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Subtype identification and pathogenicity of avian influenza isolates from pet birds imported into the United States

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	3
MATERIALS AND METHODS	11
Test Birds	11
Embryonated Eggs	11
Propagation of Viruses	12
Propagation of Virus at Limit Dilution	12
Mean Death Time (MDT) for Classification of NDV Isolates	14
Reagents Used in Preparation and Test Procedures	14
Phosphate buffered saline (PBS)	14
Rooster RBCs	14
NI test reagents	15
Agar gel with 8.0% NaCl	16
Glycine sarkosyl buffer	16
Tris buffered tryptose broth (TBTB)	17
Antigen Preparation	17
Formalinized-RBC adsorbed and eluted virus	17
Sarkosyl disrupted antigen for A-RNP antiserum production	18
Preparation of isolates for A-RNP AGID procedure	19
Chorioallantoic membrane (CAM) antigen for A-RNP positive control	19
Antiserum Production	19
Influenza A-Ribonucleoprotein Typing Procedure	24
Hemagglutination-Inhibition Procedure	24
Neuraminidase-Inhibition Procedure	26
Antigen and Antiserum Verification	28
Typing and Pathogenicity Testing of Unknowns	29
Source of samples	29
Pathogenicity of isolates for chickens	31
Identification of isolates	31
RESULTS	36
Other Observations	44
DISCUSSION	48
Conclusions	52
Summary	53

REFERENCES 54

ACKNOWLEDGMENTS 61

INTRODUCTION

The outbreak of exotic Newcastle disease in southern California in 1971-2 brought into sharp focus the vulnerability of the United States (U.S.) poultry industry to diseases of foreign origin. The unprecedented attack by the viscerotropic velogenic Newcastle disease (VVND) virus was warded off only after a massive eradication effort by the poultry industry, the California Department of Agriculture, and the United States Department of Agriculture. As a result of this costly experience, a stringent set of procedures has been instituted for all birds being imported into the United States. A part of the safeguard system involves the quarantine of all imported pet birds and testing, by virus isolation procedures (40). This process has brought to light the fact that avian influenza viruses are being imported, thus exposing a more insidious problem in the possibility of not detecting imported progenitors with the genetic potential for producing a pathogenic recombinant influenza virus.

The virus isolation procedure (40) used to screen imported pet bird submissions at the National Veterinary Services Laboratories (NVSL) is designed to detect VVND virus. It has, however, demonstrated the occurrence of other hemagglutinating (HA) viruses at the rate of about 15 per thousand imported pet birds (39). Of the typed HA isolates that have not been identified as Newcastle disease virus (NDV), a substantial proportion has been identified as type A influenza viruses. This thesis is directed primarily toward the identification and further characterization of several isolates obtained from two groups of birds which were held in

quarantine. In addition, some methods are explored which might be incorporated into the scheme routinely used for identification of virus isolates which agglutinate chicken red blood cells (RBCs).

LITERATURE REVIEW

Fowl plague was first reported in Italy in 1878 and later described in many countries around the world. Its first appearance in North America was in 1924-25. It reappeared in 1929 in New Jersey, was eradicated, and has not reappeared in the United States since (18,60). In 1955, fowl plague virus was identified as a type A influenza virus (50). Since that time other antigenically distinct strains of influenza virus have been isolated from birds with illnesses varying from severe to inapparent (6, 7,16,17,24,27,30,35,42,46,55,56,57,63,76,78,79,84). Concomitantly, Newcastle disease was described by several workers in different parts of the world between 1926 and 1930 and, by the late 1940's, had been recognized in virtually every country of the world. The first U.S. occurrence was reported in 1944. By that time there were two established types of the disease--the relatively mild form common in the U.S. and a more virulent form which was common in Europe and Asia. A highly virulent form arose in the Middle East in the early 1960's and by 1972 had spread to all continents, causing serious losses (23). In 1971 the highly virulent form reached the U.S. for the 19th time (the first 18 introductions being uneventfully eradicated) to cause a very costly disease outbreak in southern California (65,66).

The ability to identify and classify the agents which cause fowl plague and Newcastle disease grew out of some very basic discoveries made by investigators working with the agents responsible for human influenza. These discoveries have led to the present systems of identification and nomenclature for the influenza viruses and their close relatives, the

paramyxoviruses. The ability to accurately subtype myxoviruses and to study them epidemiologically has its origin in the discovery of the hemagglutination phenomenon by Hirst in 1941 (25). In the ensuing thirty years, a succession of discoveries and experiments 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" (68), and a committee of the American Association of Veterinary Laboratory Diagnosticians (15) published its recommended standard procedures for the identification of NDV. The scheme for influenza viruses requires that any isolate, to be properly classified, must be identified according to the antigenic type of its ribonucleoprotein (RNP) (A, B, or C) and, for any influenza A virus, the antigenic character of its hemagglutinin and neuraminidase subtype. Among the identification methods listed are RNP typing by the use of immunoprecipitin tests in gels and envelope antigen subtyping by hemagglutination inhibition (HI) and neuraminidase inhibition (NI) procedures. An HI test also plays a predominant role in the NDV scheme.

The first report of the agglutination of RBCs by any virus was published in 1941 by Hirst (25) who wrote:

> "When the allantoic fluid from chick embryos previously infected with strains of influenza virus was being removed, it was noted that the red cells of the infected chick, coming from ruptured vessels, agglutinated in the allantoic fluid."

He went on to show that specific antiserum could inhibit the agglutination. In 1942 Burnet (9), in an attempt to apply Hirst's discovery in a virus

classification scheme, showed that NDV had similar HA properties to influenza virus. Also, based on Hirst's discovery, Salk in 1944 (47) published the basic HA and HI test procedures in use today. Salk used a tube dilution scheme and established the HA titer as being the reciprocal of the highest dilution of infected amnionic-allantoic fluid (AAF) or mouse lung suspension that gives complete agglutination of chicken RBCs. He then used the endpoint HA dilution, which he defined as one HA unit, as the standard virus dilution to be used in the HI procedure. He defined the HI titer of a serum as being the reciprocal of the highest serum dilution causing complete inhibition of agglutination by 1 HA unit of virus. In the same work, he showed the necessity of using a standardized volume and concentration of RBC suspension in both procedures. By 1959 standard tube and plate techniques were established (69). In 1971 and in 1975, the current standard protocols for the microtitration HA and HI methods were published for NDV (15) and influenza virus (64), respectively. The only major change from Salk's method is that four HA units are used in today's HI procedure for influenza virus (64) and ten HA units for NDV (15).

At this juncture, it is appropriate to go back in time to 1940 when Francis (20) published his report describing the differences in serologic response to, and protective immunity against, two types of influenza virus recovered from man. The "Lee" strain proved to be sufficiently different from other strains of epidemic influenza virus to warrant the establishment of two categories. The epidemic strain was designated as influenza A and the Lee strain as influenza B. In 1949, Taylor (62) published results of a survey in which an isolate designated as "1233"

was recovered from throat washings taken at a boys' school. The following year Francis, Quilligan, and Minuse (21) published results of their work with an isolate recovered from a case of human respiratory disease which they designated "JJ". They found the "JJ" isolate to be identical to Taylor's 1233. The two new isolates were serologically different from influenza A and influenza B strains, and they suggested the isolates be classed influenza C.

Another significant line of investigation was unfolding during the years that procedures for HI and RNP identification of influenza viruses were being developed. As a result of experimentation which followed his initial observation of hemagglutination, Hirst in 1942 (26) suggested that influenza hemagglutinin might be an enzyme that first attaches to a receptor site on the red cell surface and then proceeds to destroy that site, thus eluting the virus. Expanding on Hirst's work, Burnet (10) reported in 1945 that NDV likewise elutes following agglutination of red cells, and in 1946 (11) he suggested that the elution of NDV and influenza virus must be enzymatic. Briody in 1948 (8) showed that influenza's "elutability" was heat labile at 52° C, but the "Hemagglutinative titer" was unaffected by the same treatment. He also demonstrated the similarity of receptor-destroying factors (now commonly known as receptor-destroying enzyme or RDE) from _Vibrio cholerae_ with the enzymic action of influenza viruses and NDV.

In 1958, Gottschalk (22) published a review of work of his own and others showing that the enzymic action of influenza viruses and RDE resulted in the release of neuraminic acid. He concluded that "the influenza virus enzyme and the isodynamic vibrio enzyme may, therefore,

be defined as neuraminidases".

Studies of neuraminidase activity required an assay method. In 1959, Warren (70) and Aminoff (2) independently developed techniques in which the enzymic 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 in 1961 (3) published a quantitative assay method for NANA which has become the basis for methods used today.

Further elucidation of the structure of myxoviruses occurred in 1961 when Mayron et al. (34), using trypsin, separated the hemagglutinin and neuraminidase activities of two influenza A viruses. Then, in 1962, Noll et al. (37) accomplished the same with the Lee strain of influenza B virus. In 1963, Laver (31), using sodium dodecyl sulphate, effectively disrupted influenza B virus (Lee strain) resulting in a subunit with neuraminidase activity which could be isolated by electrophoresis. In the same investigation, viruses treated with deoxycholate resulted in two subunits, partially separable by electrophoresis, one of which had hemagglutinin and the other neuraminidase activity. The neuraminidase subunit was physically comparable to that which had been isolated by trypsin treatment. Laver then suggested that "neuraminidase and hemagglutinin activities of the virus reside in separate covalently bonded structures" (31).

With the advent of recombinant viruses, the history of which will not be reviewed here, Laver and Kilbourne (32) in 1966 were able to use virus hybridization to separate hemagglutinin and neuraminidase subunits of two influenza A viruses. They wrote, "It is now possible to produce

antisera specific for viral neuraminidase that may be employed in divorcing the biological effect of hemagglutinin and enzyme". The possibility became reality in 1968 when Kilbourne et al. (29) reported the production of an antiserum against an isolated neuraminidase subunit. Inhibition of the neuraminidase enzyme activity by specific antiserum was developed as one of the assay methods for that work.

By 1975, Scheid and Choppin (51) had worked out the structure of the NDV envelope proteins to the point of being able to state that the hemagglutinin and neuraminidase reside in a single glycoprotein molecule thus establishing the basis for major differences between the serologic behavior of orthomyxoviruses (influenza viruses) and paramyxoviruses (includes NDV).

A detailed standard NI procedure was published in 1973 (4) by which one can make rather precise measurements of the enzyme activity of a virus and the inhibition of that activity by appropriate antiserums.

During the 1960's, a very sensitive new method for either antigen or antibody detection, known as radioimmunoassay (RIA), was developed. In 1972 (19), an innovative variation of the RIA method opened the way to development of equally sensitive serologic tests for microbial disease agents. Sufficient background for the purposes of this paper begins in 1966-7 when Catt et al. in Australia (12,13) published a method for RIA in which antibody was adsorbed to a plastic solid phase and then used to detect antigen. In an independent effort in 1966, Wide and Porath in Sweden (82) developed a solid phase system in which antibody attached to Sephadex (Pharmacia, Uppsla, Sweden) was used in an RIA antigen detection technique. Catt and Tregear (14)

went on to develop their solid-phase RIA methods to the point of using plastic tubes to which antibody was adsorbed. This innovation provided a method which "is simple, rapid, inexpensive, and suitable for automation". In all of the above RIA methods, the antigen in question was added to the solid-phase-bound antibody. Following an appropriate incubation period during which the unknown antigen had an opportunity to be bound by the antibody, the solid phase could be washed, thus removing all antigen remaining in solution. This left only specifically bound antigen attached, via the antibody, to the solid phase. A radioactive isotope-labeled antigen of known type was then added to the system. Following another incubation period, the solid phase was again washed to remove unbound, labeled antigen. If the unknown antigen was specifically attached to the antibody, the attachment of labeled antigen was blocked. This resulted in a measurable reduction in radioactivity when compared to controls.

In 1967, Wide et al. (80) went on to develop their radioallergosorbent test or "RAST" in which they attached a known antigen to a polymer, then allowed this bound antigen to react with an unknown test serum. After a wash step, an antispecies ^{125}I-labeled antiglobulin was added. After another wash, the end-product was examined for radioactivity due to ^{125}I, the presence of which indicated that the test antiserum contained antibody against the known antigen.

In 1972, Engvall and Perlmann (19) published a technique for detecting specific antibody in unknown test sera which was a combination of the solid phase tube idea of Catt and Tregear (14) and the RAST method of Wide et al. (80). The new method, however, replaced the radioactive

isotope-labeled antibody as the indicator system with an alkaline phosphatase-conjugated antibody, hence the name "enzyme-linked immunosorbent assay" or ELISA.

The test was read by determining whether or not the conjugated enzyme was present after the final wash. This detection was accomplished by adding p-nitro-phenylphosphate, a colorless substrate for the enzyme which, if present, hydrolyzed bound phosphate and liberated p-nitro-phenolate, which is yellow.

In 1974, Saunders and Wilder (49) published a technique for screening swine serums for hog cholera using a horseradish peroxidase (HRP)-conjugated antispecies antibody. The indicator system in this technique was guiacol which results in a brown reaction product upon enzyme catalysis. A number of substrates for HRP have since been evaluated with a solution of H_2O_2 and 2,2-azino-di-(3-ethylbenzo-thiazoline-6-sulphonic acid) (ABTS) emerging as a superior one (59). Saunders (48) has recently published a microtechnique adaptation of the hog cholera screening test which uses ABTS as the substrate for the indicator system.

Recently published adaptations of ELISA include a method for detection of two viruses pathogenic to plants (67)--a semi-automated technique for detection of hog cholera antibody in swine sera (58) and a method for serotyping herpes simplex virus isolates (36). Thus, ELISA has emerged as a new tool for the identification of viral antigens or serum antibodies.

MATERIALS AND METHODS

Test Birds

All chickens were from the National Animal Disease Center (NADC) closed random-bred white leghorn flock. Twenty randomly selected hens were tested for influenza HI antibodies and for evidence of anti-A RNP antibodies by HI and agar gel immunodiffusion (AGID) procedures described below. No evidence of influenza infection was found. The NADC flock is free also of NDV (for which it is regularly tested). All turkeys were from the NADC closed, random-bred, small Beltsville white flock which is regularly tested and free of NDV. Parakeets were obtained from Robinson's Pet Shop in San Francisco, CA. Conures were donated by Pet Farm, Inc., Bernard Levine, Miami, FL. The parakeets and conures were apparently healthy but were of unknown origin. All were tested for HI antibody against Hav4 influenza hemagglutinin prior to challenge and found to be negative.

All birds in this study were maintained in isolation facilities consisting of plastic cages maintained under negative air pressure with individual filtered air intakes and with individual feed and water supplies. The room containing the cages likewise was maintained under negative air pressure with approximately nine complete air changes each hour. This latter feature permitted handling of different viruses in the same room, provided 15 minutes elapsed between opening of cages.

Embryonated Eggs

Embryonated 8 to 12 day chicken eggs were purchased from Larson Lab-Vac Eggs, Inc., Gowrie, Iowa. The source flock is certified free of

NDV and type A avian influenza virus, among other things.

Propagation of Viruses

All viruses were propagated in 8 to 11 day embryonated chicken eggs inoculated via the allantoic route (AR). The dose varied from 0.05 ml to 0.3 ml depending on the dilution of the inoculum. All eggs were candled immediately prior to inoculation and daily thereafter for embryo viability. The AAF was harvested either upon the death of the embryo or (depending on the intended use of the AAF) on the third, seventh, or tenth day post inoculation (PI) if the embryo survived. All harvested fluids were maintained in a -70° C freezer.

The prototype and recombinant viruses used in this study as well as the antigenic subtype and source for each are listed in Table 1.

Propagation of Virus at Limit Dilution

To assure isolates were single subtypes, a limit dilution of each was made based on the following scheme: the AAF was first titrated by the HA method and the HA endpoint dilution selected as the starting point for five serial tenfold dilutions. Two-tenths ml of each dilution was inoculated into each of five 8 to 11 day embryonated chicken eggs by the AR method described above. The eggs were candled daily for seven days. AAF was individually harvested and saved from each embryo that died. Eventually, AAF from a single embryo from among the five receiving the highest virus dilution causing embryo death was selected and used for further propagation of virus for identification.

Table 1. Influenza viruses used in this study

Name[a]	Subtype	Source[b]
(F) A/Ty/Ore/71	Hav1 Neq2	1
(R) Ty/Ore/71-Port Chalmers/73	Hav1 N2	2
(P) A/Ck/Germany "N"/49	Hav2 Neq1	2
(F) A/Quail/Ithaca/1117/65	Hav2 Neq2	1
(F) A/Dk/Memphis/546/74	Hav3 Nav6	2
(R) Dk/Eng/56-Bel	Hav3 N1	2
(P) A/Dk/Czech/56	Hav4 Nav1	2
(F) A/Dk/Eng/62	Hav4 Nav1	1
(F) A/Mynah/Mass/71	Hav4 Neq2	1
(R) Dk/Czech/56-Bel	Hav4 N1	2
(F) A/Tern/So. Africa/61	Hav5 Nav2	2
(P) A/Ck/Scotland/59	Hav5 N1	1
(F) A/Dk/Penn/69	Hav6 N1	1
(F) A/Shearwater/Australia/1/73	Hav6 Nav5	2
(P) A/Dk/Ukr/1/63	Hav7 Neq2	1
(R) Dk/Ukr-Bel	Hav7 N1	2
(P) A/Ty/Ontario/6118/67	Hav8 Nav4	1
(P) A/Ty/Wisconsin/1/66	Hav9 N2	1
(R) Ty/Wisconsin/1/66-Bel	Hav9 N1	2
(P) A/Dk/Alberta/60/76	Hav10 Nav5	2
(R) Dk/Alberta/60/76-Bel	Hav10 N1	2
(R) Eq1-Sw/Tenn/3/76	Heq1 N1	2
(R) Nws-Eq1	HØ Neq1	2
(R) Nws-Eq2	HØ Neq2	2
(R) Nws-Dk/Eng	HØ Nav1	2
(R) Nws-Nav2	HØ Nav2	2
(R) Nws-Nav5	HØ Nav5	2
(R) Nws-Dk/546	HØ Nav6	2

[a](P) = prototype strain; (F) = field isolate; (R) = recombinant strain.

[b]1 = NVSL Repository, originally obtained from B. C. Easterday, Madison Wisconsin; 2 = WHO Typing Center, St. Jude Children's Research Hospital, courtesy of Dr. R. G. Webster.

Mean Death Time (MDT) for Classification of NDV Isolates

The MDT procedure as described by the National Academy of Sciences Subcommittee on Avian Diseases (61) was followed. It entailed inoculating two sets of twenty-five 9- or 10-day-old embryonated eggs, 5 with each of 5 dilutions of AAF (10^{-1}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}), using 0.1 ml of inoculum per egg via the allantoic route. 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 37° C, candled at 8:00 a.m. and 4:00 p.m. each day for the ensuing 5 days, and the time of death of each embryo recorded. The minimal lethal dose (MLD) was identified as the virus dilution which caused all 10 embryos to die. The MDT was then calculated by dividing by ten the sum of the hours PI that it took for those embryos to die. If the MDT was less than 60 hours, the NDV was classed as a velogenic strain; Between 60 and 90 hours, a mesogenic strain; or more than 90 hours, a lentogenic strain.

Reagents Used in Preparation and Test Procedures

Phosphate buffered saline (PBS)

Unless otherwise stated, PBS refers to 0.01 Molar phosphate buffered saline, pH 7.2.

Rooster RBCs

Rooster RBCs were obtained fresh each week from donor birds in the NADC flock. Rooster blood was collected directly into an equal volume of Alsever's solution. The cells were then washed by centrifugation and resuspension in PBS three times. Packed cells were stored at 4° C until

needed. A 0.5% suspension of RBCs in PBS was used for all HA and HI procedures.

NI test reagents

The NI test reagents listed below were modified from those outlined in the United States Public Health Service (PHS) Procedural Guide (64). Inasmuch as no spectrophotometric readings were required, sodium sulfate which is required for butanol extraction procedures was omitted.

Arsenite reagent   Ten gm of sodium arsenite was added to 100 ml of distilled water and mixed to dissolve. To this, 0.3 ml concentrated sulfuric acid was added and mixed.

Periodate reagent   A 4.28 gm quantity of sodium metaperiodate was added to 38 ml distilled water and mixed to dissolve. Sixty-two ml of concentrated orthophosphoric acid was then added and mixed. This reagent was protected from direct light during storage.

Thiobarbituric acid   Three gm of thiobarbituric acid was added to 500 ml distilled water. This was warmed in a boiling water bath and mixed to dissolve. This reagent was protected from direct light during storage.

0.4 M Phosphate buffer, pH 5.9   Four-tenths Molar Na_2HPO_4 was prepared by dissolving 56.78 gm of the dry salt in about 900 ml distilled water in a volumetric flask. Distilled water was added to make one liter of final solution. A 0.4 M $NaH_2PO_4 \cdot H_2O$ solution was made by dissolving 55.20 gm of the salt to make one liter of final solution in distilled water. The buffer was then made by mixing 190 ml of the Na_2HPO_4 solution with 180 ml of the $NaH_2PO_4 \cdot H_2O$ solution. The pH was adjusted to 5.9 by

adding 0.4 M Na_2HPO_4 to raise the pH or 0.4 M $NaH_2PO_4 \cdot H_2O$ to lower it.

Fetuin The following preparation method was provided by Hinshaw (Department of Virology, St. Jude Children's Research Hospital, Memphis, TN, personal communication, 1978): after dissolving 0.5 gm fetuin in 20 ml distilled water, 20 ml of 0.4 M phosphate buffer pH 5.9 was added. The solution was stored at -20° C.

Agar gel with 8.0% NaCl

The gel was prepared by adding 0.7 gm Oxoid Agar (Oxoid Ltd., London) and 8.0 gm NaCl to 100 ml PBS. This was heated and mixed using a heated magnetic stirrer to dissolve, then autoclaved 20 minutes at 15 psi.

Glycine sarkosyl buffer

A 10% solution of sodium lauryl sarcosinate (SLS) was prepared according to a procedure supplied by Pryzgoda (CIBA-GEIGY Corp., Greensboro, NC; personal communication; 1977) by adding 10 gm of "Sarkosyl-L" (donated by Dystuffs & Chemical Division, CIBA-GEIGY Corp., Greensboro, NC) to 70 ml distilled water. The Sarkosyl-L was dissolved by slowly adding 10% NaOH to the mixture while it was continuously stirred using a magnetic stirrer. The pH was adjusted to 7.5 by the drop-by-drop addition of either 10% NaOH to raise or 10% H_2SO_4 to lower the pH. The solution was then transferred to a 100 ml volumetric flask and distilled water added to make 100 ml final volume.

Glycine sarkosyl buffer was then prepared according to the procedure published by PHS (64). Approximately 20 ml of distilled water and 0.925 gm of glycine were placed in a 25 ml volumetric flask and swirled until the glycine was completely dissolved. The pH was adjusted to 9.0 with 1 N NaOH

and 2.5 ml of 10% SLS was added. The final volume was adjusted to 25 ml with distilled water and the buffer was stored at 4° C.

Tris buffered tryptose broth (TBTB)

Ordinary tryptose broth was buffered to pH 7.5 by the addition of 1.21 gm Trisna base (Sigma Chemical Co., St. Louis, MO), Tris, per liter. The pH was then adjusted to 7.5 by addition of 0.1 N NaOH.

Antigen Preparation

Formalinized-RBC adsorbed and eluted virus

When necessary, virus concentration and removal of most of the extraneous AAF protein was accomplished by adsorption onto and elution from formalinized rooster red blood cells (F-RBCs) prepared according to Weinbach's technique for sheep RBCs, as translated and reported by Wide and Gemzell (81), which was modified:

> F-RBCs for virus adsorption were prepared using packed rooster RBCs which were diluted to 8% in PBS. Equal volumes of 8% cells and 3% formalin (3% solution of 37% formaldehyde in PBS, pH adjusted to 7.2 with 1 N NaOH) were combined and incubated 18-20 hours at 37° C with continuous stirring. The bottom of the flask was insulated to prevent overheating due to contact with the stir plate. The cells were washed four times by centrifugation at 500-600 Xg, and resuspended in distilled H_2O, except after the fourth centrifugation. The packed cells were finally resuspended to 10% in PBS containing 1:10,000 merthiolate. The

suspension was stored at 4° C.

Adsorption and elution of virus was performed according to Pirtle's method (41):

For each 10 ml of AAF to be treated, one ml of 10% F-RBCs was centrifuged (1500 Xg/10 min) and the supernate discarded. The AAF was then added to the packed F-RBCs and the cells resuspended. The mixture was incubated at 4° C for 1 hour, during which it was shaken every 15 minutes, to allow attachment of virus to the F-RBCs. The mixture was then centrifuged (1500 Xg/10 min) and the supernate discarded. The F-RBCs were washed once with cold sterile PBS, centrifuged again, and the supernate discarded. PBS was added to make 1/10 the original volume of AAF and the cells resuspended and incubated in a 37° C waterbath for 2 hours, shaking every 15 minutes, to allow elution of the virus from the F-RBCs. The F-RBCs were then removed from the eluate by centrifugation and the supernate stored at -70° C.

<u>Sarkosyl disrupted antigen for A-RNP antiserum production</u>

Stock virus was concentrated by the F-RBC technique described above and pelleted by ultracentrifugation at 27,000 RPM for one hour. The supernate was discarded and the sides of the tube rinsed once with sterile PBS. The pellet was then resuspended in 0.1 ml of glycine Sarkosyl buffer. This mixture was left at room temperature for one hour, after which 2 ml of sterile PBS was added. The antigen was stored at -70° C until used.

Preparation of isolates for A-RNP AGID procedure

The PHS (64) procedure for isolate preparation was performed using 10 ml of chilled AAF. The pH was reduced to 4 by the addition of approximately 0.3 ml 1 N HCl. This mixture was held in an ice bath for one hour and then centrifuged at 1000 x G for 10 minutes at 4° C. The supernate was removed and the tubes inverted and drained to remove all fluid. The packed precipitate was resuspended in 0.1 ml of glycine Sarkosyl buffer which disrupted the virus, thus exposing the RNP. The preparation was frozen at -70° C until used.

Chorioallantoic membrane (CAM) antigen for A-RNP positive control

Positive control antigen used for the A-RNP AGID procedure was produced by the Avian Viruses Unit of the Diagnostic Virology Laboratory at NVSL according to a modification of Beard's (5) procedure.

Ten-day embryonated chicken eggs were inoculated with A/Ck/Scot/59 (Hav5 N1). The CAM was harvested 3 days PI, rinsed in sterile PBS, then homogenized. The homogenate was then subjected to three freeze-thaw cycles followed by 3 one-minute 20 KH_2 bursts using a Bronwill Biosonik IV (VWR Scientific, San Francisco, CA) sonicator. The suspension was then centrifuged to remove tissue debris. Formaldehyde was then added to the supernate to give a 0.1% final concentration. The mixture was incubated at 37° C for 36 hours, and finally bottled in 2 ml amounts and frozen at -70° C for storage.

Antiserum Production

Antiserums, unless otherwise indicated, were either on hand at NVSL, or prepared as part of this work. In either case they were produced in

8-week-old roosters by one of the following procedures:

1. Immunization with virulent viruses

   When using a virus which caused clinical disease in chickens, four birds were inoculated with an initial 1 ml intranasal dose using a 10^{-3} or 10^{-6} dilution of AAF in sterile PBS, depending on virulence of the subtype. Three days later a 1 ml dose containing a tenfold greater concentration of the same material was given intravenously (IV) to each bird. Ten days later a blood sample was drawn from each bird and tested for HI and/or NI activity. If the titer was above HI $\geq$ 1:64 and/or NI $\geq$ 1:10, the bird was exanguinated the following day. If the titers were below these minimums, a second IV dose of virus was administered and the bird exanguinated after an additional ten days.

2. Immunization with avirulent viruses

   For viruses that would not cause clinical disease in chickens, 5 ml of AAF virus preparation was administered IV to each of four birds. Ten days later a blood sample was drawn from each bird and an HI and/or NI test performed. The birds were either exanguinated or reinoculated based on the same criteria as for virulent viruses (above).

3. Immunization for anti A-RNP serum production

   A rooster was initially inoculated by the IV route with 5 ml of AAF containing Heql-Bel (Heql Nl) with an HA titer of 1:128. A second equivalent inoculation with the same virus was administered IV two weeks later. Following another two week period, a blood sample was taken and the resulting serum tested for

anti A-RNP activity by the AGID method. A weak positive reaction was observed at that time.

A Sarkosyl-disrupted A-RNP antigen was prepared starting with 20 ml of AAF containing A/Shearwater/Australia/1/73 (Hav6 Nav5) with an HA titer of 1:128. The entire amount of resulting antigen was administered to the bird IV. Five days later a blood sample was taken and the serum checked for anti A-RNP activity. At this time the serum gave an optimum reaction in the AGID test when diluted 1:32 and the bird was exanguinated.

Following harvest, serums were treated by one of the following methods:

1. Serums for use in HI test procedures were treated with RDE for 18-20 hours and heat inactivated at 56° C for 30 minutes according to the PHS protocol (64).
2. Serums for the NI test were heat inactivated at 56° C for 30 minutes.
3. Serum for the AGID-RNP test was diluted 1:32 with fetal calf serum.

All serums were stored at -20° C.

Table 2 lists the name and antigen subtype designation of the viruses used for inoculation of HI antiserum production birds. The table is divided to distinguish antiserums on hand at the outset of the work from those produced or obtained during this work. Table 3 lists the name and antigenic subtype designation of the viruses used for inoculation of NI antiserum production birds. All were produced as a part of this study.

Table 2. Antigens used for HI subtype antiserum production

H Type	Virus	Surface antigens
	On hand at NVSL	
Hav1	A/Ty/Ore/71	Hav1 Neq2
Hav2	A/Quail/Ithaca/1117/65	Hav2 Neq2
Hav3	A/Dk/Eng/56	Hav3 Nav1
Hav4	A/Dk/Eng/62	Hav4 Nav1[a]
Hav5	A/Ty/Wisc/68	Hav5 Nav6
Hav6	A/Ty/Ontario/63	Hav6 Neq2
Hav7	A/Dk/Ukr/1/63	Hav7 Neq2
Hav8	A/Ty/Ontario/6118/67	Hav8 Nav4
Hav9	A/Ty/Wisc/66	Hav9 N2
	Produced	
Hav1	Ty/Ore/71-Port Chalmers/73	Hav1 N2
Hav2	A/Ck/Germany "N"/49	Hav2 Neq1
Hav3	Dk/Eng/56-Bel	Hav3 N1
Hav4	Dk/Czech/56-Bel	Hav4 N1
Hav5	A/Tern/So. Africa/61	Hav5 Nav2
Hav6	Ty/Mass-Bel	Hav6 N1
Hav7	Dk/Ukr-Bel	Hav7 N1
Hav8	None produced--A/Ty/Ont/6118/67 antiserum used	
Hav9	Ty/Wisc/1/66-Bel	Hav9 N1
Hav10	Dk/Alb/60/76-Bel	Hav10 N1
Hav4	A/Mallard/Alberta/43/77[b]	Hav4 Nav1

[a]Later found to be Hav4 Neq2.

[b]Obtained from Dr. V. Hinshaw, Dept. of Virology, St. Jude Children's Hospital, Memphis, TN.

Table 3. Antigens used for NI subtype serum production

N Type	Virus	Surface antigens
N1	Eq1-Sw/Tenn/71	Heq1 N1
N2	X-7 Nws-N2/1957	HØ N2
Neq1	Nws-Eq1	HØ Neq1
Neq2	Nws-Eq2	HØ Neq2
Nav1	Nws-Dk/Eng	HØ Nav1
Nav2	Nws-Nav2	HØ Nav2
Nav4	A/Ty/Ont/6118/67[a]	Hav8 Nav4
Nav5	Nws-Nav5	HØ Nav5
Nav6	Nws-Dk/546	HØ Nav6

[a]There is no Nav4 recombinant available at present so the prototype virus was used.

Influenza A-Ribonucleoprotein Typing Procedure

The agar gel immunodiffusion procedure for influenza A-RNP antigen detection used was a modification of the procedure published by Beard (5). The test was performed using 60 mm disposable plastic Petri dishes. Five ml of hot 8% NaCl agar was poured into each plate and allowed to cool. Once solidified, three patterns, each consisting of six wells surrounding a central well, each 3 mm in diameter, were cut into the agar. The distance from the edge of the center well to the nearest edge of each peripheral well was 2 mm.

Type A influenza virus identification was accomplished by filling the center well with A-RNP antiserum, and alternately filling each peripheral well with control positive CAM antigen and unknown virus preparations. The plates were then incubated overnight at room temperature in a sealed moist chamber. The plates were examined the following morning by illuminating from below to allow easy observation of precipitin lines. A positive was interpreted as being any line that formed between the serum well and a well containing unknown antigen, provided the line was continuous with the control positive line. This included lines that were no more than slight "hooks" at the ends of the flanking control positive lines.

Hemagglutination-Inhibition Procedure

The HI procedure used for identification of hemagglutinin subtypes was essentially that published in the PHS procedural guide (64).

All unknown and positive control AAF virus preparations were diluted with PBS to a concentration of four HA units in 0.025 ml. This dilution was confirmed during the HI procedure by placing two drops (0.05 ml) of

each virus preparation in the first well of a six-well row which were later serially diluted twofold in PBS. A 0.05 ml drop of 0.5% rooster RBCs was then added and the HA pattern observed 30 minutes later.

All typing serums were diluted with PBS to obtain a 1:64 HI titer when tested in equal volumes against four HA units of control positive antigen.

The HI procedure was carried out in 96-well U-bottom microtitration trays. Using a standardized dropper, one drop (0.025 ml) of virus preparation was placed into each of 12 wells in a sufficient number of rows to allow testing with each typing serum selected. One drop (0.025 ml) of typing serum was placed in the first well of the appropriately labeled row. Twofold dilutions of serum were then made by carrying 0.025 ml serially, well-to-well, in antigen using microtitration dilution loops. The trays were then covered and left at room temperature for one hour to allow sufficient time for antibodies to attach to the virus hemagglutinin.

At the end of the hour, a 0.05 ml drop of 0.5% rooster RBCs was added to each well. The trays were then sealed with clear tape and incubated another 30 minutes at room temperature. The trays were then examined for presence or absence of hemagglutination in each well and the results recorded.

An HI subtype identification of an unknown virus was made based on certain criteria. It was assumed that the subtype was identified if hemagglutination by the isolate was inhibited by a typing serum, and if the HI titer with that serum was no less than one twofold dilution lower than that observed with the homologous control antigen. A further

requirement was that HI titers had to be at least fourfold lower with all the other typing sera when compared to the titers obtained with their respective homologous control antigens.

Steric inhibition caused by antibody to the isolate's neuraminidase could give a false positive HI result. This was avoided mainly by use of typing sera made against recombinant viruses. If steric inhibition was still suspected, a different set of HI typing serums was used based on the results of NI typing of the isolate and avoidance of that specific type of neuraminidase antibody in the HI typing serums.

Neuraminidase-Inhibition Procedure

Identification of the viral neuraminidase subtype was carried out using the NI screening procedure published by Webster and Campbell (72).

Each AAF containing an unknown isolate was first diluted with PBS using the following scheme based on HA titer:

HA titer of AAF	Dilution for NI test
<1:128	undiluted
1:128-1:512	1:2
≥1:512	1:4

The entire procedure was run in duplicate as follows:

For each isolate to be typed, two 11 x 75 mm disposable tubes were labeled and placed in a rack for each of the antiserums it was to be tested with. The test always included a negative control serum. Appropriate positive and negative control antigens were run with each antiserum to

assure specific reactivity of the antibodies. A 0.05 ml aliquot of standardized antiserum was then placed directly into the bottom of each of the appropriate tubes using a Pipetman (Gilson, France) P 200 pipetter. In similar fashion, 0.05 ml of the above virus dilution was placed directly into the bottom of each appropriate tube. The tube racks were covered to prevent evaporation and incubated for one hour at room temperature to allow antibody to attach to the viral neuraminidase. A 0.1 ml aliquot of fetuin solution was then added to each tube by means of a Cornwall (Becton-Dickinson and Co., Rutherford, NJ) syringe. All tubes were placed in a 37° C water bath for 30 minutes to allow any unbound viral neuraminidase to catalytically remove NANA from the fetuin substrate. One-tenth ml of periodate reagent was added to each tube using a Cornwall syringe and the tubes incubated for exactly 20 minutes at room temperature. This step allowed oxidation of free NANA to malenaldehyde. At the end of the 20 minutes, this reaction was stopped by the addition of 1 ml of 10% sodium arsenite reagent. The tubes were vigorously shaken until no brown color was observed in any of them. A 2.5 ml quantity of 0.6% thiobarbituric acid (TBA) solution was added to each tube and the tubes placed in a boiling water bath for 15 minutes. In this step, the aldehyde reacted with the TBA to form a pink reaction product. The test was read by observing the intensity of pink color in each tube. If a particular subtype antiserum caused a substantial reduction in color, the virus was considered to be of that subtype.

An occasional isolate had a weakly reactive neuraminidase which resulted in poor color development when tested. This was remedied by increasing the incubation time, after addition of fetuin,

to eighteen hours.

Antigen and Antiserum Verification

All antigens and antiserums on hand at the beginning of this experiment were checked for specificity of hemagglutinin or anti-hemagglutinin type. All responded as expected in the HI procedure except the virus identified as A/Turkey/Wisconsin/66 (Hav6 N2) and its corresponding antiserum. The hemagglutination by this virus was not inhibited by any other anti Hav6 serum, nor by any other of the anti-hemagglutinin subtype antiserums. It was, however, inhibited by its homologous antiserum. Likewise, this antiserum inhibited only the A/Ty/Wisc/66 virus. At the time, only subtypes Hav1 throught Hav8 had been recognized in the literature. This problem was recounted to Dr. B. C. Easterday (Vet. Sci. Dept., Univ. of Wisc., Madison, WI) with the suggestion that the virus might have been mis-identified and should perhaps be designated as subtype Hav9. Dr. Easterday agreed and pointed out that a paper (78) was in press which proposed re-classification of the virus as subtype Hav9.

A similar problem was encountered later when anti-neuraminidase serums had been produced and were being checked for specificity. A virus taken from the NVSL repository which had been sent from another laboratory identified as A/Duck/England/62 (Hav4 Nav1) was not inhibited in the NI procedure by anti NWS-Dk/Eng (HØ Nav1) serum. It was, however, inhibited by anti NWS-Eq2 (HØ Neq2) serum. Likewise, antiserum which had been produced against the virus in question only inhibited viruses possessing Neq2 neuraminidase.

No other discrepancies in the antigen or antiserum reagents were detected.

Typing and Pathogenicity Testing of Unknowns

Source of samples

Twenty isolates which had been recovered previously from two groups of imported birds held in different quarantine facilities were selected. These were each passed in four embryonated eggs. The AAF from dead embryos was harvested individually and checked for HA titer. An HI test for NDV was performed on each of the resultant HA positive AAFs. Each of the AAFs were passed in embryonated eggs at limit dilution. AAF from one dead embryo at the limit dilution was saved for further typing of each of the virus preparations. For ease of identification, these were referred to as samples 1 through 79. Original tissues from 23 different birds from the same groups under investigation were removed from storage, thawed, ground, suspended in TBTB, and inoculated into four 8-day embryonated eggs each. One specimen yielded an embryo-lethal hemagglutinating virus isolate. This agent was not inhibited by anti NDV serum in the HI procedure. The resultant two AAF harvests (one from an embryo which died 2 days PI, and the other from two 5-day PI deaths) were passed at limit dilution. One limit dilution AAF was selected from each. These were identified as M-1a and M-1b. Table 4 shows the assignment of project specimen numbers and correlates the identification of the field specimens with the limit dilution sample numbers.

Table 4. Correlation of field specimens with limit dilution AAF sample numbers

Quarantine facility	Accession number	Bird number	Type	AAF sample numbers	Project specimen number
A	11988	8	Finch	1, 2, 3, 6	1
		19	Cackatoo	7, 64, 65, 66	2
		23	Mynah	8, 9, 10, 67	3
	12231	1	Rosella	36, 37, 38, 39	4
		5	Mynah	26, 40, 41, 42	5
		7	Finch	27, 43, 44, 45	6
		35	Lorie	28, 46, 47, 48	7
		37	Lovebird	29, 30, 49, 50	8
		38	Finch	31, 32, 33, 34	9
	12564	26	Mynah	20, 21, 22, 23	10
		59	Cockatoo	68, 69, 70	11
		60	Cockatoo	24, 25, 71, 72	12
	12785	2	Cockatoo	51, 52, 53, 54	13
		3	Rosella	11, 12, 55, 56	14
		4	Finch	13, 14, 15, 57	15
		14	Parrot	16, 17, 18, 58	16
		18	Lorie	4, 19, 59, 60	17
		20	Lovebird	5, 61, 62, 63	18
B	14518	2	Mynah	35, 73, 74, 75	19
		8	Mynah	76, 77, 78, 79	20
		1	Mynah	M-1a, M-1b	21

Pathogenicity of isolates for chickens

Samples or sample pools representing all of the original specimens were selected and each was inoculated via the posterior air sac route into four 4 to 6-week-old chickens. Each group of four birds was maintained in isolation for 10-12 days following inoculation. All surviving birds were then euthanatized and necropsied by a pathologist. Birds that did not survive were necropsied within 18 hours of the time of death. Tissues were collected from each bird at necropsy, portions of them ground in TBTB, and inoculated into four 10 to 11-day embryonated eggs each. Isolates identified as NDV were tested by the mean death time procedure.

Identification of isolates

All samples were passed via the allantoic route in 10 to 11-day embryonated eggs for purposes of virus propagation. AAF was harvested from all embryos on the third day PI and tested for HA activity. Three of the samples (nos. 51, 52, and 55) proved to be nonviable when removed from storage. Inasmuch as there were other viable samples representing the same original specimens, no attempt was made to repropagate these three. Upon repropagation, a portion of each of the AAFs which were HA positive was treated by the acid precipitation procedure and subjected to the A-RNP, AGID test procedure. Thirty-seven of the type A positive samples were selected and checked for influenza hemagglutinin type by the HI procedure using antiserums available at NVSL. The antiserums had been produced in chickens by inoculation with prototype or field isolate influenza viruses (see Table 2).

A 5 ml alliquot of each sample (1-79, M-1a, and M-1b, except 51, 52, 55, and 73) was treated with beta propriolactone (BPL) (0.1% final concentration) to inactivate the virus. (There was insufficient material to prepare sample number 73). The inactivated samples were taken to the WHO reference laboratory at St. Jude Children's Research Hospital, Memphis, Tennessee. The neuraminidase type of these samples was established using monospecific antiserums produced at St. Jude Hospital in goats hyperimmunized with isolated viral neuraminidase subunits. Hemagglutinin typing by the HI procedure using monospecific serums from goats hyperimmunized with isolated viral hemagglutinin subunits was carried out on samples 1, 6, M-1a, M-1b, and thirteen of the remaining samples which were selected at random. By this procedure, AAFs representing 11 of the 18 original specimens from one quarantine facility and all 3 original specimens from the second quarantine facility were selected.

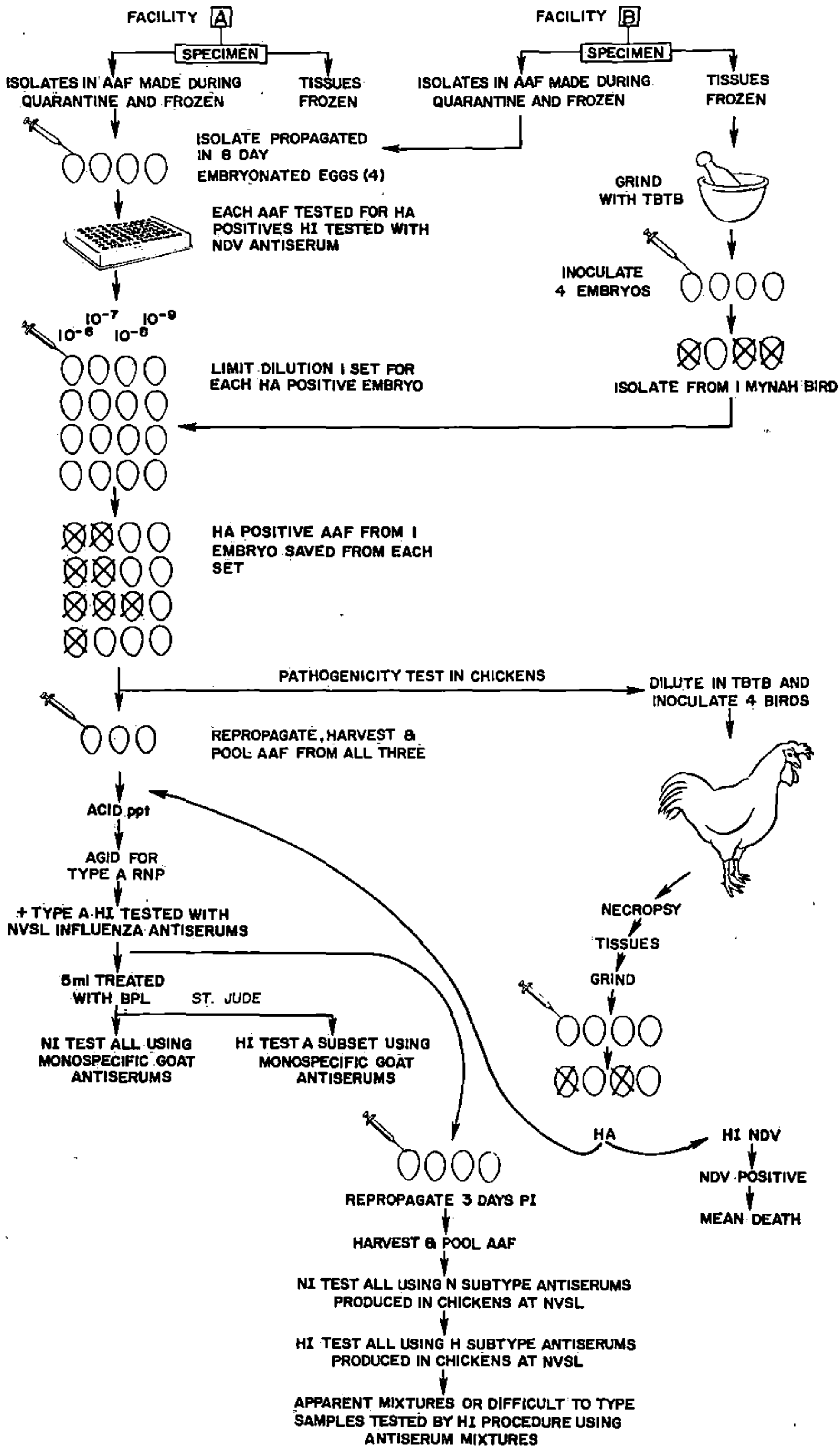
Time did not permit hemagglutinin typing of all the samples taken to St. Jude Hospital. However, recombinant and prototype viruses were supplied from the St. Jude repository by Dr. R. G. Webster (Dept. of Virology, St. Jude Children's Hospital, Memphis, TN). These were used at NVSL to establish a complete set of reference antigens for all of the known hemagglutinin and neuraminidase subtypes and to prepare specific subtype antiserums in roosters. Once all of the necessary reagents were prepared and validated, all of the samples under study were again reinoculated into embryonated eggs in preparation for HI and NI procedures to establish the subtype of each. Samples 27, 43, and 73 were not viable. An NI test was performed on all the repropagated samples using the antiserums listed in Table 3. The same samples were also

subjected to the HI procedure using the antiserums listed in Table 2.

At the suggestion of Dr. V. Hinshaw (Dept. of Virology, St. Jude Children's Research Hospital, personal communication, 1979), samples suspected of having mixtures of two or more types of virus were tested by the HI procedure using antiserums against each of the individual hemagglutinin subtypes suspected of being present and mixtures of anti-serums against these same subtypes. Anti-NDV serum was included in this trial. Figure 1 is a flowchart depicting the virus characterization procedures described above.

An attempt was made to determine if either of the two antigenic types of influenza virus isolated and identified in this study were pathogenic for parakeets or conures. Sample M-1a and sample 43 were prepared as 10^{-2} dilutions in PBS. This material was then used to create an aerosol inside the cages containing the birds to be exposed. For each of these viruses, two conures and 4 parakeets were thus inoculated.

Figure 1. Flowchart of isolation, identification, and pathogenicity testing procedures



FACILITY A
SPECIMEN
ISOLATES IN AAF MADE DURING QUARANTINE AND FROZEN
TISSUES FROZEN
FACILITY B
SPECIMEN
ISOLATES IN AAF MADE DURING QUARANTINE AND FROZEN
TISSUES FROZEN
ISOLATE PROPAGATED IN 8 DAY EMBRYONATED EGGS (4)
GRIND WITH TBTB
EACH AAF TESTED FOR HA POSITIVES HI TESTED WITH NDV ANTISERUM
INOCULATE 4 EMBRYOS
10-6 10-7 10-8 10-9
LIMIT DILUTION I SET FOR EACH HA POSITIVE EMBRYO
ISOLATE FROM I MYNAH BIRD
HA POSITIVE AAF FROM I EMBRYO SAVED FROM EACH SET
PATHOGENICITY TEST IN CHICKENS
DILUTE IN TBTB AND INOCULATE 4 BIRDS
REPROPAGATE, HARVEST & POOL AAF FROM ALL THREE
ACID ppt
AGID FOR TYPE A RNP
+ TYPE A HI TESTED WITH NVSL INFLUENZA ANTISERUMS
NECROPSY
TISSUES
GRIND
5ml TREATED WITH BPL
ST. JUDE
NI TEST ALL USING MONOSPECIFIC GOAT ANTISERUMS
HI TEST A SUBSET USING MONOSPECIFIC GOAT ANTISERUMS
HA
HI NDV
NDV POSITIVE
MEAN DEATH
REPROPAGATE 3 DAYS PI
HARVEST & POOL AAF
NI TEST ALL USING N SUBTYPE ANTISERUMS PRODUCED IN CHICKENS AT NVSL
HI TEST ALL USING H SUBTYPE ANTISERUMS PRODUCED IN CHICKENS AT NVSL
APPARENT MIXTURES OR DIFFICULT TO TYPE SAMPLES TESTED BY HI PROCEDURE USING ANTISERUM MIXTURES

RESULTS

On initial HI test with NDV antiserum, none of the isolates were inhibited.

The results obtained upon inoculation of chickens are presented in Table 5, which lists the number of birds in each test group failing to survive the challenge, which groups showed any pathologic changes at necropsy, and the nature and severity of the changes found. Five of the samples or sample pools (nos. 1, 6, 21, M-1a, and M-1b) proved to be sufficiently pathogenic to cause fatal illness. Twenty of the samples were associated with a variety of gross pathologic changes observed at the time of euthanasia. The remaining 16 samples failed to elicit any changes. Except for the birds that became moribund and died, there were no clinical signs of illness in any of the test birds.

Tissues from the three groups of dead birds inoculated with samples 1, 6, and 21 yielded isolates identified as Newcastle disease virus by the HI procedure. The combined results of the mean death test and the gross pathology observed in the test birds indicated that samples 1, 6, and 21 were VVND.

One bird died in each of the groups of chickens inoculated with samples M-1a and M-1b, but all eight test birds showed moderate to severe gross pathologic changes upon necropsy. In each case, a virus was isolated from the tissues of the birds which died during the test. Both re-isolated viruses possessed type-A RNP and were subtyped as Hav4 by HI test using the serums available at NVSL.

Table 5. Summary of gross pathologic findings (Lesions tended to indicate respiratory or GI tract infection and are so indicated. Other significant lesions are individually listed. Results listed are a compilation of findings among the 4 test birds inoculated with each isolate or pool.)

Project specimen number	Sample number	Number dead	Resp.[a]	GI[a]	Other
1	1	4	++	++++	-
	3	0	+++	+	Excess pericardial fluid
	6	3	++	+	-
2	7	0	-	-	-
3	9	0	-	-	-
	67	0	-	-	-
4	38	0	-	+	-
	39	0	-	-	-
5	26	0	-	-	-
	40, 42	0	-	+	-
6	27	0	+	+	-
	43	0	+	++	-
7	46	0	-	-	-
	28, 47	0	+	+	-
8	29	0	-	-	-
	30, 49	0	-	+	-
9	31	0	-	-	-
	32, 34	0	+	++	-
10	20	0	-	-	-
	21	4	++	+++	-

[a]+ = mild lesions; ++++ = most severe lesions.

Table 5. (Continued)

Project specimen number	Sample number	Number dead	Resp.[a]	GI[a]	Other
11	69	0	-	-	-
	70	0	++	-	Excess pericardial fluid; congestion of epicardium
12	25	0	+	-	-
	24, 71, 72	0	-	-	-
13	54	0	-	+	-
14	11	0	-	+	-
15	14	0	+	++	-
	13, 15	0	-	-	-
16	17	0	-	-	-
	18, 58	0	-	-	-
17	4	0	+	-	-
	19	0	++	+	-
	60	0	+	+	Epicardial hemorrhage
18	5	0	-	+	Pericarditis
	63	0	+	-	Excess pericardial fluid
19	35	0	-	+	Pericarditis
	73	0	+++	-	Peritonitis; myocardial edema
20	76	0	-	-	Pericarditis
	78	0	-	-	-
21	M-1a	1	++++	++	Excess pericardial fluid; epicardial hemorrhage; swollen liver
	M-1b	1	++++	++	Excess pericardial fluid; swollen liver

Upon repropagation and testing by the AGID test procedure, all but six of the viable samples were positive for type A-RNP. Three of the six negatives (numbers 1, 6, and 21) originated from specimens previously identified as containing VVND virus.

The results of HI tests using serums on hand at NVSL are shown in Table 6. Samples 13, 19, and 60 appeared to be subtypes Hav4, Hav7, and Hav7, respectively. The remaining 32 samples, while reacting with some of the antiserums, did not meet the criteria for hemagglutinin identification outlined in Materials and Methods and, therefore, could not be conclusively subtyped by this method.

When tested by the NI procedure with monospecific goat antiserums, all but samples 1, 6, 21, and 22 were inhibited only by Neq2 antiserum. Samples 1 and 6 were inhibited by NDV neuraminidase antiserum as well as Neq2 antiserum. Samples 21 and 22 were inhibited by NDV neuraminidase antiserum only.

The results of HI tests on 17 selected samples using monospecific goat anti-Hav4 and anti-Hav7 serums are shown in Table 7. Three samples, numbers 35, M-1a, and M-1b proved to be subtype Hav4. The remaining samples were identified as subtype Hav7.

After fifth passage repropagation, when the entire set of viable samples was tested by the NI procedure with newly produced antiserums (see Table 3), all samples except 21, 22, and 23 were inhibited by anti-Neq2 serum only. The same samples, when tested in the HI procedure using newly produced or obtained antiserums (see Table 2), resulted in inhibition by Hav4 and Hav7 antiserums only. The HI titers obtained with the latter two antiserums are shown in Table 8. Most of the samples could

Table 6. Results of HI tests using antiserums against field isolates (Results are expressed as the reciprocal of the highest dilution that inhibited hemagglutination.)

Sample number	Antigenic subtype of antiserum								
	Hav1 Nav2	Hav2 Neq2	Hav3 Nav1	Hav4[a] Neq2	Hav5 Nav6	Hav6 Neq2	Hav7 Neq2	Hav8 Nav4	Hav9 N2
1	-	-	-	16	-	-	2	-	-
2	-	-	-	2	-	-	2	-	-
3	-	2	-	4	-	-	2	-	-
4	-	2	-	32	-	-	8	-	-
5	-	-	-	-	-	-	2	-	-
7	-	-	-	8	-	-	2	-	-
9	-	-	-	4	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
13	-	2	-	512	-	-	4	-	-
14	-	2	-	16	-	-	2	-	-
15	-	2	-	8	-	-	4	-	-
17	-	2	-	8	-	-	4	-	-
19	-	4	-	32	-	-	32	-	-
20	-	2	-	32	-	-	4	-	-
21	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	2	-	-
26	-	-	-	8	-	-	2	-	-
27	-	-	-	-	-	-	2	-	-
29	-	2	-	8	-	-	8	-	-
30	-	-	-	-	-	-	2	-	-
31	-	-	-	-	-	-	-	-	-
32	-	-	-	8	-	2	4	-	-
33	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-
36	-	-	-	8	-	-	4	-	-
37	-	-	-	8	-	-	2	-	-
38	-	-	-	8	-	-	4	-	-
39	-	-	-	-	-	-	4	-	-
41	-	-	-	-	-	-	2	-	-
42	-	-	-	-	-	-	2	-	-
45	-	2	-	8	-	-	4	-	-
49	-	2	-	4	-	-	2	-	-
59	-	2	-	32	2	-	8	-	-
60	-	2	2	128	4	2	16	-	-
66	-	-	-	-	-	-	2	-	-
67	-	2	-	16	-	-	4	-	-
72	-	-	-	4	-	-	4	-	-
Homologous control	256	128	64	512	256	512	32	32	128

[a]This antiserum was produced against a virus which was originally labeled as Hav2 Nav1 when stored in the NVSL repository. Many of the weak reactions are probably due to steric inhibition by Neq2 antibody.

Table 7. HI test results using monospecific goat antiserums (Results are expressed as the reciprocal of the highest antiserum dilution that inhibited hemagglutination.)

Project specimen number	Sample number	HI titer with antiserum against:	
		Hav4	Hav7
1	1	<10	40
	6	<10	80
4	39	<10	40
5	26	<10	40
6	43	<10	80
	45	<10	80
7	28	<10	40
8	29	<10	40
9	32	<10	80
12	71	<10	40
14	56	<10	80
16	58	<10	160
17	60	<10	160
19	35	160	<10
20	78	<10	80
21	M-1a	>1280	80
	M-1b	80	<10

Table 8. Results of HI test of samples with Hav4 and Hav7 antiserums (Results are expressed as the reciprocal of the highest antiserum dilution that inhibited hemagglutination.)

Project specimen number	Sample identification	Results with antiserum against: A/Mal/Alb/43/77 (Hav4 Navl)	Results with antiserum against: Dk/Ukr-Bel (Hav7 N1)
1	1	4	8
	2	2	2
	3	2	2
	6	0	64
2	7	0	32
	64	0	32
	65	0	32
	66	0	32
3	8	0	4
	9	0	8
	10	0	8
	67	0	4
4	36	2	16
	37	2	32
	38	0	64
	39	0	64
5	26	0	32
	40	0	32
	41	0	32
	42	0	16
6	44	0	64
	45	0	64
7	28	2	16
	46	0	64
	47	2	128
	48	0	32
8	29	2	64
	30	0	16
	49	2	32
	50	0	32
9	31	16	0
	32	16	2
	33	4	2
	34	16	4
10	20	0	4
	21	4	4
	22	4	4
	23	4	2

Table 8. (Continued)

Project specimen number	Sample identification	Results with antiserum against: A/Mal/Alb/43/77 (Hav4 Nav1)	Dk/Ukr-Bel (Hav7 N1)
11	68	0	32
	69	0	16
	70	0	16
12	24	0	16
	25	0	32
	71	0	64
	72	0	32
13	53	0	32
	54	0	64
14	11	0	32
	12	0	16
	56	0	32
15	13	0	32
	14	0	16
	15	2	64
	57	0	128
16	16	2	64
	17	0	32
	18	0	64
	58	2	32
17	4	16	32
	19	32	32
	59	4	64
	60	2	64
18	5	0	32
	61	0	16
	62	0	32
	63	0	16
19	74	2	4
	75	2	2
20	76	0	32
	77	0	32
	78	0	32
	79	0	64
21	M-1a	4	4
	M-1b	2	4

definitely be subtyped as Hav4 or Hav7. Some, however, were only partially inhibited by anti-Hav7, anti-Hav4, or both of these antiserums. Among these were samples which were already known to contain NDV.

These uncertain findings were resolved upon HI testing with serum mixtures. The sample numbers, the serums or serum mixtures used, and the results of these HI tests are shown in Table 9. When the single serum HI titers are compared to the mixed serum titers, it is apparent in most instances that the titer differences are commensurate with the dilution of one serum with equal parts of another. Notable exceptions to this effect were observed when samples 6, 20, 21, and 22 were tested with mixed Hav7 and NDV antiserums which resulted in an increase in titer over that observed with either single serum.

When parakeets and conures were exposed to the Hav4 Neq2 isolate identified as M-1a or to the Hav7 Neq2 isolate (sample 43), no illness or death occurred in any of the test birds. There was no evidence of seroconversion when serum samples taken before and after exposure were tested by the HI method with homologous antigen.

Other Observations

A probe was initiated in an attempt to explore a faster, more efficient way to identify hemagglutinating isolates. This dealt with the application of the ELISA technique to influenza virus identification and subtyping. Materials required for the probe were available in this laboratory. They included horseradish peroxidase (HRP)-conjugated rabbit anti-chicken globulin (produced by Reagents Section, Scientific

Table 9. Results of HI tests of selected samples using various mixtures of Hav4, Hav7, and NDV antiserums (Results are expressed as the reciprocal of the highest dilution of antiserum that inhibited hemagglutination.)

Sample number	Hav4	Hav7	Hav4 Hav7	Hav4 NDV	Hav7 NDV	Hav4 Hav7 NDV
1	2	32	16	0	16	8
2	2	8	4	0	8	8
3	4	16	8	2	8	8
6	0	0	0	0	4	2
31	2	16	8			
32	2	32	8			
33	2	32	8			
34	2	16	8			
20	2	4		32	64	32
21	2	16		0	32	8
22	2	2		32	128	32
23	2	32		0	8	8
4	2	128	32			
19	4	128	32			
59	4	128	32			
60	2	16	8			
74	16		4			
75	8		4			
M-1a	16		4			
M-1b	8		4			

Services, NVSL, Ames, IA), 4 mM ABTS, 0.05 M citrate buffer (pH 4.0) with 0.05% Tween 80 (Fisher Scientific Co., Fair Lawn, NJ), 1.25% H_2O_2 (mixed fresh for each use), and 0.42% hydroflouric acid (HF). All were used according to the procedure published by Snyder and Stewart (59).

The probe was conducted by first adsorbing influenza virus to the surface of a well in a polystyrene 96-well microtitration tray and then removing any excess virus by washing with PBS. See Figure 2a. The attached virus was then exposed to specific antiserum produced in roosters. If the serum contained antibodies against the attached virus, they would attach to the available antigenic sites as indicated in Figure 2b. The excess (or unattached) antibody was removed by washing with citrate buffer. HRP conjugate was then added. If antibodies had attached to the adsorbed virus, the anti-chicken globulin conjugate would attach to the complex as in Figure 2c. Any unattached conjugate was removed by washing with citrate buffer. ABTS reagent was then added. If HRP was present, oxygen was released (from the H_2O_2) which would oxidize the ABTS to a blue product as indicated in Figure 2d.

The results of this effort were not conclusive, and time did not permit the resolution of variables.

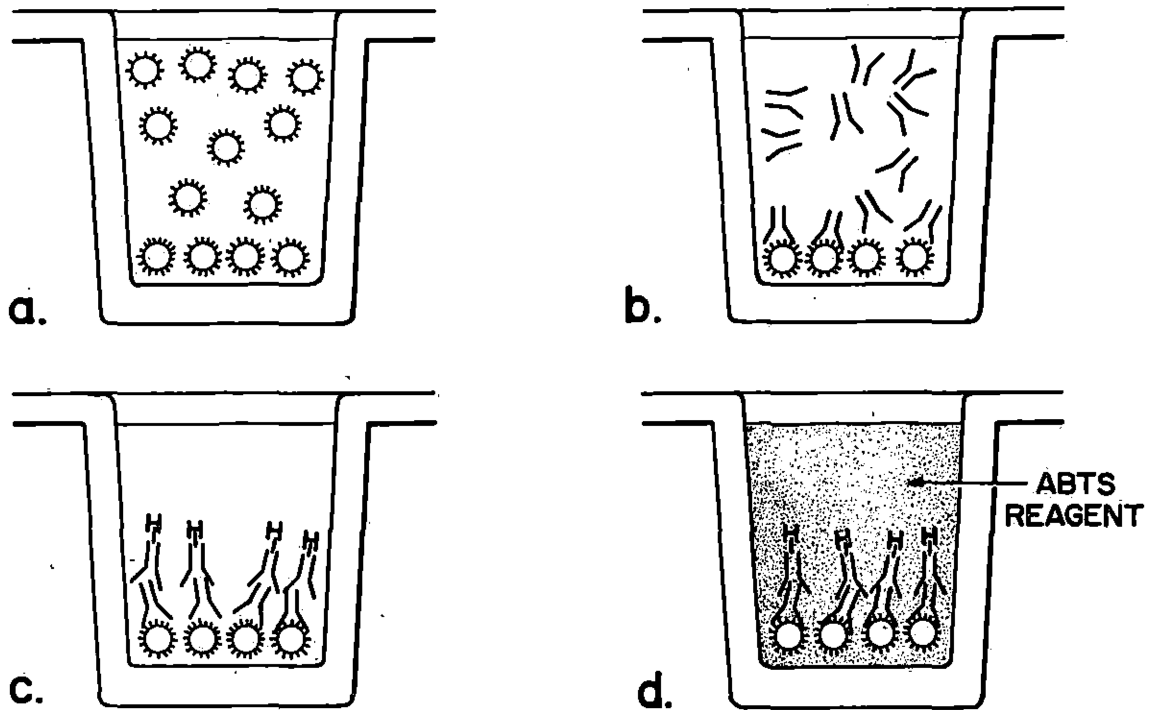


Figure 2. Schematic representation of the ELISA procedure: a) adsorption of virus particles (✪) to the surface of a polystyrene well; b) chicken-anti-virus globulin (≻=) attaching to adsorbed virus; c) HRP-conjugated rabbit-anti-chicken globulin (≻=-ɪ) attached to chicken-anti-virus globulin; d) oxygen catalytically released from H_2O_2 by HRP oxidizes clear ABTS to give a blue product

DISCUSSION

Two clearly distinct antigenic subtypes of type A influenza virus, Hav4 Neq2 and Hav7 Neq2, have been identified in this study. Both were associated with mortality in exotic birds being held in quarantine prior to entry into the United States. One Hav4 Neq2 isolate from a mynah bird at facility B was sufficiently pathogenic to kill two of eight test chickens and to produce extensive gross pathologic changes in the six surviving chickens. It is suggested that this isolate be named A/Mynah/Ames/14518/76.

A second Hav4 Neq2 isolate, also from a mynah bird at the same quarantine facility, was somewhat less pathogenic in that it did not kill any test chickens, but it did cause fairly severe gross pathologic changes. The several Hav7 Neq2 isolates from a variety of species of birds at facility A and one from a mynah bird at facility B showed a range of pathogenicity from virtually nil to moderate. None of the Hav7 Neq2 isolates killed test chickens.

The significance of these findings lies in the fact that the potential exists for influenza viruses, possibly pathogenic for domestic poultry, to enter the United States via imported pet birds. This situation is compounded by the possibility that individual virus subtypes, which are not in themselves pathogenic, could be carrying part of the viral RNA code for pathogenicity which might be expressed if such a virus were to recombine with another which possessed the remainder of the code necessary for full virulence. Webster (71) and Webster et al. (73,74) have shown that influenza viruses of different antigenic subtypes freely

recombine under laboratory conditions and that there is good evidence recombination can occur under natural conditions. Such recombination during quarantine might result in a progeny virus capable of destroying the pet birds themselves, or in the production of a virus, not lethal to the pet bird species, which could be highly pathogenic for domestic poultry.

There has been one instance in which a virus of the Hav4 Neq2 subtype has been associated with disease and mortality in chickens in the United States (28). The source of that viruswas never identified, nor was the disease ever reproduced in laboratory chickens. That report, along with the results of this study, indicates that we do not yet understand the circumstances under which an influenza virus' virulence can be expressed. Recent work on the genetics and molecular biology of influenza viruses has just begun to elucidate how the virus adapts for survival and interacts with host species to cause disease (38,44,53,54,75,83). At the same time, there is speculation about the possibility of new strains of human influenza originating from strains which normally infect other mammals and birds (33,52,77,83).

It has been clearly demonstrated that virulence and surface antigen subtype are independent (1,6,38,44,45). Careful identification and characterization of all influenza subtypes found in quarantined birds as well as those isolated from domestic poultry is one way to help monitor changes the virus is undergoing in nature. Such monitoring, coupled with rapid remedial measures such as quarantine and vaccination programs which have been suggested (43), may help lessen the effect of a new pathogenic subtype in its host population, should one appear.

Consideration of the findings reported here gives rise to the following questions and thoughts. Why the apparent difference in pathogenicity of the two Hav4 Neq2 isolates recovered from two mynah birds at facility B? Are the observed differences in pathogenicity for chickens due to real variation in the viruses recovered from the two birds, or might they be due to the techniques used to isolate these viruses possibly selecting, by chance, two different variants from what originally were two identical populations of virus? We tend to presume that the isolated viruses were responsible for the observed illness in the exotic bird hosts. Our techniques did not, however, tell us if only one subtype of influenza was present to start with. Why, for instance, did a population of mynahs, enclosed in a single building, yield two subtypes--Hav4 Neq2 and Hav7 Neq2? The source birds had died in each instance. Had these hosts "pre-filtered" a mixed virus population, and, if so, what is the nature of that process? Considering the ease with which influenza viruses recombine, there were opportunities for such an occurrence in the quarantine facility. If a mixed population was originally present, recombination could likewise have occurred in the embryonated eggs used in the isolation process.

The standard method in use at NVSL of pooling untyped hemagglutinating viruses isolated from several different birds in the same quarantine facility in order to test their pathogenicity in chickens and turkeys should be reconsidered. A better approach might be to first identify which isolates are type A influenza viruses, then to pool the influenza isolates separately according to subtype for the pathogenicity test. This would reduce the possibility of giving rise to any recombinant viruses in the test birds, and obtain a better assessment of the pathogenicity of each subtype

isolated.

Subtype identification of influenza isolates can be a useful tool for tracing the spread of viruses during disease outbreaks or to identify recombination events occurring in nature. This author proposes the following procedures be adopted for routine use in influenza isolate identification:

1. Most veterinary diagnostic laboratories that are capable of virus isolation can first eliminate the possibility of NDV by the standard NDV-HI procedure.
2. The AGID technique is one that most diagnostic laboratories can use to establish that isolates are type A influenza and should be the next test used on HA isolates other than NDV.
3. The neuraminidase subtype should be identified next using the NI procedure.
4. Hemagglutinin subtype should be determined using the HI procedure. Accurate hemagglutinin subtyping is often not possible if steric inhibition due to neuraminidase antibodies is not avoided. To avoid this problem, none of the antiserums used should have antibodies against the neuraminidase subtype determined in step 3 above.

Application of the techniques herein described has already produced some rather interesting information (NVSL unpublished data). During the fall of 1978, an influenza outbreak in turkeys was studied. Serum collected from the diseased flock on the first day of the outbreak had detectable HSw1 HI antibody.

Swabs from moribund birds collected the same day yielded an Hav6 N1 isolate. Serums collected 20 days later from some of the survivors yielded antibodies against HSw1 and Hav6 subtypes by HI test and N1 and Neq2 subtypes by NI test. This information leads the author to suggest that the flock could have been previously exposed to swine influenza virus (HSw1 N1). An Hav6 Neq2 subtype could then have been introduced in some undetermined way, hence the presence of Neq2 antibodies. The two virus types might then have given rise to an Hav6 N1 recombinant which could have been responsible for the observed clinical disease and subsequent Hav6 and N1 antibody titers.

Such thinking, while highly speculative, does have some foundation in fact. But many more such incidents must be similarly studied and documented before much credence will be established. To this end, identification of influenza subtypes to the full extent of our capabilities is the best tool available in the diagnostic laboratory to follow the evolution of virulence. Without such identification, there is no way to develop information on the epidemiology of influenza virus subtypes or to associate subtype with virulence.

Conclusion

This work has established that imported birds can carry type A influenza viruses into the United States. One of the isolates was pathogenic for test chickens. Antigenic subtyping of influenza isolates is the only tool presently available for logically grouping isolates for pathogenicity testing.

Summary

Methods for influenza subtype identification are presented in detail. The methods were used to identify two antigenic subtypes of Type A influenza virus, Hav4 Neq2 and Hav7 Neq2, isolated from imported pet birds being held in quarantine. An Hav4 Neq2 isolate from a mynah bird was capable of causing death in test chickens. The name A/Mynah/Ames/14518/76 is suggested for this isolate.

The implications of recombinant influenza viruses possibly arising in quarantined pet bird populations are discussed. Routine subtyping of influenza isolates by the methods described is recommended.

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