of the virus particle. Within the core, there are eight segments of single-stranded genome RNA which are associated with the nucleoprotein forming a helical structure. The polymerase has RNA-dependent RNA polymerase activity which is necessary to initiate transcription

The genes of influenza virus The genome of influenza viruses is comprised of eight segments. The segments vary in length from approximately 850 to 2400 nucleotides (depending on the virus strain) which code for at least ten proteins. Seven of these proteins are structural proteins. Details of the gene segments and their coding assignments have been summarized by Air and Laver (1986) for the A/PR/8/34 influenza virus (Table 1).

The hemagglutinin of influenza virus The surface glycoproteins of influenza viruses are responsible for many of the biological functions of the virus. The HA, for example, is involved in attachment of the virus to cells, penetration of the viral components into the host cell, and in immunity (antibodies to the HA are the protective antibodies). It accounts for approximately 25% of the viral protein in the virus and is coded by the fourth largest RNA segment. The HA is synthesized as a single polypeptide chain with a molecular weight (MW) of approximately 80 kilodaltons (Kd). The HA is glycosylated and transported across the plasma membrane in intact form, but remains anchored to the plasma membrane by a sequence of hydrophobic amino acids at the carboxyl end of the molecule. Once the HA is inserted into the plasma membrane, the molecule must undergo post-

Table 1. RNA gene segments and coding assignments for human influenza virus A/PR/8/34[a]

Gene Segment	Gene length (nucleotides)	Protein encoded	Protein length (amino acids)	Approx. no. of molecules per virion	Function
1	2,341	PB2	759	30-60	RNA transcription.
2	2,341	PB1	757	30-60	RNA transcription.
3	2,233	PA	716	30-60	RNA transcription.
4	1,778	HA	566	500	Receptor-binding and fusion activity; Major antigenic surface glycoprotein.
5	1,565	NP	498	1000	Part of the ribonucleoprotein complex; structural component of RNA transcriptase; type-specific antigen.
6	1,413	NA	454	100	Neuraminidase activity; antigenic surface glycoprotein.
7	1,027	M	252	3000	Major structural protein of virus; type-specific antigen.
		M2	96		Nonstructural protein; function unknown.
8	890	NS1	230		Nonstructural protein; function unknown.
		NS2	121		Nonstructural protein; function unknown.
Total	13,588				

[a]Air and Laver (1986).

translational cleavage into disulfide-bonded HA1 (50 Kd) and HA2 (30 Kd) subunits for the virus particle to be infectious. The cleavage of the HA is thought to be necessary to facilitate fusion of the virus to the endosomal membrane following attachment and uptake of the virus by the cell, thus initiating infection. The mechanism of fusion is poorly understood at present. However, previous studies have indicated the HA undergoes a conformational change at a pH of approximately 5.5, resulting in the expression of the previously buried fusion protein (N-terminus of HA2) at the surface of the HA molecule (Figure 2). Noninfectious virus can be converted into infectious virus by *in vitro* treatment with trypsin or trypsin-like enzymes to facilitate cleavage of the HA (Klenk et al., 1975; Lazarowitz and Choppin, 1975).

The HA spike is divided into two distinct regions comprised of a long fibrous region (stalk), containing residues from both HA1 and HA2, and a globular region which sits on top of the fibrous region and contains residues entirely from HA1 (Wilson et al., 1981) (Figure 2). The HA glycoprotein spike of the virus is formed as a trimer of three disulfide-bonded HA1 and HA2 subunits. Antibody binding sites on the HA have been determined (Wiley et al., 1981) (Figure 2) and the HA molecule of several type A influenza viruses have been sequenced (Ward, 1981; Skehel et

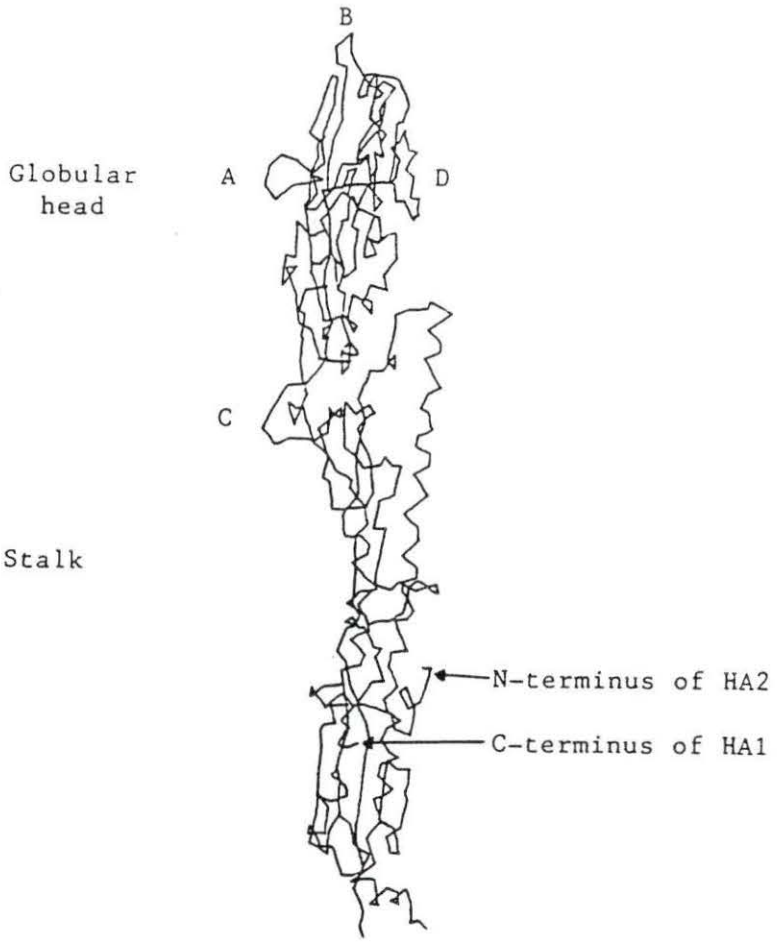


Figure 2. α-carbon drawing of the A/Hong Kong/68 HA monomer (Wilson et al., 1981) showing the globular head and stalk. Cleavage of this molecule results in the formation of the C-terminus of HA1 and the N-terminus (fusion peptide) of HA2. The location of antibody binding sites are designated as A, B, C, and D (Wiley et al., 1981)

al., 1983; Both et al., 1983; Kawaoka et al., 1984, 1987). The percent homology between subtypes of influenza A viruses is generally lower (24% to 80%) than viruses within a particular subtype (90% or greater).

The neuraminidase of influenza virus The second surface glycoprotein of influenza viruses, the neuraminidase, has enzymatic activity which permits transport of the virus through mucin and destroys the HA receptor (Markoff and Lai, 1982) on host cells, thus allowing elution of progeny virus particles from infected cells. The N also probably plays a role in preventing self-aggregation of newly synthesized virus by removing the terminal sialic acid residues from the carbohydrate moiety of the HA and N (Palese et al., 1974). The N spike consists of four coplanar spherical subunits which sit on top of a centrally attached stalk and has a total molecular weight of 240 Kd (Figure 1). Unlike the HA molecule, the N is attached to the virus membrane by the trans-membrane segment at the amino-terminus.

Other proteins of influenza virus The remaining five proteins which are coded by the virus are the nucleoprotein (NP), matrix (M) protein, and three polymerases (P), designated PA, PB1, and PB2. The nucleoprotein and the matrix protein are structural proteins which have been highly conserved and are both common to all viruses of the same type

(A, B, or C). The polymerases are involved in the initiation of virus transcription (Krug, 1983). In addition to the structural proteins, there are at least three virus-coded nonstructural proteins (M2, NS1, and NS2) which can be found in infected cells, but which are not incorporated into the virus.

Antigenic drift and shift Influenza viruses have demonstrated a tremendous capability of undergoing antigenic variation. Two distinct kinds of antigenic variation have been recognized: antigenic drift and major antigenic shift. Both the HA and N glycoproteins can undergo these changes independently. Antigenic drift in influenza A viruses is the result of minor changes in the structure of the surface proteins caused by point mutations in the viral genome. These changes may result in the appearance of antigenic variants, which are common during epidemics of influenza, or they may have a more significant consequence, as was the case when a point mutation in the HA was believed to have been the cause of the conversion of the avirulent H5N2 virus in chickens in Pennsylvania in 1983 into a highly pathogenic virus (Kawaoka et al., 1984). Antigenic shift, on the other hand, is the result of substantial alterations of the chemical structure of the protein as a result of the replacement of the entire gene for the HA or N glycoprotein,

by genetic reassortment (Webster and Laver, 1975; Webster et al., 1982); an event which can occur when a single cell is simultaneously infected with two different influenza viruses. Examples of antigenic shift in human influenza viruses occurred with the emergence of the "Asian flu" in 1957 (a change from H1N1 to H2N2), and the "Hong Kong flu" in 1968 (a change to H3N2).

In 1970, Webster published a laboratory procedure for the production of stable reassortant viruses (hybrids) with surface antigens which could be specified by the investigator Webster, 1970). These reassortant viruses derived the hemagglutinin antigen from one parent and the neuraminidase antigen from the other parent. According to Webster (1970), reassortant viruses have been a valuable tool in research and diagnostic laboratories for several reasons: 1) they have allowed for physical separation of antigens for biochemical characterization that could not be done using the original or parental viruses (Laver and Kilbourne, 1966); 2) they have permitted evaluation of the immune response to one surface antigen without the possibility of steric interference with antibody to the other antigen (Schulman and Kilbourne, 1969); 3) they have permitted studies on the possible origin of new influenza viruses following mixed infection of animals or birds (Webster, 1970); 4) they have been useful in studies to

determine the genetic control of virulence (Rott et al., 1976; Scholtissek et al., 1977; Ogawa and Ueda, 1981).

Diagnosis

A field diagnosis of avian influenza infection in poultry is extremely difficult considering the variability in kind and severity of lesions associated with the different isolates. For this reason, the diagnosis of AI requires the use of virologic and/or serologic procedures. Virus isolation is the method of choice, since this method would permit pathogenicity studies and molecular characterization of the virus.

Virus isolation

Collection of specimens Specimens for the diagnosis of influenza are collected as early as possible after onset of symptoms. Virus can be isolated from most tissues collected from dead birds, and from tracheal and cloacal swabs from live birds. The efficiency with which influenza viruses are recovered from these specimens vary with different strains of virus. During the 1983/1984 outbreak of H5N2 virus in Pennsylvania, virus was isolated more often from tracheal swabs than cloacal swabs submitted for surveillance. A review of 92 positive cases revealed that virus was isolated from only tracheal swabs from 42 of those cases, from only cloacal swabs from one case, and from both

tracheal and cloacal specimens in 49 cases. In those same cases, virus was isolated from 88 percent of the tracheal swabs and 38 percent of the cloacal swabs (Pearson et al., 1987). Alexander, on the other hand, has reported that with some H5 isolates, cloacal swabs were more sensitive than tracheal swabs (Alexander et al., 1986). Recovery of virus from birds infected with AI varies with the infecting strain, but virus can usually be recovered from up to 13 to 16 days following exposure to the virus. However, isolation of influenza virus for up to 30 days has been reported in experimentally-inoculated chickens (Pearson et al., 1987)

Influenza viruses have been isolated from a variety of sources and seem to be quite stable in the environment if kept from desiccation and heat. For example, Webster has reported that influenza virus will survive in lake water for up to 30 days at 0 °C, and for 4 days at 22 °C (Webster et al., 1978). Influenza viruses have frequently been recovered from water samples collected from lakes inhabited by waterfowl and from fecal samples collected on the shores of these lakes (Alexander et al., 1980; Hinshaw, 1987). During the Pennsylvania outbreak, numerous isolations of AI were made from fecal samples from premises at the time of depopulation and for approximately 100 days following depopulation (Pearson et al., 1987). Virus was also isolated

from the yolk and albumin of eggs laid from infected hens (Cappucci et al., 1985), insects, egg flats, egg belts, and the floors of the egg room, and air samples collected within 25 feet of exhaust fans of the buildings (Pearson et al., 1987).

Embryo inoculation The inoculation of 9- to 11-day-old embryonating chicken eggs is the most commonly used system to isolate AI viruses. Both amnionic and allantoic routes of inoculation are satisfactory. The embryos are usually incubated for 3 to 4 days at 33 to 37 °C following inoculation. The allantoic and amnionic fluid (AAF) is harvested from dead and live embryos to check for hemagglutinating activity using chicken erythrocytes. Most isolations can be made from the first passage, however, a second passage may be required to detect some isolates.

Egg bit The egg bit (allantois on eggshell) procedure has been used in the isolation and propagation of influenza viruses (Fazekas de St. Groth and White, 1958). Fragments of the allantoic membrane of the embryonating egg, still attached to the eggshell, are cultivated *in vitro*. The eggshell serves to buffer the medium and anchor the membrane. Infectivity titers with most influenza A viruses in the egg bit system are similar to those obtained in embryos. This procedure has has its best application in performing virus

infectivity assays and neutralization tests and for rapid isolation and purification of reassortant viruses (Webster, 1970).

Cell culture Cell cultures have been used to isolate influenza viruses, but there are serious limitations to their use due to variations in sensitivity of different types of cells to different viruses (Dowdle and Schild, 1975). Furthermore, not all AI viruses are produced in infectious form when grown in cell culture, a topic which will be discussed in another section.

Serological assays

A comprehensive manual describing the techniques used for influenza diagnosis has been published (Palmer et al., 1975). Procedures used for identification of influenza A viruses are based on characterization of the type-specific internal antigens (RNP and M) and the subtype-specific surface antigens (HA and N). Likewise, the serological diagnosis of AI is based on the detection specific antibodies to these same antigens. The preferred, and most commonly used techniques to identify surface antigens are the hemagglutination-inhibition (HI) test and the neuraminidase-inhibition (NI) test (Kendal, 1982). The agar-gel immunodiffusion (AGID) test is the standard procedure for identifying the type-specific RNP and M antigens of AI

viruses and for detection of the corresponding antibodies (Beard, 1970). Several additional techniques have also been described for identification of the antigens and/or antibodies of influenza virus.

Hemagglutination-inhibition test With the discovery of the hemagglutination phenomenon in 1941 (Hirst, 1941), the HI test has become the most widely used method for identifying the HA or its corresponding antibody. The basic principles of the HI test in current use were published in 1944 (Salk, 1944). Inhibition of hemagglutination is the result of attachment of antibody to the virus HA, thus preventing attachment of the virus to receptors on the surface of erythrocytes. Care must be taken in the selection of reference reagents used in the HI test to avoid steric interference with the neuraminidase.

Neuraminidase-inhibition test The NI test is the recommended procedure for the identification of neuraminidase antigen or its corresponding antibody. Warren (1959) and Aminoff (1959) independently developed a technique in which the enzymatic release of N-acetylneuraminic acid (NANA) could be detected by the formation of an aldehyde with periodate which then forms a chromophore with thiobarbituric acid. Aminoff later published a quantitative assay to determine the enzyme potency of a virus preparation based on the rate of

hydrolysis of the substrate, fetuin (Aminoff, 1961). The inhibition of enzymatic activity, by specific antibody, is the basis of the NI procedure. Both macro (Palmer et al., 1975) and micro (Van Deusen et al., 1983) procedures have been published.

Neutralization test The basic principles of the neutralization test have been reviewed (Lafferty, 1963). Neutralization assays provide a high sensitivity for detecting antibody and the best index of protection (Easterday and Beard, 1984); however, this test is not frequently used as a routine diagnostic procedure.

Double immunodiffusion test The double immunodiffusion or agar-gel immunodiffusion (AGID) has been extensively used to determine antigenic relationships of influenza virus antigens and for detecting type-specific antibody (Dowdle et al., 1974; Schild and Pereira, 1969; Beard, 1970). The principal of the test was first described by Ouchterlony in Sweden in 1949, and involves concurrent migration of antigen and antibody through a gel matrix. A line of precipitation is observed in the gel matrix where optimal proportions of antigen and homologous antibody occur. This procedure can be used as a type-specific test to screen avian serums for the presence of antibody to any subtype of influenza A virus (Beard, 1970).

Single radial diffusion test The single radial diffusion test has been used to assay both the HA and N antigens of influenza viruses (Schild et al., 1971). The test is a simple, two-component system based on the principles first described by Mancini in 1965. One of the two reactants is incorporated uniformly throughout the gel matrix while the second reactant is placed in a well cut in the matrix. As the reactants diffuse, antigen-antibody complexes form and are trapped as a precipitate in the gel. Quantitation of the reaction is based on the size of the disc of precipitate and concentration of reactants.

Complement fixation test The complement fixation (CF) test has been used for the serological detection of antibodies to both subtype-specific surface antigens (HA and N) and type-specific internal antigens (RNP and M) (Lief and Henle, 1959). This procedure is not used in the diagnosis of AI, since avian serums fix complement poorly.

Immunoassays Several immunoassays have been described for the diagnosis of influenza infections. These would include the fluorescent antibody (Hers, 1963; Skeeles et al., 1984); radioimmunoassay (Kendal, 1982); and the enzyme-linked immunoassay (Snyder et al., 1985). The usefulness of these tests as a subtype-specific identification tools have not been thoroughly evaluated;

however, they have provided increased versatility and sensitivity in studies with monoclonal antibodies (Kendal, 1982).

Pathogenicity

The pathogenic nature of AI viruses isolated from poultry has not only been of concern to the poultry producer, but has also had a major impact on the formulation of control measures at the state, national, and international levels. This impact is reflected in the regulations of many countries that require slaughter of infected poultry to prevent the spread of the pathogenic form of the disease.

Definition of pathogenicity

Pathogenicity is the ability of an organism to produce disease in a given host and is a measure of the degree of transmissibility, or the ability of an agent to pass from one host to another. It has been well documented that transmissibility of AI viruses between like species and different species is extremely variable. The most convincing evidence for this variability lies in the fact that waterfowl are generally completely resistant to viruses which are highly pathogenic to domestic poultry (Alexander et al., 1978; Pearson et al., 1987; Kawaoka et al., 1987). In most cases, pathogenic viruses which cause sudden deaths are the viruses least likely to spread to birds in contact,

apparently because the host dies before the virus is shed into the environment in significant quantities (Alexander, 1987). For this reason, many of the outbreaks of highly pathogenic AI have been self-limiting.

Clinical signs and lesions

Presently, factors which regulate or contribute to pathogenicity are, at best, poorly understood. The clinical disease in domestic poultry as a result of infection with AI virus can vary from inapparent to an acute, generalized disease characterized by high morbidity and mortality. Clinical signs which are observed with AI infections are affected by species, age, and sex of the host and would include coughing, lacrimation, emaciation, huddling, sinusitis, subcutaneous hemorrhage (cynanosis) of the unfeathered skin, edema of the head and face, ruffled feathers, nervous disorders, reproductive failure, and diarrhea (Easterday and Beard, 1984). Some of the first signs of trouble in flocks infected with nonpathogenic strains of AI are reproductive failure, decreased activity of the birds and a decrease in food consumption. The expression of clinical disease in chickens and turkeys may also be affected by the immunocompetence of the birds, as well as environmental factors and exacerbative agents which may be present in the flock at the time of infection with AI (Newman

et al., 1981). Because of the broad spectrum of clinical signs and lesions, for both highly pathogenic and nonpathogenic strains of AI, a differential diagnosis should be conducted to rule out infections with Newcastle disease virus (NDV), other paramyxoviruses, chlamydia, mycoplasmas, and bacteria (Easterday and Beard, 1984).

The need for defining pathogenicity

Historically, FP was considered to be caused by viruses of the H7 subtype. For many years FP was easily distinguished from other AI viruses based on the clinical disease. However, may countries went so far as to adopt a definition of FP based on identification of an H7 avian influenza virus despite isolations of highly pathogenic viruses in 1959, 1961, and 1966 which were identified as members of the H5 subtype--a subtype completely unrelated to the classical FP virus (Alexander, 1987). It was not until 1971, when an avirulent H7 virus was isolated from turkeys in Oregon (Beard and Easterday, 1973) that the serological definition of FP was questioned. For several years there did not seem to be a clear definition of what was meant by FP. The need for such a definition became more critical as AI viruses of subtypes H5 and H7 were being isolated from poultry and waterfowl from many countries of the world (Alexander, 1982). To date, all AI virus which have

demonstrated the ability to cause fulminating disease have been members of the H5 and H7 subtypes. It is not known why these viruses have been unique in this respect.

Definition of highly pathogenic AI

In 1981, at the First International Symposium on AI held in Beltsville, Maryland, it was recommended the term FP be discarded, except for historical purposes, and replaced by the term highly pathogenic. In addition, highly pathogenic AI was defined as

> any virus that results in not less than 75% mortality within 8 days in at least 8 healthy susceptible chickens, 4-8 weeks old, inoculated by the intramuscular, intravenous, or caudal airsac route with bacteria-free infectious allantoic or cell culture fluids. This assumes the use of standard operating procedures to assure specificity (Easterday, 1981).

This definition has become known as the "Beltsville criterion" for classifying highly pathogenic AI viruses.

The "Beltsville criterion" was first used in the United States to identify the pathogenicity of viruses isolated during the 1983/1984 outbreak of H5N2 AI in Pennsylvania, Virginia, New Jersey, and Maryland (Fichtner, 1984). Throughout the outbreak, discrepancies and inconsistencies were frequently noted between the field disease and the laboratory characterization of the isolated viruses. For example, Utterback indicated that nearly one third of the flocks which clinically appeared to be infected with the

highly pathogenic virus did not yield viruses which fulfilled the "Beltsville criterion" for classification as highly pathogenic (Utterback, 1984). In addition, nonpathogenic viruses were isolated from some flocks experiencing 60-80% mortality, while highly pathogenic viruses were isolated from flocks experiencing less than 10% mortality. Problems were compounded by the fact that viruses representing a spectrum of pathotypes, from nonpathogenic to highly pathogenic, could occasionally be isolated from the same flock (Utterback, 1984).

In 1986, following the Second International Symposium on Avian Influenza in Athens, Georgia, a subcommittee of the Committee of Transmissible Diseases of Poultry and Other Avian Species of the United States Animal Health Association (USAHA) was formed to re-evaluate the definition of highly pathogenic AI and make recommendations as to changes needed to accurately assess pathogenicity of AI viruses. The committee recommended the abandonment of the term "fowl plague" except for historical references, and that the terms "nonpathogenic" or "low pathogenic" be discarded, with future isolates being classified as either "avian influenza" (AI) or "highly pathogenic avian influenza" depending upon the results of laboratory pathotyping. In addition, it was recommended that a combination of *in vivo* and *in vitro*

methods be used to completely evaluate the pathogenic potential of AI viruses (McCapes, in press).

In vivo pathogenicity tests

Pathogenicity testing of AI viruses has traditionally been accomplished using chickens as the host of choice. This may seem presumptuous considering the number of AI viruses which have also been isolated from wild birds (Hinshaw et al., 1981), and captive birds (Slemons et al., 1973a, 1973b; Alexander et al., 1977, 1980; Senne et al., 1983). It is not clear as to how, or why, the chicken was selected as the host of choice for pathogenicity studies. Alexander has suggested that even though both chickens and turkeys are highly susceptible to infections with AI, chickens were selected because they are generally available as specific pathogen-free laboratory animals, and the turkeys are not reared in all counties with domestic poultry industries and therefore may not be available (Alexander, 1987). This choice can also be supported by the fact that since the chicken is the most economically important domestic species world wide, it would be important to know the pathogenicity of AI viruses for this species. It has also been demonstrated that some strains of AI which are not pathogenic for ducks are pathogenic for chickens. The routes of inoculation used for chicken

pathogenicity testing have been quite varied, as reflected by the "Beltsville criterion."

Virulence index tests The use of virulence index tests for assaying avian influenza viruses for their ability to cause disease in chickens have been reported (Allan et al., 1977). Allen used the intracerebral pathogenicity index (ICPI) in day-old chicks, and the intravenous pathogenicity index (IVPI) in six-week-old chickens. These tests had previously been used for characterizing NDV. Allen reported that these tests could confirm differences in pathogenicity that was commonly seen with AI viruses and would be a better guide in determining the seriousness of an isolate than to infer disease potential based on the antigenic characteristics of the surface antigens, which, at the time was a popular practice. The principal behind the virulence index tests was to obtain a numerical value for pathogenicity which could then be used to accurately compare one virus to another. One important aspect of the virulence index tests was that sick birds, in addition to dead birds were reflected in the numerical score, a factor which is sometimes overlooked using other methods.

Embryo mortality The use of chicken embryo mortality as a method to evaluate the pathogenicity of AI viruses has also been used. This test, referred to as the mean death

time (MDT) has been routinely used to characterize NDV (Hanson and Brandly, 1955; Allan et al., 1978). The principle of the test is to titrate the virus in embryos to determine the time it takes for the minimum lethal dose of virus to kill embryos. Isolates which are more pathogenic kill embryos faster than less pathogenic viruses. In 1981, however, Bennejean reported that the use of the MDT for AI was generally unreliable (Bennejean, 1981). The MDT is not routinely used by many laboratories for AI characterization.

In vitro pathogenicity tests

Since the early 1970s, much of the emphasis on research on influenza viruses centered around understanding the molecular basis of pathogenicity. From the molecular studies of reassortants between the virulent FP virus and avirulent avian or human influenza viruses, it has been demonstrated that virulence is polygenic (controlled by more than one gene), but that the HA gene is a key determinant of virulence (Rott et al., 1976; Scholtissek et al., 1977; Ogawa and Ueda, 1981). In 1975, it was reported that proteolytic cleavage of the 80 Kd HA glycoprotein into smaller disulfide-bonded glycoproteins of 50 Kd (HA1), and 30 Kd (HA2), at the connecting peptide region, was required for virus infectivity (Klenk et al., 1975; Lazarowitz and Choppin, 1975). Cleavage of the HA, however, was not required for hemagglutinating

activity and virus assembly. All influenza viruses are produced with cleaved HA (infectious form) when propagated in chick embryos or cells of the chorio-allantoic membrane, but in other cell systems, such as chick embryo fibroblast (CEF), or Madin-Darby canine kidney (MDCK) cells, not all viruses are produced with cleaved HA (Rott, 1979). Infectivity of virus particles assembled with uncleaved HA can be restored artificially, *in vitro*, using the protease trypsin (Klenk et al., 1975). In 1979, Bosch demonstrated that influenza A viruses which were produced in infectious form, when propagated in a wide range of host cells, were also pathogenic for chickens (Bosch et al., 1979; Rott, 1981). The correlation of pathogenicity with cleavage of the HA in a wide variety of cells was an important step leading to the use in *in vitro* methods as a possible means of determining pathogenicity of influenza viruses.

Plaque formation in cell culture Plaque formation in cell culture is dependent on the spread of infectious virus to adjacent cells. Consequently, in cells such as CEFs and MDCKs, only viruses with highly cleavable HA (highly pathogenic viruses) have the capability to produce infectious virus and form plaques (Klenk et al., 1975, 1977). Nonpathogenic viruses can be induced to form plaques by incorporating the proteolytic enzyme trypsin in the overlay

medium. Plaquing efficiencies can therefore be calculated by determining the number of plaques observed when the virus is grown in the presence versus absence of trypsin. Determination of plaquing efficiencies can be used to determine if mixtures of both highly pathogenic and nonpathogenic viruses exist in the same culture. A strict correlation between plaquing ability and pathogenicity for chickens has been reported (Bosch et al., 1979; Rott, 1981).

Radioimmunoprecipitation Sodium dodecyl sulfate-polyacrylimide gel electrophoresis (SDS-PAGE) procedures have been used for many years to separate proteins according to MW (Laemmli, 1970). One application of this technique, relating to *in vitro* pathogenicity determination of AI viruses, has been developed to determine if cleavage of the 80 Kd HA glycoprotein into the 50 Kd HA1 and the 30 Kd HA2 fragments has occurred (Webster et al., 1983). Using this procedure, radio-labeled (^{35}S-methionine) viral proteins can be prepared by inoculating MDCK cells with virus in the presence and absence of trypsin. The HA can be separated from other viral and cellular proteins by precipitation with monoclonal antibodies directed against the HA. The intact HA molecule or HA1 and HA2 subunits can be separated by SDS-PAGE and detected by autoradiography. As previously discussed,

cleavage of the HA has been correlated with pathogenicity of AI viruses for chickens.

Nucleotide and amino acid sequencing Differences in the ability of H7N7 viruses to be cleaved, has been correlated with the amino acid sequence at the connecting peptide region of the HA (Bosch et al., 1981). Bosch also reported that highly pathogenic H7N7 viruses (fowl plague) all had additional basic amino acids at the connecting peptide region. Kawaoka, however, found that the virulent H5N2 viruses isolated from Pennsylvania in 1983 did not possess these additional amino acids, and that both the virulent and avirulent viruses had the same sequence at the connecting peptide region (Kawaoka et al., 1984). Although the present implications of the amino acid sequence at the cleavage site may not be totally understood, it was recently reported that the presence of two pairs of basic amino acids at that site is a common finding in all virulent viruses which have been analyzed so far; the exception being the avirulent H5N2 virus isolated from Pennsylvania in April 1983 (Kawaoka et al., 1987). The exception of the avirulent Pennsylvania virus to this common finding can be explained by the fact that all of the genes for virulence were apparently present when the virus first appeared in April, 1983, and that a single point mutation in the HA gene, resulting in a

conformational change in the tertiary structure of the HA, was all that was required for acquisition of virulence (Kawaoka et al., 1984). Kawaoka also suggested that virulence of the influenza virus in chickens may be correlated with the distribution of the cleavage enzyme in the host, and that enzymes which recognize a single basic amino acid may occur less frequently than those cell types which possess enzymes capable of recognizing pairs of basic amino acids (Kawaoka et al., 1987). That could explain why most AI infections are restricted to the respiratory and intestinal tract of birds. Since the expression of a highly pathogenic disease in chickens requires a systemic infection (Rott, 1987), it appears that virulence may depend on the genetics of both the host and the virus (Kawaoka et al., 1987).

MATERIALS AND METHODS

Embryonating Chicken Eggs

Embryonating (9-day-old) chicken eggs were purchased from Larson Lab-Vac Eggs, Inc., Gowrie, Iowa. The source flock was certified to be free of most avian bacterial and viral pathogens, including avian influenza (AI). All embryos used in this study, for purposes of virus propagation, or virus characterization, were inoculated at 9- to 11-days of age. All eggs were candled immediately prior to inoculation and daily thereafter for embryo viability.

Test Chickens

All chickens used in this study were of the White Leghorn breed between 4 and 8 weeks of age. Chickens were either obtained from the closed chicken flock at the National Animal Disease Center (NADC), Ames, Iowa, or were purchased from outside vendors: Welp Hatcheries, Bancroft, Iowa, and Hy Cross, Mason City, Iowa.

Quality control

Quality control measures used to assure validity of chicken inoculation results consisted of routine flock monitoring of the NADC flock for antibodies to AI, NDV, and other diseases of poultry. Purchasing specifications for chickens obtained from outside vendors required that chickens

be negative for antibodies to AI, and approximately 10% of the chickens purchased were sampled upon arrival at NVSL and tested for antibodies to AI using the AI agar-gel immunodiffusion (AGID) test (described below). All tests for antibody to AI were negative.

Avian influenza agar-gel immunodiffusion test The procedure used was similar to a procedure described by Beard (1970). The gel was prepared by adding 0.9 gm agarose (Seakem ME, Marine Colloids Division, Rockville, ME) and 8.0 gm NaCl to 100 ml PBS. The solution was autoclaved for 20 minutes at 15 psi to dissolve and sterilize.

Plates for the AGID were prepared by dispensing 17 ml of melted agar into a plastic petri dish (100 × 15 mm). The agar was cut using a seven-well template with a center well and six wells in a circle around it. The wells were 2.4 mm apart and 5.3 mm in diameter. For serological testing, antigen was placed in the center well and positive control serum was placed on either side of the test serum samples. Using this procedure, three samples could be tested on each pattern.

Viruses

Source of viruses

Virus isolates used in these studies were obtained from diagnostic specimens collected by members of the Avian Influenza Task Force during the early part of the 1983/1984

outbreak of H5N2 avian influenza in Pennsylvania. Virus isolation, identification and characterization was performed in high security facilities by personnel of the Diagnostic Virology Laboratory at the National Veterinary Services Laboratories, Ames, Iowa, using standard procedures (Pearson and Senne, 1987).

Selection and classification of viruses

Selected viruses were first- or second-passage chicken-embryo isolates which had been identified as AI subtype H5N2, pathotyped by chicken inoculation and stored at -70 °C for approximately 2 1/2 years. Classification on the isolates was based on the following criteria: if six or more of the eight inoculated chickens died with characteristic lesions of AI, the isolate was classified as highly pathogenic avian influenza (HPAI). If one to five chickens died, the isolate was classified as pathogenic avian influenza (PAI). If clinical disease was not observed in the inoculated chickens, the isolate was classified as nonpathogenic avian influenza (NPAI). Fourteen NPAI, 17 PAI, and 17 HPAI viruses were selected based upon the original pathotyping results obtained from chicken inoculations. Three prototype viruses were also included: nonpathogenic, A/chicken/Pa/21525/83 (index case, isolated in April 1983); pathogenic, A/chicken/Pa/4104/83; and highly pathogenic, A/chicken/Pa/1370/83.

Propagation of viruses

Isolates were diluted 1:1000 in Tris-buffered tryptose broth (TBTB) containing antibiotics (described below) and inoculated into the chorio-allantoic sac of 10- to 11-day-old embryonating chicken eggs. Inoculated eggs were incubated at 36 °C for 72 hours. Allantoic-amnionic fluid (AAF) was harvested from dead embryos or, if no embryos died, from the surviving embryos after chilling the eggs overnight at 4 °C. The AAF was checked for hemagglutinating activity to assure viability of the isolate. Allantoic-amnionic fluids which were hemagglutination positive were divided into aliquots and frozen at -70 °C until needed.

Tris-buffered tryptose broth (TBTB) with antibiotics

Ordinary tryptose broth was buffered to pH 7.5 by the addition of 1.21 gm trisma base (Sigma Chemical Co., St. Louis, MO), Tris, per liter. The concentration of antibiotics was 10,000 units/ml penicillin G, 2,000 µg/ml streptomycin sulfate, 650 µg/ml kanamycin sulfate, 1,000 µg/ml gentamicin sulfate and 20 µg/ml amphotericin B.

Hemagglutination test

The procedure used for the hemagglutination test was essentially that described by Carbrey et al. (1974). Fifty microliters of AAF was placed in the first well of a 96-well U-bottom microtiter plate. Fifty microliters of PBS was

added to the first well and all remaining wells of the row. Beginning with the first well, serial two-fold dilutions of AAF were prepared using a 50 µl microtiter diluting loop. Fifty microliters of a 0.5% chicken RBC suspension was added to each well. The plates were agitated and incubated at room temperature for 30 minutes before results were recorded.

Chicken red blood cells (RBC) Chicken RBCs used in hemagglutination tests were obtained fresh each week from donor roosters in the NADC flock. Chicken blood was collected aseptically and mixed with an equal volume of Alsever's solution. The cells were washed by centrifugation and resuspension in PBS three times. Washed, packed RBCs were stored at 4 °C until needed. A 0.5% suspension of RBCs diluted in PBS was used for the hemagglutination test procedure.

Pathogenicity Testing of Isolates in Chickens

Caudal-thoracic-air-sac (CTAS) inoculation of chickens

Routine pathotyping of isolates required the inoculation of eight 4- 8-week-old chickens (Pearson et al., 1987). Chickens were inoculated via the CTAS route with 0.2 ml of a 1:10 dilution of virus-infected, bacteria-free, AAF. The injection site was ventral to the junction of the sternal and vertebral portion of the last rib. The tip of the needle (25

gauge 5/8-inch) was pointed in an anterior direction. Chickens were held in isolation cages and observed daily for eight days. Chickens which died were necropsied for evidence of lesions consistent with avian influenza. Isolates were classified as previously specified in the "viruses" section of the materials and methods.

Intravenous pathogenicity index (IVPI)

The IVPI was performed and calculated as described for Newcastle disease virus (Allan et al., 1978). Ten 6-week-old chickens were inoculated by the intravenous route with 0.1 ml of a 1:10 dilution of virus-infected egg AAF and observed daily for 10 days. Each day, the chickens were scored as healthy, sick, paralyzed, or dead. Scores for dead birds were recorded cumulatively. A weighted value was given for each observation: healthy chickens were weighted zero, sick chickens were weighted 1, a paralyzed chicken 2, and a dead chicken 3. The IVPI equals the weighted value divided by the number of observations made. A maximum score of 3 would be obtained if all 10 chickens died within 24 hours.

Biosecurity for inoculated chickens

Following inoculation, chickens were maintained in plastic isolation cages under negative pressure with individual filtered air intakes and individual feed and water supplies. The room containing the cages was also maintained

under negative air pressure with approximately nine complete air changes each hour. This latter feature permitted working with different isolates in the same room, provided some time had elapsed between the opening of cage doors. Inoculated chickens were observed daily for the required length of time as specified in the test procedure being used. Once the chickens were inoculated, the cage doors were not opened except to remove dead birds.

Mean Death Time (MDT) in Embryonating Chicken Eggs

The MDT for each isolate was determined as previously described for Newcastle disease virus (Allan et al., 1978). Two sets of twenty-four 9- to 11-day embryonating chicken eggs were inoculated via the chorio-allantoic sac with dilutions of AAF (10^{-1}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}) using 0.1 ml per egg. These dilutions were chosen because most isolates of influenza virus will have titers between 10^{-6} and 10^{-9} when titrated in embryonating chicken eggs. Five eggs were used for dilutions 10^{-6} through 10^{-9} and four eggs for the 10^{-1} dilution. One set was inoculated shortly after 8:00 a.m. and the second set at 4:00 p.m. of the same day. The eggs were incubated at 35 °C, candled at 8:00 a.m. and 4:00 p.m. each day for 8 days, and the time of death of each embryo was recorded. The minimum lethal dose (MLD) was

identified as the highest virus dilution (least amount of virus) which caused the death of all embryos at that dilution. The MDT was then calculated by dividing by ten, the sum of the hours post inoculation that it took for the MLD dilution embryos to die.

Calculation of fifty percent endpoints

The fifty percent embryo lethal dose (ELD_{50}) for each isolate was determined from the MDT titrations. To establish whether surviving embryos were infected with virus, AAF was harvested from surviving embryos following the 8-day incubation period and checked for hemagglutinating activity. The ELD_{50} endpoint was calculated using the Kärber method of determining fifty percent endpoints, with embryo mortality or hemagglutinating activity being used as evidence of virus infection.

Plaque Formation in Cell Culture

Preparation of chicken embryo fibroblasts (CEF)

Primary cultures of CEF cells were prepared from 9- to 11-day chicken embryos. The surface of the eggs were disinfected with 70% ethanol and allowed to dry. Eggs were opened and embryos removed and placed in sterile petri dishes. Embryos were decapitated and the bodies rinsed two times in PBS and two times in trypsin solution (0.25% in PBS). Following the last trypsin rinse, the embryos were

placed into a 50 ml sterile disposable syringe and forced through the tip of the syringe into a sterile trypsinizing flask. The minced cells were rinsed two times in trypsin solution (cells were allowed to settle between rinses) followed by a 20- to 30-minute digestion with trypsin. The resulting cell suspension was filtered through 4 layers of gauze and centrifuged to pellet the cells. The cells were resuspended in PBS and centrifuged again. The cells were then resuspended in growth medium (1:1 ratio of M199 and F10) containing 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin G and 100 µg/ml streptomycin sulfate) and the cell concentration adjusted to approximately 3.0×10^6 cells per milliliter. Sterile 60 mm tissue-culture-treated petri dishes were each seeded with 5 ml of standardized cell suspension. Cultures were incubated for 48 hours at 37 °C in a humidified atmosphere with 5 percent CO_2 to allow cells to reach confluency.

Plaque assay procedure

Confluent monolayers were washed two times with 4 ml Eagle's minimum essential medium (MEM) with antibiotics to remove residual fetal bovine serum (FBS). Six culture dishes were then inoculated with 0.2 ml of a virus dilution (previously determined for each virus) which would produce 20 to 200 plaque-forming units (PFU). Following inoculation,

the dishes were incubated for 45 to 60 minutes at 37 °C with occasional rocking to keep cells moist. All cells were then overlaid with 5 ml of overlay medium (see Appendix B) containing 1 percent Bacto-agar. Three of the cultures received overlay medium with trypsin (10 µg/ml) and three received overlay medium without trypsin. Cultures were incubated at 37 °C for 48 hours and overlaid a second time with 3 ml of overlay medium containing 0.004 percent neutral red and 1 percent Bacto-agar. Plaques were counted after an additional 24 to 48 hours of incubation at 37 °C.

Calculation of plaquing efficiency

The efficiency of plaquing for each virus was calculated as the ratio of the average number of PFUs observed on plates where trypsin was added to the overlay versus the average number of PFUs observed when trypsin was omitted.

Radioimmunoprecipitation (RIP) Assay

Preparation of radio-labeled viral proteins

Confluent monolayer cultures of Madin-Darby canine kidney (MDCK) cells, grown in tissue-culture-treated 24-well cluster plates, were washed twice with PBS to remove residual FBS. Duplicate wells of cells were inoculated with 0.5 ml of undiluted, virus-infected AAF. Virus adsorption was carried out at room temperature for one hour, with occasional agitation of the plates. Unabsorbed virus was removed by

aspiration and the cells were washed two times with PBS. One of the duplicate wells of cells received 0.5 ml Eagle's MEM (methionine deficient) containing 4% BSA (fraction V), antibiotics, and 0.05 mCi of ^{35}S-methionine. The second well of cells received the above medium with the addition of 1 µg/ml trypsin. The virus-infected cells were incubated 18 to 24 hours at 37 °C in a humidified atmosphere with 5% CO_2.

Fluid and cells from wells where the virus-infected cells had detached from the plate were transferred to microfuge tubes. The microfuge tubes were centrifuged at 2000 xg for two minutes to pellet the cells. The supernatant was discarded and 0.05 ml of lysis buffer (see Appendix A) was added to the cell pellet. The tubes were vortexed vigorously to resuspend the cells in the lysis buffer. In wells where the cells were not detached from the plate, the lysis buffer was added directly to the plate well. Tubes and plates were incubated at 4 °C for 30 minutes during cell lysis. Cell lysates from that plates were transferred to microfuge tubes and all tubes were centrifuged at 4 °C for 10 minutes using an Eppendorf microfuge. The resulting supernate was then transferred to a new microfuge tube and frozen at -70 °C until needed.

Rabbit anti-mouse- (RAM-) coated Sepharose beads

A 1.5 gm quantity of lyophilized protein A-coated Sepharose CL-4B beads (Pharmacia Inc., Piscataway, NJ) was rehydrated with 10 ml PBS. One drop of an 8% solution of sodium azide was added to retard bacterial growth. The beads were gently mixed by inverting several times, then allowed to swell overnight at 4 °C.

An appropriate quantity of rehydrated Sepharose beads was transferred to a 15 ml graduated conical centrifuge tube and centrifuged at 800 xg to pack the beads. The supernatant was removed by aspiration and RAM serum (provided by Dr. R. G. Webster, Department of Virology, St. Jude Children's Research Hospital, Memphis, TN) was added to the beads in a ratio of 4 volumes packed beads to 1 volume RAM. The tube was gently inverted several times to mix the beads and RAM. The mixture was incubated at room temperature for 30 minutes with occasional mixing. Unreacted RAM was removed by washing the beads 4 times using RIP buffer (see Appendix A). Following the last wash, the RAM-coated beads were prepared as a 30% suspension in RIP buffer, and stored at 4 °C.

Monoclonal antibodies, produced as ascites fluids, were kindly provided by Dr. R. G. Webster, Department of Virology, St. Jude Children's Research Hospital, Memphis, TN. The monoclonal antibodies were produced against the hemagglutinin

of A/Chicken/Pa H5N2 viruses. Equal volumes of three monoclonal antibodies were pooled for use in the RIP procedure. The individual monoclonal antibodies were identified as CP62, Ck/Pa/83-8125-777/1, and CP22-Ck/Pa/132.

RIP procedure

The RIP procedure used was essentially that described by Webster et al. (1983). Fifty microliters of 30% RAM-coated Sepharose beads were added to microfuge tubes (two tubes per isolate). Five microliters of the monoclonal antibody pool were added to each microfuge tube. The tubes were placed on a vibrating platform for one hour at room temperature. Unreacted ascites fluid was removed by washing 3 times with RIP buffer. Tubes were centrifuged for 30 seconds between washes.

Ten microliters of radio-labeled antigen (propagated in the presence and absence of trypsin) was added to respective tubes. Tubes were placed on a vibrating platform and incubated for one hour at room temperature to permit adsorption of hemagglutinin. Unabsorbed proteins were removed by washing the beads three times with RIP buffer as before. After the final wash, 50 µl of sample buffer (see Appendix A) was added to each tube. Tubes were vortexed vigorously, centrifuged, heated at 100 °C for three minutes, and centrifuged again to separate the adsorbed hemagglutinin

from the beads. The supernatant, containing the radio-labeled hemagglutinin, was then transferred to a new microfuge tube and stored at 4 °C until needed.

Preparation and processing of SDS-Polyacrylamide gels

The lower and upper gels were prepared as specified in Appendix A using standard procedures (Laemmli, 1970). Eight millimeter spacers and a 20-toothed sample comb were used in forming the gels. Twenty-five microliters of precipitation-purified radio-labeled hemagglutinin were added to respective sample wells and electrophoresed 14 hours at 70 volts (constant voltage). The gel was removed from the glass plates, placed in a pyrex dish, covered with sodium salicylate solution (see Appendix A), and placed on a rocking platform for one hour. The gel was then dried onto filterpaper using a combination of heat and vacuum. Autoradiography was performed using Kodak XAR-5 X-ray film at -70 °C for 96 hours.

Interpretation of RIP results

Each sample (prepared in the presence and absence of trypsin) was evaluated for the presence of cleaved or uncleaved hemagglutinin using the following criteria: if the majority of the detectable radioactivity was located in a band of approximately 80,000 MW, the HA was classified as uncleaved; if the majority of radioactivity was located in

two bands of approximately 50,000 MW and 30,000 MW, the HA was classified as being cleaved; and when approximately equal amounts of radioactivity was detected in all three MW bands, the HA was classified as being partially cleaved and partially uncleaved. However, for purposes of classification, partially cleaved HA was recorded as being cleaved.

RESULTS

During the 1983/1984 outbreak of AI in Pennsylvania, H5N2 viruses were pathotyped using eight 4- to 8-week-old chickens inoculated via the caudal-thoracic-air-sac route (CTAS). This was one of three routes of inoculation specified by the "Beltsville criterion" for pathotyping AI viruses isolated from poultry. Using this classification system, 51 virus isolates were selected for use in this study. Fifteen isolates were classified as nonpathogenic avian influenza (NPAI), eighteen were classified as pathogenic avian influenza (PAI), and eighteen were classified as highly pathogenic avian influenza (HPAI).

Virus Activity

The 51 virus isolates selected for this study were passaged one time in embryonating chicken eggs, bottled, and frozen at -70 °C. Fifty percent endpoint determinations and HA titers were performed on all viruses to compare infectivity titers with HA activity (Tables 2, 3, and 4). Infectivity titers ranged from 1.6×10^{6} for PAI isolate 24 (Table 3), to 1.6×10^{8} for HPAI isolate 50 (Table 4). The mean titer for NPAI, PAI, and HPAI viruses was 1.8×10^{7}, 2.5×10^{7}, and 3.6×10^{7}, respectively. Although the mean titer of HPAI viruses was twice that of NPAI viruses, this difference was not statistically significant ($p>0.15$) using the F test. If

the atypical NPAI viruses (3, 6, 7, and 8) were excluded, the difference was greater, but still not statistically significant ($p>0.05$).

Table 2. Nonpathogenic[a] isolates: Comparison of hemagglutinating activity and virus titer

Isolate No.	HA[b] titer	EID_{50}[c]/ml
1	1:128	4.0×10^7
2	1:64	no test
3	1:16	2.0×10^6
4	1:8	4.0×10^7
5	1:8	2.5×10^6
6	1:8	7.9×10^6
7	1:8	6.3×10^6
8	1:8	7.9×10^6
9	1:32	5.0×10^6
10	1:32	6.3×10^6
11	1:16	3.2×10^6
12	1:16	7.0×10^7
13	1:16	1.3×10^7
14	1:8	1.6×10^7
15	1:32	2.5×10^7
Mean	1:27	1.8×10^7

[a]Did not kill any of the eight chickens.

[b]Hemagglutination.

[c]Fifty percent embryo infectious dose.

Table 3. Pathogenic[a] isolates: Comparison of hemagglutinating activity and virus titer

Isolate No.	HA[b] titer	EID_{50}[c]/ml
16	1:32	1.6×10^7
17	1:32	5.0×10^7
18	1:16	1.6×10^7
19	1:32	7.9×10^6
20	1:8	3.2×10^7
21	1:16	4.0×10^7
22	1:32	1.6×10^7
23	1:8	2.0×10^7
24	1:32	1.6×10^6
25	1:2	1.6×10^7
26	1:16	5.0×10^7
27	1:8	3.2×10^6
28	1:32	2.5×10^7
29	1:8	7.0×10^7
30	1:16	2.0×10^7
31	1:8	1.6×10^7
32	1:8	2.5×10^7
33	1:16	1.6×10^7
Mean	1:18	2.5×10^7

[a]Killed one to five of eight chickens.

[b]Hemagglutination.

[c]Fifty percent embryo infectious dose.

Table 4. Highly pathogenic[a] isolates: Comparison of hemagglutinating activity and virus titer

Isolate No.	HA[b] titer	EID_{50}[c]/ml
34	1:128	1.0×10^{7}
35	1:8	2.5×10^{7}
36	1:8	1.6×10^{7}
37	1:4	1.6×10^{7}
38	1:8	4.0×10^{7}
39	1:16	5.0×10^{6}
40	1:64	4.0×10^{7}
41	1:16	2.0×10^{7}
42	1:8	1.3×10^{8}
43	1:16	7.9×10^{6}
44	1:8	2.0×10^{7}
45	1:16	7.9×10^{6}
46	1:4	4.0×10^{6}
47	1:16	5.0×10^{7}
48	1:2	7.9×10^{6}
49	1:2	7.0×10^{7}
50	1:8	1.6×10^{8}
51	1:4	1.6×10^{7}
Mean	1:19	3.6×10^{7}

[a]Killed six to eight of eight chickens.

[b]Hemagglutination.

[c]Fifty percent embryo infectious dose.

The hemagglutinating activity of these viruses were quite variable, with titers ranging from 1:2 to 1:128 (Tables 2, 3, and 4). The average HA titer for NPAI, PAI, and HPAI viruses was 1:27, 1:18, and 1:19, respectively. If the four atypical viruses (3, 6, 7, and 8) were removed, the average hemagglutination titer of the NPAI viruses would have been 1:33. There was no significant difference ($p>0.10$) between the HA titers of the NPAI, PAI, and HPAI viruses when the F test statistic was used. Because of the dramatic differences in the hemagglutination titers for these viruses, it was also of interest to see if there was a correlation between hemagglutination activity and infectivity titers. There was no correlation ($r = -0.05$) between hemagglutinating activity and infectivity.

Pathogenicity in CTAS Inoculated Chickens

Chicken inoculations (CI) were repeated on all 18 of the PAI viruses to determine the reproducibility of results when compared to the original pathotyping (Table 6). Considerable differences in mortality were observed when CIs were repeated; mortality ranged from 0 to 100 percent. The classification of 7 of the 18 PAI viruses would have changed if the repeat CI results would have been used to classify these viruses. Four of the 18 viruses failed to produce disease and would have been classified as NPAI, and three

would have changed from PAI to HPAI. Pathogenic isolates 16, 19, 21, and 26 were inoculated a third time because of the marked differences observed between results of two bird inoculations (Table 6). The observed chicken mortality on the third inoculation were similar to those of the second inoculation. The overall mortality rate of the first and second trials were 43 percent and 31 percent, respectively. This reduction of 12 percent in mortality was statistically significant ($p<0.05$), using the chi-square test statistic.

Four NPAI viruses (3,6,7, and 8) and one HPAI virus (43), were also reinoculated into chickens because of inconsistencies between *in vivo* and *in vitro* results (Tables 5 and 7). Nonpathogenic isolates 3, 6, 7, and 8 appeared to be more characteristic of the PAI and HPAI viruses as determined by MDT, RIP results, and the ability of the viruses to produce plaques when grown in CEK cells without added trypsin (Table 5). However, when chicken inoculations were repeated, two of the four isolates (3 and 6) still did not produce disease in the inoculated birds. Isolate 7 killed one of eight chickens; isolate 8 also killed one of eight chickens, but the remaining cage mates were all severely depressed at the end of 8 days. Contrariwise, isolate 43 appeared to be more characteristic of NPAI isolates as determined by RIP and plaquing assays (Table 7).

Table 5. Nonpathogenic[a] isolates: Summary of *in vivo* and *in vitro* results

Isolate	1st, 2nd Chicken Inoculation Dead/Inoc.	MDT[b]	IVPI[c]	Cleaved HA Present[d]	Plaque Formation Without Trypsin[e]	Plaquing Efficiency[f]
1	0/8,ND[g]	74	0.00	-	-	>100,000
2	0/8,ND	86	ND	-	-	>10,000
3	0/8,0/8	60	1.08	+	+	0.9
4	0/8,ND	92	0.00	-	-	>100,000
5	0/8,ND	115	ND	-	-	>10,000
6	0/8,0/8	63	0.02	+	+	1.1
7	0/8,1/8	67	0.14	+	+	1.1
8	0/8,1/8	69	0.22	+	+	0.9
9	0/8,ND	96	ND	-	-	>100,000
10	0/8,ND	104	0.00	-	-	>100,000
11	0/8,ND	97	0.00	-	-	>10,000
12	0/8,ND	126	ND	-	-	>100,000
13	0/8,ND	82	0.00	-	-	>100,000
14	0/8,ND	124	ND	-	-	>100,000
15	0/8,ND	100	ND	-	-	>100,000

[a]Did not kill any of the eight chickens.

[b]Mean death time in embryonating chicken eggs (hours).

[c]Intravenous pathogenicity index in 6-week-old chickens.

[d]Determined by radioimmunoprecipitation.

[e]Performed in chicken embryo fibroblasts.

[f]Ratio of plaques formed in chicken embryo fibroblasts when trypsin was added versus plaques observed without trypsin.

[g]Not done.

Table 6. Pathogenic[a] isolates: Summary of *in vivo* and *in vitro* results

Isolate	1st, 2nd, 3rd Chicken Inoculation Dead/Inoc.	MDT[b]	IVPI[c]	Cleaved HA Present[d]	Plaque Formation Without Trypsin[e]	Plaquing Efficiency[f]
16	2/8,0/8,0/8	72	0.00	+	+	1.5
17	2/8,2/8,ND[g]	66	1.41	+	+	1.1
18	4/8,8/8,ND	84	2.06	-	+	1.7
19	5/8,0/8,0/8	67	0.35	+	+	1.9
20	4/8,2/8,ND	69	ND	+	+	1.6
21	4/8,0/8,1/8	63	0.65	+	+	1.2
22	1/8,1/8,ND	76	1.12	+	+	5.5
23	4/8,3/8,ND	90	1.68	+	+	1.9
24	4/8,8/8,ND	45	ND	+	+	1.2
25	5/8,7/8,ND	66	ND	+	+	0.8
26	5/8,0/8,1/8	57	1.00	+	+	1.0
27	5/8,5/8,ND	79	ND	+	+	1.4
28	2/8,1/8,ND	65	1.14	+	+	0.8
29	1/8,0/8,ND	68	ND	+	+	1.1
30	5/8,5/8,ND	68	1.64	+	+	2.3
31	2/8,0/8,ND	78	0.73	+	+	1.7
32	2/8,1/8,ND	66	ND	+	+	2.4
33	5/8,1/8,ND	75	ND	+	+	1.5

[a]Killed one to five of eight chickens.

[b]Mean death time in embryonating chicken eggs (hours).

[c]Intravenous pathogenicity index in 6-week-old chickens.

[d]Determined by radioimmunoprecipitation.

[e]Performed in chicken embryo fibroblasts.

[f]Ratio of plaques formed in chicken embryo fibroblasts when trypsin was added versus plaques observed without trypsin.

[g]Not done.

Table 7. Highly pathogenic[a] isolates: Summary of *in vivo* and *in vitro* results

Isolate	1st, 2nd Chicken Inoculation Dead/Inoc.	MDT[b]	IVPI[c]	Cleaved HA Present[d]	Plaque Formation Without Trypsin[e]	Plaquing Efficiency[f]
34	8/8,ND[g]	63	1.83	+	+	1.6
35	8/8,ND	54	1.83	+	+	1.1
36	8/8,ND	67	ND	+	+	1.0
37	8/8,ND	68	2.05	+	+	0.9
38	8/8,ND	65	0.33	+	+	1.1
39	8/8,ND	51	ND	+	+	1.6
40	7/8,ND	70	ND	+	+	3.0
41	6/8,ND	64	1.46	+	+	3.5
42	8/8,ND	74	1.94	+	+	1.2
43	7/8,0/8	72	0.00	-	-	>10,000
44	8/8,ND	65	ND	+	+	1.0
45	8/8,0/8	74	0.6	+	+	0.9
46	8/8,ND	71	1.92	+	+	1.1
47	6/8,ND	44	ND	+	+	2.5
48	7/8,ND	68	ND	+	+	1.4
49	6/8,ND	68	1.74	+	+	0.9
50	7/8,ND	68	ND	+	+	1.7
51	8/8,ND	52	ND	+	+	1.7

[a]Killed six to eight of eight chickens.

[b]Mean death time in embryonating chicken eggs (hours).

[c]Intravenous pathogenicity index in 6-week-old chickens.

[d]Determined by radioimmunoprecipitation.

[e]Performed in chicken embryo fibroblasts.

[f]Ratio of plaques formed in chicken embryo fibroblasts when trypsin was added versus plaques observed without trypsin.

[g]Not done.

Misidentification or a mixup of samples would be the most likely explanation for the discrepancies noted with isolate 43.

Chickens were reinoculated with highly pathogenic isolate number 45 because of the low IVPI value (Table 7). The reinoculated chickens did not show clinical signs of disease. The *in vitro* results, however, were consistent with PAI an HPAI isolates.

Intravenous Pathogenicity Index

The intravenous pathogenicity index (IVPI) was determined for 9 NPAI, 11 PAI, and 10 HAPI viruses. Except for the four atypical NPAI viruses (3, 6, 7, and 8) and the atypical HPAI virus (43), the viruses were selected at random for IVPI testing. A score of zero on the IVPI test would indicate that all chickens remained healthy throughout the 10-day observation period. A maximum score of three would have been assigned if all chickens died within 24 hours post inoculation. Critical values for IVPI have not been formally established. However, viruses with IVPI values of 1.2 or greater are usually common with viruses which can cause significant mortality in chickens. The IVPI scores for the 30 isolates which were tested are presented in Tables 5, 6, and 7. When IVPI scores were compared to the CTAS inoculation of chickens, there was a better correlation with

results from the second and third trials than with the first trial. One of the atypical NPAI isolates (3) did not kill chickens when inoculated via the CTAS route, but had a IVPI of 1.08; this value was high when compared to other isolates which killed none, or one, of eight chickens. Likewise, PAI isolate 31, and HPAI isolate 45, failed to kill chickens inoculated via the CTAS route on the second trail, but had IVPIs of 0.73 and 0.60, respectively; these were also higher than other viruses which did not kill chickens. On the other hand, HPAI isolate 38 killed all eight chickens on the original pathotyping but only had an IVPI of 0.33, which was considerably lower than other HPAI isolates. If one compares the IVPI results to the original CI results, there were at least nine isolates (3, 16, 19, 21, 26, 31, 38, 43, and 45) viruses which stand out as being considerably different from expected values. A summary of the average IVPI values for each group are presented in Table 11. The IVPI values of NPAI viruses were significantly lower ($p<0.05$) than PAI and HPAI viruses when evaluated by the Least Significant Difference (LSD) statistic.

Mean Death Time

Results of MDT determinations are given in Tables 5, 6, and 7. There was no significant difference in MDTs between PAI and HPAI viruses. The MDTs of the NPAI viruses, however,

did show significant differences from PAI and HPAI viruses ($p<0.05$), particularly if the equivocal isolates (3, 6, 7, and 8) were excluded (Table 11). The MDTs of NPAI viruses ranged from 60 to 126 hours, with a mean of 90 hours. The range of nonpathogenic viruses, excluding equivocal isolates 3, 6, 7, and 8, was 74 to 126 hours, with a mean of 100 hours (Table 11). The MDT of viruses in the PAI group ranged from 45 to 90 hours, with a mean of 70 hours. Viruses in the HPAI group had a range from 51 to 74 hours and a mean of 64 hours. The MDT would not appear to be totally reliable in classifying these viruses.

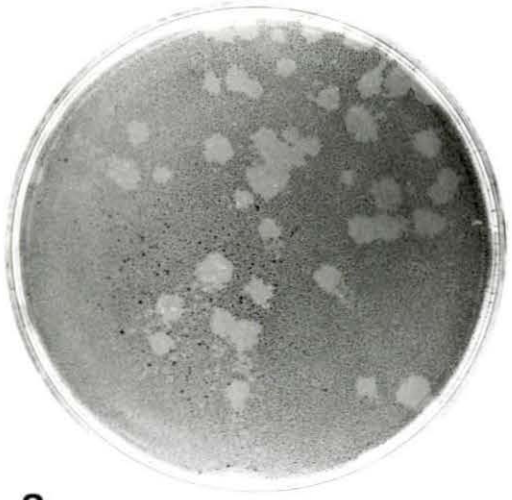
Plaque Formation

Plaque-forming ability of isolates grown in CEF cells in the presence and absence of trypsin was determined. Four of 15 NPAI, 18 of 18 PAI, and 17 of 18 HPAI viruses demonstrated the ability to form plaques without the addition of trypsin (Tables 5, 6, and 7). Examples of typical plaques for two viruses, one NPAI (7) and one HPAI (49) is provided in Figure 3. Plaque sizes for all viruses grown in the presence of trypsin varied from 1 to 3.5 millimeters in diameter when measured at 96 hours post inoculation. Plaque sizes for all viruses which demonstrated the capability of producing plaques without added trypsin were much smaller, and varied from 0.5 to 1.5 millimeters in diameter. The shape of

plaques were round to irregular, and were opaque in appearance. Although there were size differences noted in the plaques expressed within a single culture, there did not appear to be significant overall differences in size and shape of plaques produced by viruses within a group or between the viruses of different groups.

Plaquing efficiency of each virus was determined and results expressed as the ratio of plaques observed in the presence versus the absence of trypsin (Tables 8, 9, and 10). Among the isolates which had the ability to plaque in the absence of added trypsin, there was no significant difference in plaquing efficiency between viruses of the different pathotype groups; the range being 0.8 to 5.5. However, plaquing efficiencies of the nonpathogenic isolates was significantly different ($p<0.05$) than PAI and HPAI viruses, with the exception of NPAI viruses 3, 6, 7, and 8 and HPAI virus 43; the range being >10,000 to >100,000 (Table 11). This test would appear to be reliable in detecting viruses which are capable of producing highly cleavable HA in the absence of trypsin, as determined by comparison of results obtained using RIP assays (Tables 5, 6, 7).

Figure 3. Effect of trypsin (10 μ/ml) on plaque formation in chick-embryo fibroblasts overlaid with agar. Highly pathogenic isolate 49 is pictured in A (with trypsin) and B (without trypsin), 120 hours post inoculation. Nonpathogenic isolate 3 is pictured in C (with trypsin) and D (without trypsin) 96 hours post inoculation



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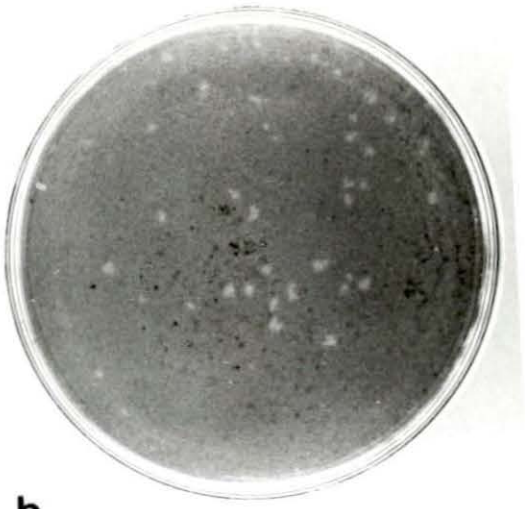

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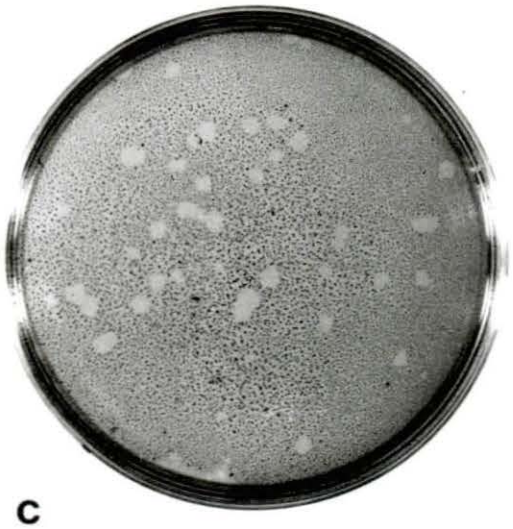

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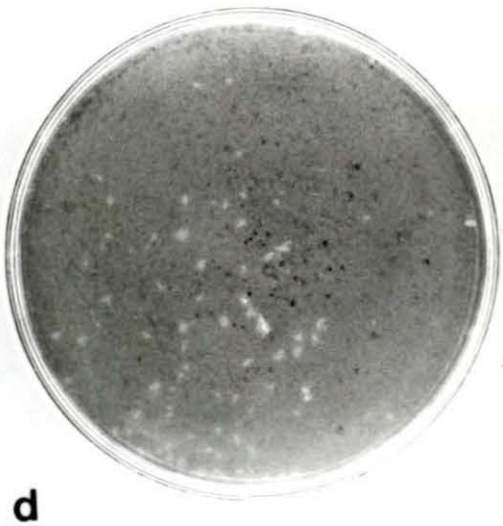


d

Radioimmunoprecipitation

The RIP procedure was used to directly evaluate whether cleaved HA was produced when these viruses were grown in MDCK cells in the presence and absence of trypsin. Radio-labeled HA was precipitated with monoclonal antibody and subjected to SDS-PAGE. The presence of cleaved or uncleaved HA was detected by autoradiography. Examples of typical SDS-PAGE gels employing radioimmunoprecipitated HA are presented in Figure 4. Radioimmunoprecipitation results indicated that 11 of the 15 NPAI viruses were unable to produce cleaved HA when the viruses were grown in MDCK cells without the addition of trypsin (Table 5). The remaining four NPAI viruses which demonstrated cleaved HA were the atypical NPAI isolates (3, 6, 7, and 8). Thirty-four of 36 PAI and HPAI viruses were capable of producing cleaved HA when grown without trypsin (Tables 6, and 7). Of the two isolates which did not produce cleaved HA by the RIP procedure, one was a PAI isolate (18) which demonstrated plaquing ability but did not produce cleaved HA, and the other isolate was the atypical HPAI isolate 43, which appears to be more like NPAI viruses. There was 98 percent agreement with the ability to plaque in cell culture and the presence of cleaved HA as determined by RIP assays (Table 11).

Table 8. Nonpathogenic[a] isolates: Effect of trypsin (10μg/ml) in overlay on plaque formation in CEF[b] cells

Isolate No.	PFU[c]/ml with trypsin	PFU/ml without trypsin	Ratio with/without trypsin
1	2.0×10^6	$<1.0\times10^1$	>100,000
2	1.1×10^5	$<1.0\times10^1$	>10,000
3	6.1×10^5	7.1×10^5	0.9
4	2.4×10^6	$<1.0\times10^1$	>100,000
5	2.3×10^5	$<1.0\times10^1$	>10,000
6	7.2×10^5	6.4×10^5	1.1
7	7.0×10^5	6.2×10^5	1.1
8	8.0×10^4	9.4×10^4	0.9
9	1.2×10^6	$<1.0\times10^1$	>100,000
10	8.5×10^5	$<1.0\times10^1$	>10,000
11	3.0×10^5	$<1.0\times10^1$	>10,000
12	7.5×10^6	$<1.0\times10^1$	>100,000
13	1.7×10^6	$<1.0\times10^1$	>100,000
14	1.4×10^6	$<1.0\times10^1$	>100,000
15	2.2×10^6	$<1.0\times10^1$	>100,000

[a]Did not kill any of the eight chickens.

[b]Chicken embryo fibroblast.

[c]Plaque forming units.

Table 9. Pathogenic[a] isolates: Effect of trypsin (10µg/ml) in overlay on plaque formation in CEF[b] cells

Isolate No.	PFU[c]/ml with trypsin	PFU/ml without trypsin	Ratio with/without trypsin
16	3.9×10^5	2.6×10^5	1.5
17	4.8×10^6	4.5×10^6	1.1
18	7.3×10^5	4.3×10^5	1.7
19	8.5×10^5	4.5×10^5	1.9
20	8.5×10^5	5.3×10^5	1.6
21	1.9×10^6	1.6×10^6	1.2
22	8.3×10^5	1.5×10^5	5.5
23	1.7×10^6	8.8×10^5	1.9
24	9.8×10^5	8.0×10^5	1.2
25	9.0×10^5	1.1×10^6	0.8
26	1.2×10^6	1.2×10^6	1.0
27	4.3×10^5	3.0×10^5	1.4
28	1.6×10^6	2.0×10^6	0.8
29	4.5×10^5	4.0×10^5	1.1
30	1.5×10^6	6.5×10^5	2.3
31	8.8×10^6	5.3×10^6	1.7
32	6.0×10^5	2.5×10^5	2.4
33	5.3×10^6	3.5×10^6	1.5

[a]Killed one to five of eight chickens.

[b]Chicken embryo fibroblast.

[c]Plaque forming units.

Table 10. Highly pathogenic[a] isolates: Effect of trypsin (10μg/ml) in overlay on plaque formation in CEF[b] cells

Isolate No.	PFU[c]/ml with trypsin	PFU/ml without trypsin	Ratio with/without trypsin
34	7.5×10^5	4.8×10^5	1.6
35	2.0×10^6	1.8×10^6	1.1
36	1.2×10^6	1.2×10^6	1.0
37	1.0×10^6	1.1×10^6	0.9
38	4.8×10^6	4.4×10^6	1.1
39	1.3×10^5	8.0×10^4	1.6
40	1.4×10^5	4.5×10^4	3.0
41	2.3×10^6	6.5×10^5	3.5
42	3.0×10^6	2.5×10^6	1.2
43	7.7×10^5	$<1.0\times10^1$	>10,000
44	2.8×10^5	2.8×10^5	1.0
45	4.5×10^5	4.8×10^5	0.9
46	1.6×10^5	1.5×10^5	1.1
47	1.0×10^5	4.0×10^4	2.5
48	3.6×10^5	2.5×10^5	1.4
49	2.0×10^5	2.3×10^5	0.9
50	2.5×10^6	1.5×10^6	1.7
51	6.0×10^5	3.5×10^5	1.7

[a]Killed six to eight of eight chickens.

[b]Chicken embryo fibroblast.

[c]Plaque forming units.

Table 11. Summary of *in vivo* and *in vitro* results for all viruses

Group (atypical isolates)	1st/2nd CI[a] % dead	Mean[b] MDT	Mean IVPI[c] (No.tested)	Isolates with Cleaved HA[d]/ plaques[f]	Plaquing Efficiency[e] (No.tested)
NPAI[g]	0/ND[h]	100	0.00 (5)	0/0	>10,000(11)
(Nos.3,6,7,8)	0/6	65	0.37 (4)	4/4	1.0 (4)
PAI[i]	43/31	70	1.07(10)	17/18	1.7(18)
HPAI[j]	93/ND	64	1.52 (9)	17/17	1.5(17)
(No.43)	88/0	72	0.00 (1)	0/0	>10,000 (1)

[a]Chicken inoculation.

[b]Mean death time in embryonating chicken eggs (hours).

[c]Intravenous pathogenicity index in 6-week-old chickens.

[d]Cleaved hemagglutinin as determined by radioimmunoprecipitation.

[e]Ratio of plaques observed when trypsin was added versus plaques observed without trypsin.

[f]Plaques observed in chicken embryo fibroblasts.

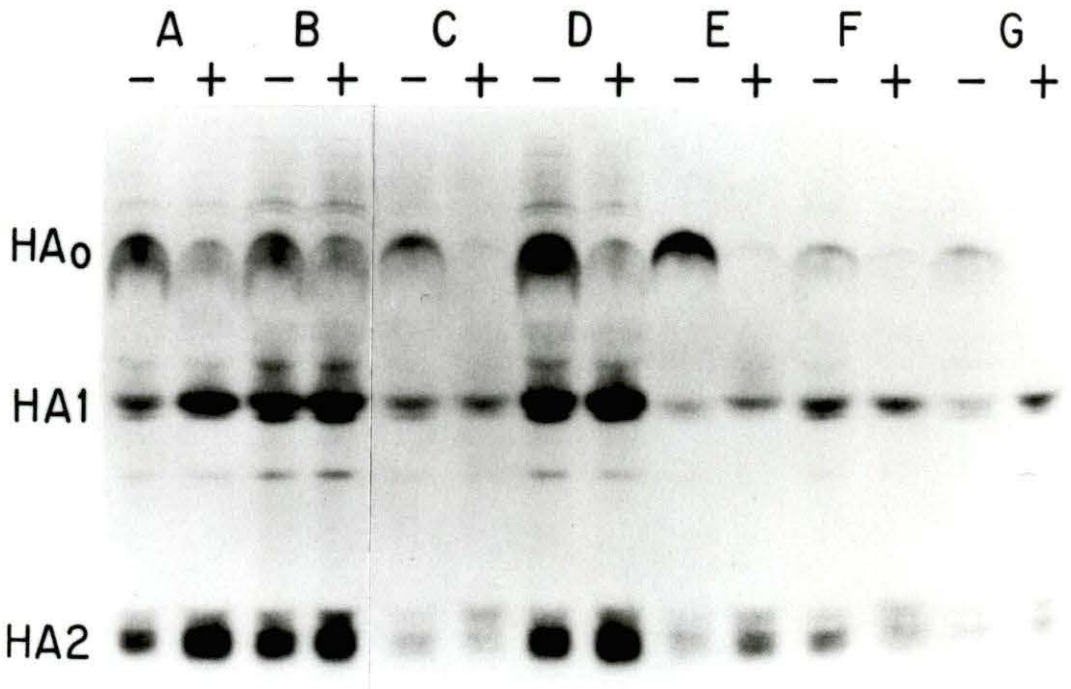
[g]Nonpathogenic avian influenza; did not kill any of the eight chickens.

[h]Not done.

[i]Pathogenic avian influenza; killed one to five of the eight chickens.

[j]Highly pathogenic avian influenza; killed six to eight of the eight chickens.

Figure 4. Radioimmunoprecipitation results of seven isolates grown in MDCK cells. Each virus was grown in the presence (+) and absence (-) of trypsin. Uncleaved (HA0) and cleaved (HA1 and HA2) hemagglutinin (HA) was separated by SDS-PAGE and detected by autoradiography. Lanes A-D, F, and G are examples of pathogenic or highly pathogenic isolates demonstrating partial or total cleavage of HA when grown in the absence of trypsin. Sample E is a nonpathogenic isolate demonstrating the lack of cleaved HA when grown in the absence of trypsin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DISCUSSION

The results of this study have indicated that *in vitro* correlates of pathogenity may be important in determining the pathogenic potential of H5N2 avian influenza viruses isolated from chickens in Pennsylvania in 1983. It has been well established that pathogenicity of avian influenza viruses is dependent upon the property of the HA polypeptide to be cleaved in a wide range of cell types (Bosch et al., 1979; Rott, 1981). The *in vitro* assays used in this study, therefore were aimed at detecting cleaved HA for viruses grown in CEF cells; a system usually nonpermissive for avirulent AI viruses unless the proteolytic enzyme trypsin is added. When results obtained from *in vitro* (plaque assay and RIP) assays were compared to the original pathotyping results in chickens, there was a good correlation between the presence of cleaved HA and pathogenicity; the results of 46 of 51 (90%) isolates were in agreement in this respect. In addition, *in vitro* assays detected cleaved HA with some isolates which did not kill chickens on subsequent inoculations (7 and 8).

There was very good agreement between results of the plaque assay test and the RIP procedure. These two *in vitro* tests were in agreement on 98% (50 of 51) of the isolates. The implications of this are significant in that it would

allow either test to be used to determine cleavability of HA, however, the plaque assay would be more practical for use as a diagnostic procedure when testing large numbers of viruses; such was the case during the 1983/1984 outbreak in Pennsylvania. Some of the limiting factors of the plaque assay would be the necessity of having cell cultures available at the correct stage of growth, and the need to titrate the virus before or at the time of testing (Alexander, 1987).

Although the ability of AI viruses to produce plaques in cell culture in the absence of trypsin has been correlated with the cleavage of the HA polypeptide and pathogenicity, other implications of this property can be postulated. Plaque size, for example, could relate to the speed of replication of a particular isolate; highly virulent viruses would be likely to spread rapidly from cell to cell, resulting in the formation of larger plaques compared to viruses which are less virulent. In this study, however, there was no significant difference in plaque size between NPAI, PAI, and HPAI viruses which demonstrated the ability to plaque without the addition of trypsin; although there were differences in the size of plaques observed for a given sample. In addition, the size of the plaques produced by the H5N2 viruses from Pennsylvania were increased 2 to 3 fold by

the addition of trypsin in the overlay (Figure 3). These results are in contrast to another study where the size of plaques formed by antigenically different AI viruses were related to virulence; i.e., highly virulent viruses produced larger plaques (Alexander et al., 1981). They also reported that the addition of trypsin in the overlay did not increase significantly the size of plaques formed by the highly pathogenic viruses, but that trypsin enhanced the size of plaques produced by viruses with intermediate and low virulence by 3 to 5 fold.

In addition to plaque size, plaquing efficiency values, obtained by determining the ratio of the number of plaques observed with and without added trypsin, can be used to determine the relative proportion of the progeny virus produced with cleaved HA. Viruses used in this study could be placed into two distintive categories based upon the plaquing efficiency data. All viruses which were unable to produce plaques in the absence of trypsin had plaquing efficiencies greater than 10,000 and would make up the first category. The second category would be made up of viruses with plaquing efficiencies between 0.8 and 5.5. There was no statistical difference in the plaquing efficiencies between the PAI and HPAI viruses; with mean plaquing efficiencies of 1.7 and 1.5, respectively (Table 11). These results were

similar to plaquing efficiencies of 1.4 and 1.2 reported for other HPAI viruses such as A/Ck/Germany/34, and A/Ck/Australia/75 (Alexander et al., 1981).

It has been reported that mixtures of AI viruses with different pathogenicity characteristics can affect expression of disease *in vivo*. Bean et al. (1985) reported that when the avirulent A/Ck/Pa/1/83 and virulent A/Ck/Pa/1370/83 H5N2 viruses were mixed (10^7 and 10^4, respectively) and inoculated topically into the nasal cleft of chickens, a considerable reduction in mortality was observed, when compared to the virulent virus inoculated by itself. However, in a similar experiment, when the same avirulent and virulent viruses were mixed as a 3:1 and 10:1 ratio (based on the observed plaquing efficiency ratios from this study) and inoculated into chickens via the CTAS route, no significant differences in mortality was observed (unpublished data). The discrepancy in observations in these two studies may reflect the difference in concentrations of virus used or the route of exposure. Although mixtures of viruses probably did occur in some AAF samples, the plaquing efficiency data presented in this study would not support interference as a significant factor contributing to the variability in chicken mortality, since most of the virus progeny of plaque forming isolates were produced with cleaved HA. Some of the variation in

plaquing efficiencies reported may, to some extent, be related to the difficulty in counting the very small plaques produced by these viruses when grown without the addition of trypsin.

The IVPI test also appears to be a good method for determining the pathogenicity of these viruses. The major advantage of the IVPI test would be that sick chickens, as well as dead chickens, are included in the numerical score. This is especially important with isolates which may produce severe clinical disease but are not lethal for chickens. On the other hand, the IVPI test is very labor-intensive because of the time required for IV inoculations, daily observations of test chickens and calculation of scores. In addition, this test also specifies the use of ten 6-week-old chickens, which would make it difficult to have adequate numbers of the correct age of chickens available at all times for pathotyping new isolates.

The results of this study have raised some serious questions as to whether the use of chicken inoculations alone should have been utilized in determining pathogenicity of chicken Pennsylvania H5N2 avian influenza viruses. Four nonpathogenic isolates (3, 6, 7, and 8) used in this study clearly had characteristics of PAI and HPAI isolates as determined by the ability to form plaques and produce HA in a

cleaved form when cell culture propagated viruses were examined. The plaquing efficiency of these isolates would also indicate that they are more characteristic of PAI and HPAI isolates (Table 11). In addition, there were some PAI and HPAI viruses which demonstrated *in vitro* characteristics consistent with other PAI and HPAI viruses but failed to kill chickens when inoculations were repeated. There does not appear to be an obvious explanation for this lack of pathogenicity on repeated inoculations of chickens. When the original CI mortality rate of PAI viruses was compared with the mortality rate of the second CI, there was a 12 percent decrease, from 43 percent to 31 percent (Table 11). Although, for a given PAI virus, the expected variation in the number of chickens dying on repeated inoculations could be as much as plus or minus three chickens, this overall decrease was significant ($p<0.05$) when evaluated using the chi-square statistic.

There were also two HPAI isolates in which conflicting results were observed. Isolate 43, by *in vitro* methods, appeared to be typical of NPAI isolates (Table 7). Results of the second CI would also support this classification. The most probable explanation for this discrepancy would be misidentification of the sample or a mixup in samples when the original CI was done. Contrariwise, HPAI isolate 45

exhibited the ability to produce plaques and cleaved HA but was not pathogenic for chickens when inoculations were repeated (Table 7). The IVPI and MDT on this isolate would place it in the PAI group.

The question of why there was such a variation in repeated chicken inoculation results remains an enigma. The implications of cleavage of the HA, and virus dose relating to pathogenicity have already been discussed. Other factors such as the generation of defective interfering (DI) particles; heterogeneous populations of pathotypes; genetic alterations; and prolonged storage of viruses may be important in expression of disease in the host. Bean et al. (1985) have reported the presence of subgenomic RNA (DI virus) in the original nonpathogenic H5N2 isolate from Pennsylvania. They have proposed that the presence of these defective viruses may affect the expression of disease *in vivo*.

Heterogeneity of virus populations has also become a popular concept to explain variability in chicken mortality. Brugh and Beard (1986) have isolated virulent subpopulations of virus from isolates previously characterized as nonpathogenic. These studies would suggest that perhaps expression of disease in the host may be related to the relative concentration of the subpopulation pathotypes

present in the isolate, i.e., if the predominant pathotype is pathogenic, then the isolate will be pathogenic, and vice versa. The concept of heterogeneity of AI viruses may be similar to that which has been recognized with NDV for many years. One last factor relating to the differences observed in chicken pathogenicity between the first and second inoculations, may be related to the length of time which had elapsed between the two trials, approximately 2 1/2 years. Allan (1981) has reported that after prolonged storage of highly pathogenic viruses either frozen at -70 °C or freeze-dried, the viruses may not exhibit their full virulence until passed several times through chickens. Similar observations have been made at the National Veterinary Services Laboratories, both with virulent AI and NDV (unpublished data). Although prolonged storage may be important, it cannot be the only factor responsible since several of the isolates killed more chickens on repeated than on the original inoculation.

The problems encountered with pathotyping H5N2 viruses isolated during the 1983/1984 outbreak in Pennsylvania points out the unreliability of the use of chicken inoculations alone to determine the pathogenic potential of these viruses. Additional studies will be needed to determine the reason for the lack of consistent mortality observed in replicate CIs

and to further evaluate *in vitro* assays as correlates of pathogenicity using other influenza A viruses.

Conclusion

It would appear that a combination of *in vivo* and *in vitro* assays may be necessary to adequately evaluate the pathogenic potential of H5N2 influenza viruses isolated from chickens during the 1983/1984 epizootic in Pennsylvania.

Summary

The pathogenic characteristics of 51 viruses isolated during the 1983/1984 outbreak of H5N2 avian influenza were determined using *in vivo* and *in vitro* procedures. Based on the original pathotyping results obtained by chicken inoculation, 15 of these viruses were classified as nonpathogenic avian influenza (NPAI); 18 were classified as pathogenic avian influenza (PAI); and 18 were classified as highly pathogenic avian influenza (HPAI). Results of chicken inoculations, and mean death time of virus-infected embryos were compared to the capability of the isolate to produce cleaved hemagglutinin and produce plaques in cell cultures without trypsin. Plaquing efficiency ratios of isolates were determined in cell cultures with and without added trypsin. Considerable variation in chicken mortality was observed when chicken inoculations were repeated. *In vivo* versus *in vitro* results agreed on 46 of 51 isolates; four of the isolates

with conflicting results were NPAI isolates which demonstrated *in vitro* characteristics compatible with PAI and HPAI isolates, and the fifth isolate was a HPAI virus which was more characteristic of NPAI isolates as determined by both *in vivo* and *in vitro* assays. There was 98 percent agreement between *in vitro* procedures, i.e., the ability or inability of the isolates to plaque in cell culture and the presence of cleaved or uncleaved hemagglutinin as determined by radioimmunoprecipitation assays. The findings of this study would indicate that a combination of chicken inoculation and plaque assay may be necessary to evaluate the true potential of H5N2 avian influenza virus to cause serious disease in poultry.

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APPENDIX A

Radioimmunoprecipitation (RIP) Reagents, Buffers, and Gels

Lysis buffer

Lysis buffer was prepared as a 0.05 M Tris (pH 7.5), 0.6 M KCL, and 0.5% Triton X-100 solution.

RIP Buffer

RIP buffer was prepared by dissolving 1.06 gm Tris (base), 8.77 gm NaCl, 0.37 gm EDTA, and 2.50 gm BSA (faction V) in approximately 950 ml distilled water. The mixture was then adjusted to pH 8.0 using HCl and the total volume adjusted to one liter. Sodium Azide was added to a final concentration of 0.1%.

Sample buffer

Sample buffer was prepared by adding 1.5 ml glycerol, 0.5 ml 2-mercaptoethanol, 3 ml of a 10% solution of sodium dodecal sulfate (SDS), 1.25 ml upper Tris buffer (4×), bromophenol blue (only to add color), and distilled water q.s. to 10 ml.

Reservoir buffer

Three hundred milliliters of Tris-glycine buffer (4×), 16 ml SDS (10%) and distilled water q.s. to 1.2 liters were combined and mixed.

Lower Tris buffer (4x)

Lower Tris buffer was prepared as a 1.5 M Tris-HCl (Ph 8.8), and 0.4% SDS solution.

Upper Tris buffer (4x)

Upper Tris buffer was prepare as a 0.5 M Tris-HCl (pH 6.8), and 0.4% SDS solution.

Tris-glycine reservoir buffer (4x)

The Tris-glycine reservoir buffer was prepared as a 0.1 M, pH 8.3 solution by combining 24 gm Tris, 115.2 gm glycine, and distilled water q.s. to two liters.

Sodium salicylate

Eighty grams of sodium salicylate was dissolved in 500 ml distilled water and stored in a brown bottle at room temperature.

Preparation of acrylamide-bis acrylamide

Thirty grams acrylamide (electrophoresis grade) and 0.8 gm methylene bis acrylamide were dispensed in 100 ml distilled water. The mixture was briefly heated to dissolve, then filtered through filter paper.

Lower gel

The 12% lower gel was prepared by adding 12.7 ml distilled water, 10.0 ml lower Tris (4x), 16.7 ml acrylamide-

bis acrylamide, 300 µl 2% ammonium persulphate and 25 µl TEMED.

Upper gel

The upper (loading) gel was prepared by adding 6.3 ml distilled water, 2.5 ml upper Tris (4×), 1.0 ml acrylamide-bis acrylamide, 150 µl 2% ammonium persulphate, and 34 µl TEMED.

APPENDIX B

Overlay Medium for Plaque Assay

Overlay medium was prepared as a 2× solution by combining the amounts of the following additives for every 100 ml of medium needed: 40 ml of 5× Eagle's MEM; 6 ml of sodium bicarbonate solution (7.5%) 4 ml MEM amino acids (50× concentrate); 2 ml MEM vitamin solution (100×); 2 ml L-glutamine (100× concentrate); 2 ml penicillin G-Streptomycin sulfate solution (5,000,000 units penicillin G and 1 gm streptomycin sulfate dissolved in 100 ml sterile distilled water); 2 ml mycostatin solution (500,000 units dissolved in 100 ml sterile distilled water); and 42 ml sterile distilled water. Prior to use, the 2× solution was added to an equal volume of sterile 2% Bacto-agar and equillibrated to 46 °C.