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of the

**FIRST
INTERNATIONAL SYMPOSIUM
ON
AVIAN INFLUENZA**

BELTSVILLE, MARYLAND, U.S.A.

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CONTENTS

Officers of the Symposium	v
Sponsoring Contributors and Contributors	vi
Introduction and Objectives of the Symposium	vii
List of Participants	xv
Report of the Panel on Development of Uniform Identification and Terminology of Avian Influenza Viruses	viii
Report of the Panel on Development of Uniform International Understanding Concerning Import-Export Requirements	viii
Report of the Panel on Development of Methods for Prevention and Control of Outbreaks Associated with Low Virulent Strains	xi
Research Priorities Recommended by the Panels	xii
Avian Influenza 1981 — A Silver Anniversary, A Centennial, or A Millennium? — B. C. Easterday	1
Uncomplicated Infection with Virulent Strains of Avian Influenza Viruses — W. H. Allan	4
Complications Associated with Avian Influenza Infections — J. Newman, <i>et al</i>	8

CURRENT WORLDWIDE SITUATION OF AVIAN INFLUENZA

Avian Influenza in the United States — (1964-1981) — B. S. Pomeroy	13
Avian Influenza in Australia — A. Turner	18
Avian Influenza in Belgium — G. Meulemans	19
A Review of Influenza in Canada Domestic and Wild Birds — G. Lang	21
Current Situation of Avian Influenza in France — G. Bennejean	28
Avian Influenza in Hong Kong — K. F. Shortridge	29
Studies on the Ecology of Avian Influenza Viruses in Israel — M. Lipkind, <i>et al</i>	30
Current Situation in Italy — M. Petek	31
Current Situation of Avian Influenza in Poultry in Great Britain — D. J. Alexander	35
Occurrence of Avian Influenza Virus Type A in Germany — K. Ottis and P. A. Bachman	46
Studies on Avian Influenza in Ducks in the Republic of China — Y. S. Lu, <i>et al</i>	52

EPIDEMIOLOGY OF AVIAN INFLUENZA AND SOURCES OF INFECTION

Epidemiology of Avian Influenza and Sources of Infection in Domestic Species — K. F. Shortridge	54
Epidemiologic Relationships of Influenza A Viruses in Domestic and Feral Species — V. S. Hinshaw, <i>et al</i>	214
Review of the Three-Year Studies on the Ecology of Avian Influenza Viruses in Israel — M. Lipkind, <i>et al</i>	69
Isolation of Influenza A Viruses from Exotic Birds in Great Britain — D. J. Alexander	79
Influenza A Virus Infection of Domestic Ducks — T. Sandhu and V. Hinshaw	93
Economic Impact of Avian Influenza in Domestic Fowl in the United States — P. Poss, <i>et al</i>	100
Economic Impact of Avian Influenza in Domestic Fowl on International Trade — R. E. Anderson	112

Economic Impact of Avian Influenza in Australia — A. J. Turner	114
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MOLECULAR BASIS OF PATHOGENICITY AND VIRULENCE OF AI VIRUSES

The role of the Hemagglutinin in Infectivity and Pathogenicity of Avian Influenza Viruses — R. Rott	116
Application of Recently Developed Techniques to Determine the Origin of Influenza A Viruses Appearing in Avian and Mammalian Species and to Develop Potent Avian Influenza Vaccines — V. S. Hinshaw, <i>et al</i>	134
Plaque Forming Ability in MDCK Cells and Structure of the Haemagglutinin of Influenza A Viruses which Differ in Virulence for Chickens — D. J. Alexander	148

DIAGNOSIS AND IMMUNIZATION

Avian Influenza Diagnostic Procedures in the United States — J. E. Pearson and D. A. Senne	151
Diagnostic Procedures — Response — W. H. Allan	167
Immunization Approaches to Avian Influenza — C. W. Beard	172
Commercial Avian Influenza Vaccines — R. J. Price	178
Avian Influenza in the Control of Disease with Inactivated Vaccines in Oil Emulsion — A. Zanella, <i>et al</i>	180

CONTROL OF AVIAN INFLUENZA

International Responsibility for Control of Avian Influenza — J. E. Lancaster	184
Is International Regulation of Avian Influenza Feasible? — G. Bennejean	198
Regulatory Problems Associated with Avian Influenza — J. K. Atwell ...	211

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INTRODUCTION AND OBJECTIVES OF THE SYMPOSIUM

R. A. Bankowski

Avian influenza, or "fowl plague," is a major disease of poultry. Most diseases in domestic poultry are controllable, but the plurality of antigenic, and thus immunologic types, of the influenza virus and the mode of introduction and spread into flocks makes this disease one of the most potentially economically disruptive maladies affecting the poultry industry.

At the 1979 United States Animal Health Association Meeting, the Committee on Transmissible Diseases of Poultry recognized the need for international discussions on this problem and it was resolved to hold an international symposium on avian influenza in the Spring of 1981. It is of international importance to understand how regulatory agencies would respond to the antigenic makeup and differing levels of virulence of avian influenza isolates when they consider embargoes and other control measures. After several informal meetings, an international organizing committee was established to assemble a number of scientists with expertise in this field, representatives from government regulatory agencies and poultry industry representatives to participate in this symposium.

The aims and objectives of this International Symposium were to:

1. Assemble information from scientific and regulatory people from several countries to present their problems and losses.
2. Establish the need for an international agreement and understanding regarding the terminology of "influenza" and "fowl plague."
3. Explore the possibility of an international agreement and understanding on control measures of influenza in the avian species, particularly in domestic turkeys, chickens, ducks, and geese.
4. Seek a common understanding among countries regarding the epidemiology and severity of the disease to prevent unnecessary embargoes associated with AI infections.
5. Evaluate the role of research and official regulatory actions on the control and prevention of AI.
6. Document the economic effects of viruses of differing levels of virulence.
7. Consider the importance of exotic birds in commerce and their role in the introduction and perpetuation of AI.
8. Establish the extent of AI viruses in migratory and other wild birds around the world.

On the last day of the symposium the committee has requested that scientific, regulatory, and industrial participants be divided into three panels. Each panel is assigned to deliberate and summarize specific areas of the internationally significant avian disease problem and make recommendations regarding:

1. Development of uniform identification and terminology of avian influenza viruses.
2. Development of uniform international understanding concerning import-export requirements.
3. Development of methods for prevention and control of outbreaks associated with low-virulent strains.
4. Define research priorities in each area.

It is hoped that the participants of this symposium can reach an agreement that will be acceptable to and adapted by regulatory agencies around the world.

REPORT OF THE PANEL ON DEVELOPMENT OF UNIFORM IDENTIFICATION AND TERMINOLOGY OF AVIAN INFLUENZA VIRUSES

Panel members: B. C. Easterday (U.S.), *Chairman*
P. A. Bachman (West Germany), R. A. Bankowski (U.S.), V. S. Hinshaw (U.S.), G. Lang (Canada), J. E. Pearson (U.S.), and R. Rott (West Germany).

Objective 1: Discard the term Fowl Plague?

RECOMMENDATIONS

That the term Fowl Plague be discarded, except for historical purposes.

Objective 2: Develop criteria for highly pathogenic isolates causing outbreaks that may need government intervention.

RECOMMENDATIONS

That influenza virus highly pathogenic for avian species be considered any influenza 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.

REPORT OF THE PANEL ON DEVELOPMENT OF UNIFORM INTERNATIONAL UNDERSTANDING CONCERNING IMPORT- EXPORT REQUIREMENTS

Panel members: J. E. Lancaster (Canada), *Chairman*, W. H. Allan (U.K.), J. K. Atwell (U.S.), G. Bennejean (France), L. Campbell (U.S.), G.

Ghazikhanian (U.S.), G. Meulemans (Belgium), M. Petek (Italy), I. L. Peterson (U.S.), B. S. Pomeroy (U.S.), A. J. Turner (Australia), J. Walker (U.S.), G. L. Walts (U.S.), G. West (U.S.)

Objective 1: Define government activities when an outbreak is defined as being caused by a highly pathogenic isolate.

RECOMMENDATIONS

- A. That a stamping-out policy be adopted by either:
 - 1. Destruction and burial or incineration, or
 - 2. Effective processing (e.g., cooked, canned)
- B. That this policy require:
 - 1. Statutory authority
 - 2. Resources, e.g., personnel and funding
 - 3. Diagnostic support
 - 4. Adequate compensation program
 - 5. Quarantine measures and control of movements
 - 6. Inspection premises
 - 7. Surveillance both inside and outside the quarantine area
 - 8. Recognition of the above control activities by the industry.

Objective 2: Placement, size and duration of quarantine

RECOMMENDATIONS

Objective 2(a): Determine placement of quarantine

- A. Quarantine to be placed with the minimum of delay and released as soon as sound control measures permit
- B. Authority to utilize a presumptive diagnosis (action or suspicion)

Objective 2(b): Determine size of quarantine

- A. That infected premises and area be defined
- B. That radius of surveillance be established at an early date with restriction of animal movement except when licensed for movement
- C. That regionalization or zone of a country be areas within a country defined by the National Government and not necessarily confined to State or Provincial lines within a country. Government definition of regionalization or zone of a country is needed together with the utilization of regionalization for the purpose of export trade
- D. That valuable genetic material be protected
- E. That size and scale of the quarantine will depend on the ability of all agencies and industry to respond to the quarantine measures.

Objective 2(c): Determine duration of quarantine

- A. That a stamping-out policy be in effect according to these guidelines:
 - 1. Duration of quarantine: a minimum of 4 weeks of freedom from the disease within the *quarantine area*
 - 2. An additional 4 weeks (total 8 weeks minimum) freedom from the disease before *a country* is declared free from the disease
 - 3. Determination of freedom from disease, plus absence of recrudescence of disease, depends on surveillance measures.
 - 4. Quarantine should be in effect for as short a period as possible.
- B. That studies be conducted on the role of vaccination on the duration and release of quarantine

Objective 2(d): General considerations

- A. That the definition of disease, the epidemiology and practical control measures be studied
- B. That national credibility be maintained regarding diagnosis, control, and national disease-control measures
 - 1. That assistance be provided to developing countries
 - 2. That embargoes and other limitations to trade be placed only on a strictly scientific basis
- C. That quarantine be limited sound control measures, that early resumption of trade be facilitated (see Objective 2(c) (4) above) and that economic management of the slaughter policy to be achieved
- D. That quarantine measures protect gene pools by industry and by government in order to maintain continuity of the genetic material (see Objective 2(b) (D) above) and that within each country, the legal authorities must recognize the above measures
- E. That legislation and legal authority be available for the prompt imposition of quarantine
 - 1. Legal definitions of quarantines may be required
 - 2. Recognition of national authority by other countries
 - 3. Availability of assets, including financial and technical support (see Objective 1(B) (2,3) above)
- F. That value of quarantine measures depends on diagnostic capability, adequate surveillance, and reporting (see Objective 1(b) and Objective 2(c) (3) above).

Objective 3: Effect on international trade or embargoes.

RECOMMENDATIONS

- A. That regionalization or zone of a country be recognized by the governments of other countries (see Objectives 2(b) (c) above)

- B. That all embargoes be based on scientific knowledge (see Objective 2(d) (b) (2) above)
- C. That regionalization be based on epidemiological information
- D. That the export of different poultry products from an infected region to be examined
- E. That international trade be reinstated as soon as possible (see Objective 2(a) (A) above)
- F. That communications between countries to be maintained by the National Governments and the international organizations.

REPORT OF THE PANEL ON DEVELOPMENT OF METHODS FOR PREVENTION AND CONTROL OF OUTBREAKS ASSOCIATED WITH LOW-VIRULENT STRAINS

Panel members: C. W. Beard (U.S.), *Chairman*.
K. Esklund (U.S.), L. C. Grumbles (U.S.), D. D. King (U.S.), M. Lipkind (Israel), R. Munson (U.S.), J. A. Newman (U.S.), P. Poss (U.S.), R. Price (U.S.), K. F. Shortridge (Hong Kong).

Objective 1: Develop recommendations to reduce the impact of the disease caused by mild, low-virulence strains.

RECOMMENDATIONS

- A. Initiate educational programs directed toward turkey growers through their associations and state and federal governments to inform them of the probable risk of avian influenza in those flocks without adequate biosecurity practices.

Detailed recommendations on flock security should then be outlined in considerable detail for each specific segment of the operations, e.g., feed delivery, poult delivery and placement, egg collection and pick-up, contaminated house clean-up, etc.

The probable increased risk proposed for turkeys on range vs. turkeys properly housed should be presented in those recommendations.

Great emphasis should be placed on the movement of personnel from farm-to-farm. The definition of personnel should include both management and service employees.

The improved flock security should be in place and in operation before any outbreaks and should be followed continuously.

- B. A national and international system for avian influenza reporting should be established, into which all reports of virus isolation and positive serology would go and out of which frequent incidence

reports would be sent to all concerned parties. Perhaps it can be made a reportable disease in the United States.

- C. Efforts should continue to expand the capability for the use of inactivated, oil-emulsion vaccines. A collection should be made of representative strains of each subtype, known to be antigenically and immunologically acceptable, recombined, if necessary, with high-growth strains to provide vaccine manufacturers with proven seed viruses for the production of vaccines.
- D. There should be intensified discussions and trials, if necessary to develop efficacy standards mutually acceptable to regulatory agencies and the poultry industry which will facilitate the continued availability of inactivated avian influenza vaccines. Well-designed and controlled field evaluations of commercial vaccine should be considered.

Vaccines should be presented to the industry only as a second line of defense against avian influenza in problem areas in either breeders or market turkeys. Vaccine should not be a substitute for proper flock biosecurity. The difficulties of predicting the needed subtype should be well publicized.

- E. Monitoring programs designed to detect avian influenza antibodies should be conducted continuously in turkey flocks to aid in the early detection of subtypes in an area. Proper training and reagents should be made available to assist in this effort.

Objective 2: Determine the significance of avian influenza viruses in migratory and other wild birds.

RECOMMENDATIONS

- A. That the wild bird populations and other mammals and livestock, especially swine, in the vicinity of avian influenza flocks should be monitored for the presence of avian influenza virus. Attempts should be made to define the mechanisms, if any, that link avian influenza-positive birds and mammals to outbreaks in poultry.
- B. That the international efforts to isolate and identify avian influenza viruses from all species should continue, with the information being collected and disseminated to all concerned with avian influenza.

RESEARCH PRIORITIES RECOMMENDED BY THE PANELS*

- A. On Transmission
 1. To study possibility of egg transmission
 2. To develop a laboratory test to determine spreading potential of the virus

*Not listed in order of priority

3. To study possibility of transmission by meat:
 - a. Whole fresh carcass
 - b. Frozen finished whole product (processed)
 4. To determine how long virus is viable under field conditions
 5. To determine how long the virus persists in recovered birds
- B. Virulence and Pathogenicity**
1. To determine the factors affecting the virulence of AI viruses
 2. To evaluate pathogenesis and associated laboratory procedures to achieve a rapid diagnosis
 3. To research pathogenesis of influenza complicated by other infections and the environment
 4. To conduct pathogenicity studies on various strains of influenza virus isolates in turkeys and chickens
- C. Diagnosis**
1. To develop methods for rapid surveillance of influenza
 2. To research testing procedures for determining comparative pathogenicity of strains
 3. To diagnose criteria for determining status of freedom from highly pathogenic strains of avian influenza in avian species in any country
 4. To determine if parainfluenza viruses are being missed and if a disease is erroneously attributed to AI or to other agents.
- D. Pet Birds, Wild Birds and Other Reservoirs**
1. To study the role of pet birds, wild birds and mammals as reservoirs and sources of infection
 2. To investigate wild bird populations, other mammals and livestock in the vicinity of AI-infected flocks for the presence of AI and attempt to define the role and mechanisms that link AI-positive birds and mammals to outbreaks in poultry.
 3. To define possible role of human infection and particularly, swine (Hsw1) on dissemination of AI
- E. Vaccines & Immunization**
1. To define the quality and duration of vaccine-induced immunity in chickens and turkeys
 2. To determine the effect of vaccine on AI infection and shedding of AI virus in immunized chickens and turkeys
 3. To explore the possible use of genetic engineering to produce AI HA antigens in bacteria

4. To develop acceptable laboratory means of determining AI vaccine efficacy
 4. To explore the feasibility of viable vaccines against AI
- F. To Investigate Antiviral Therapeutic Agents for Control of AI

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**AVIAN INFLUENZA 1981:
A SILVER ANNIVERSARY, A CENTENNIAL, OR A MILLENNIUM?**

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Where are we in this year of 1981 with our understanding of avian influenza and where should we be five, ten and twenty years from now? What have we learned in the past one hundred years? Many significant events have been recorded in the chronicle of influenza during that time. Perhaps the most significant events have gone unrecognized and/or unrecorded. It is not appropriate to compile a comprehensive list and commentary on the important events in influenza for this symposium.

There are at least three events that relate to this symposium; 1) the recognition of a serious disease among poultry in 1878; 2) a global pandemic of influenza in human beings and the appearance of a new influenza-like disease in swine in 1918, and 3) the beginning of an avian influenza era in 1955-56; events of 100 years ago, 60 years ago and 25 years ago. In 1878 there was a very serious disease among poultry described in Italy. That disease became known as fowl plague. Just over 20 years after it was described, its cause was determined to be a "filterable virus." Along with foot and mouth disease and african horse sickness, it was one of three "filterable viruses" causing severe disease in animals at the turn of the century. The records indicate that fowl plague was responsible for extensive losses in domestic poultry operations throughout the world for the next several decades. Whether that specific disease remains today is a moot point.

The second event was a combination of a devastating pandemic of influenza in human beings in 1918 and the appearance of a new disease in swine. It is estimated that some 21 million died throughout the world with influenza during that pandemic. Its viral nature was not known at that time. The new influenza-like disease in swine, called swine influenza, was described in north central United States and in Hungary during the time that the pandemic was raging in human beings. Thus, a devastating serious disease of human beings of unknown etiology was related to a newly observed disease of swine also of unknown etiology. That the swine might have received their infection from human beings or *vice versa* was a matter of great speculation. The possibility that both human beings and swine might have received their infections from a common third species was not considered at that time. It was not until 1930 and 1933 respectively that the first influenza viruses of swine and human beings were identified.

The third event in the influenza chronicle that relates to this symposium is, as some choose to indicate, the beginning of the avian influenza

era in 1955-56. It was during that time that a fowl plague virus was determined to be a type A influenza virus. It was also during this time that type A influenza viruses different from any other type A influenza virus were recovered from ducks with acute respiratory disease almost simultaneously in England and in Czechoslovakia.

When did the avian influenza era really begin? How many hundreds (or thousands) of years have avian species been infected with influenza viruses? Has that really been the case or are these viruses only recently introduced among avian species and have become readily adapted so rapidly? There seem to be so many avian species and so many influenza viruses so well adapted that the seemingly happy ecologic family of birds and influenza viruses must be the result of a very long evolutionary process. A group of viruses once thought to be limited to human beings, swine, and horses are now found throughout the world in many avian species. How many of the more than 8,500 different species of birds throughout the world with an estimated total population of 100,000 million have been or are infected with influenza viruses? If one examines the contents of this symposium it is clear that it is inappropriate to attempt to summarize the state of the art of avian influenza because much of that information follows.

With regard to the nature of the influenza viruses there has been an extraordinary explosion of the understanding of the viruses in the past ten years and especially in the last 5 years. We know how the virus is put together, we know about the major component parts, we know how it replicates and we know how to classify it. If we only knew as much about the *disease* influenza as we do about the *virus* influenza we would have a considerable understanding of how to control these diseases in the mammalian species as well as in the avian species. It is not unreasonable to speculate that more is known about influenza viruses than any other group of viruses. It is also safe to say that we know relatively little about the disease influenza. While many other devastating diseases have been controlled, influenza remains an uncontrolled major disease problem among human beings and lower animals.

While we don't do very well in understanding the disease influenza we do nevertheless do very well in the diagnosis and identification of the viruses. We do very well in the genetic and antigenic analysis of the viruses and we know that we can successfully vaccinate birds and other species.

Despite the world wide occurrence of these viruses we know very little about their real threat and/or potential to cause significant economic losses in avian populations. And we know very little about their public health aspects. What is the threat of avian influenza? In the past there have been some very considerable losses in various parts of the world. We do have losses today, but considering the large populations at risk there seems to be relatively little loss in economic terms. Can we determine accurately the magnitude of the losses?

Certainly we know a considerable amount about avian influenza. At the same time there is a considerable amount that we don't know. The time is now. It is my firm conviction that the participants in this symposium should not leave until there is some consensus with regard to the goals, priorities and recommendations on an international level for addressing and understanding avian influenza.

UNCOMPLICATED INFECTION WITH VIRULENT STRAINS OF AVIAN INFLUENZA VIRUSES

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Defining the pathogenicity of an avian influenza isolate cannot be done in precise terms although the Intravenous Pathogenicity Index (IVPI) detailed in Poultry Biologics allows comparative tests to be carried out which their categorisation in much the same way as has been done for Newcastle disease virus.

With the IVPI test only a few isolates give an absolute maximum score of 3.0 or levels approaching it. In addition to this, virus that has been stored in the wet state at -70°C or as a freeze dried specimen may not exhibit its maximum potential until it has been passaged through SPF chickens several times. When this has been done isolates can be compared by injecting isolates into groups of birds from the same source housed in identical conditions and observed daily by the operator. Except for the most lethal of strains, the test may be considered a comparative one so that a new isolate is rated in comparison with reference strains. In our laboratory A/Chicken/Germany/34 (Rostock) (H7N1 or Hav1 N1) gives the most regular pattern of acute death with a value of 3.00 on almost all occasions with A/Chicken/Egypt/45 yielding similar results. The reference strain FPV/Dutch/27 (H7N7, Hav1 N7) has always resulted in a slightly slower pattern of death with a proportion of the injected birds dying a day later. Other isolates tested in our laboratory have included A/Turkey/England/63 (H7N3, Hav1 N3) giving slightly lower results of approximately 2.7. These are all strains which have been defined as Fowl Plague Virus.

The only other haemagglutinin type that has given similar results in our hands has been the H5 or Hav5 group including A/Chicken/Scotland/59 and A/Tern/South Africa/61 which have given values of slightly over 2.5. In summary, our collection comprises only two haemagglutinin types which regularly kill six-week old birds in one or two days.

In 1979 when a case of acute avian influenza was reported in Norfolk we obtained a series of isolates from the infected premises that yielded a maximal value of 3.00 on at least two tests although epidemiologically associated isolates taken from the same premises at the same period gave somewhat lower values. This, the newest of our collection of virulent strains was passaged in chickens and repeat lethality tests showed a significant variation in the observed IVPI value, showing that the isolate comprised a mixture of clones each of which had slightly different lethality indices.

In the laboratory when six-week old chickens are used for the test, the

clinical signs are only slight and in general sudden death at or slightly after 24 hours without any well defined external pathological signs predominates. On post-mortem examination the most evident changes are confined to congestion and inflammation of the heart fat, serous surfaces and intestinal wall.

When older birds are injected with these viruses, especially those that have a lethality index of under 3.00, oedema round the eyes and in the wattles may be seen. In most cases these changes can only be demonstrated in older birds when viruses are used which allow birds to remain alive for at least 48 hours; isolates which kill more quickly, do so without obvious signs.

Natural disease in a turkey farm with a virus which included clones of maximal virulence gave a clinical picture of an acute febrile disease in which the infected birds huddled together, were disinclined to move or feed and amongst which birds were seen to fall over onto their backs, kick several times and die within a few minutes. These, when examined showed the same pattern of congestion with some congestion of the lungs and a constant air sacculitis, petechial hemorrhages occurred in the inner breast wall and to a variable extent in leg and breast muscles. A few birds showed the presence of mucous plugs in the trachea and in some of these were also seen in the nasal cavity. Spleens were moderately enlarged and mottled in some birds and in hens, ruptured follicles and early egg peritonitis were to be seen.

Clinically the disease appeared to be more severe in some pens in affected houses and to vary slightly from house to house. (There is an overall impression which cannot now be confirmed that the clinical picture became more severe once the disease had developed.)

In Britain there has not been a case of infection in chickens with virulent virus since 1959 when disease caused by A/Chicken/Scotland/59 caused a very high mortality level. This virus H5N1, Hav5N1 is the only one other than the H1 haemagglutinin type which has possessed this quality. It may be of note that the investigator, the late Dr. J. E. Wilson made the comment that had the disease spread to other farms, consideration might have been given at that time to broadening the definition of Fowl Plague in order to control it. It would appear that a quality of highly virulent avian influenza virus is that of low transmissibility such that in rural areas where poultry populations are low, the disease may be self limiting.

In this context it may be permissible to compare virulent avian influenza virus with its equivalent paramyxovirus Velogenic Newcastle disease. The two conditions shared the name Fowl Pest for many years and the possibility of confusing the two diseases has always been recognised by poultry pathologists. In the Newcastle disease epidemic of 1970 in Britain, the author visited several farms where chickens showed the same pattern of sudden death after flipping onto their backs and kick-

ing for a few moments. The important difference was that the Essex '70 strain of Newcastle disease also gave rise to acute respiratory lesions and the epidemiology of the disease showed it to be highly transmissible. In contrast the highly virulent isolates of Newcastle disease that have had a psittacine origin and which may properly be called Velogenic Viscerotropic Newcastle disease have not, in our experience, shown the high ability to spread that the pneumotropic Essex '70 strain had which is believed to have spread from disease in chickens which was recognised in the Near East in 1968.

An important question that has yet to be answered is whether the highly virulent strains of avian influenza are always of limited transmissibility or whether some may mimic the Essex '70 type of Newcastle disease virus and show both high virulence and high transmissibility.

DR. ROSENWALD: I would like to ask Dr. Allan a question with regard to the intravenous pathogenicity index (IVPI). He posed the differences in the IVPI with a maximum of 3, I presume, dropping down to 0 if there is no mortality and no signs. However, in each case he also mentioned the transmissibility, or to use your own term, the diffusibility of the virus. Do you have any feeling that this might also be useful in defining an outbreak as being subject to regulatory action or simply handled otherwise?

DR. ALLAN: I think I am right in saying that the IVPI test was developed by yourself many years ago. I think one of the problems we have is that we had used this for Newcastle disease, and we found that it was fairly reproducible. When we have used this for avian influenza where we have a maximum kill of 3, then I think we know where we are. When we get down to lower levels, the type of pattern we see is that some of the 6-week old chicks die and others stay perfectly healthy. That is to say if we repeat the test, the standard deviation of this test may begin to get larger. Therefore, I would not want to put the weight on this test that we can confidently put on it for the use of Newcastle disease. I think this is a problem. Now as regards to diffusibility, again Rosie, I think you hit the nail right on the head. We are all, I think in this room, going to be comparing influenza and Newcastle disease because in some ways the epidemiological features are similar. And as you probably know that here in the States you refer to VVND, whereas in Britain I have always said that the highly lethal virus we have that we call Essex 70 was more pneumotropic; and although it took longer to kill than some of your parrot-origin Newcastle, its diffusibility or spread factor was such that in the long term it was much more dangerous. Professor Cummings from Australia I think had made this comment to me very often—please try and make some good method of assessing the diffusibility of a virus. I haven't managed to spend very much time on this, but I know that there are one or two scientists now working on exactly this subject in this area. That is to say, they are testing the isolates not only for lethality but

further ability to spread. I think this work is very much in its infancy. I doubt if it could be carried out in 5 minutes. I mean a test like this probably requires 2 or 3 weeks planning and 10 to 12 days observation. But I would agree with you in this. If we can find a fast spreading virus then we may want to act very much more quickly than if we say — ah yes, the virus killed so fast that the virus and the animals tend to die at the same time. I think this is one of the big problems ahead.

COMPLICATIONS ASSOCIATED WITH AVIAN INFLUENZA INFECTIONS

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Avian Influenza Viruses (AIV) like Newcastle Disease Viruses (NDV) are extremely variable in their disease producing ability (pathogenicity). Fowl Plague Virus (a strain of AIV) has been known to be a very pathogenic strain of AIV. Strains of AIV having the same or different antigenic composition have also been shown to be pathogenic for chickens and turkeys. Still other isolates might have the same antigenic composition as Fowl Plague but be completely apathogenic for chickens.²

This fact is further substantiated by the results obtained by Allan et.al.¹ in a study in which they virulence indexed several AIV isolates, using a system similar to that used for virulence testing NDV isolates. They reported the pathogenicity indices obtained with 13 influenza isolates. The indices ranged from 3.0 to 0.0. Studies conducted by Dr. Karunakaran⁶ on AIV isolates from ducks and turkeys revealed that isolates from ducks are of low pathogenicity and that the pathogenicity of isolates of the same virus subtype (based on hemagglutination and neuraminidase) vary in their disease producing ability.

Factors which have been associated with enhancement of the disease expression in addition to the pathogenicity of the virus isolate include age of exposure, environmental factors (ventilation, litter condition, temperature, crowding), physiological activity (egg laying), and concurrent infections.

Because of the low pathogenicity of many of the AIV isolates and the limited environmental stress factors which occur on range, infection in range reared market turkeys is often subclinical. For this reason a producer may not be aware of the infection until the flock is marketed. AIV infected flocks generally experience an increased condemnation loss at the processing plant. The birds are condemned because of the airsacculitis and septicemia-toxemia associated with the infection and/or its complications. Because of this limited expression of the disease in many range flocks of turkeys, producers report that the outbreak of AI first occurred in confinement reared birds. However, in testing range flocks in these operations, one often finds serological evidence that the range flocks became infected first.

Chickens and turkeys raised in confinement appear to be more likely to

develop clinical signs of the infection. The reason for this is assumed to be related to the added stresses on the respiratory system (physical, chemical, microbial) associated with confinement rearing. The social stresses and population dynamics of crowding may also play a role in allowing the disease to express itself.

Two outbreaks of Avian Influenza have occurred in egg laying strains of chickens while in production in the United States since the Fowl Plague outbreak in 1929. In both of these outbreaks the viruses were of low pathogenicity as determined by the intra-cerebral pathogenicity index and the lack of any signs of disease in subsequent laboratory studies with the isolated viruses. In the Alabama outbreak⁴, the virus was an Hav4Neq2. The virus infected chickens were located on three different farms. These flocks experienced up to 69% mortality. One flock experienced 31% mortality in a single day.

It had been suggested that some bacteria, virus, or stress was in combination with the AIV to produce the disease syndrome⁵. All culturing attempts failed to reveal any other pathogenic organisms. There were several environmental stresses on these three flocks. A hurricane passed through the area a short time before the break. It did not damage the three farms, but resulted in damp chicken houses. One flock was subjected to a calcium deficient diet earlier. These stress conditions were aggravated by temperatures varying by as much as 40°F in one day.

The second outbreak of AIV in laying chickens occurred in Minnesota in 1978³. The virus isolated from this outbreak was Hav6N¹. The mortality in this outbreak was minimal (maximum of 2.6% weekly for one week). Egg production was severely affected in two of the three affected flocks. Attempts were made to determine why the disease was more severe in houses 1 and 3 than in house 2. It was observed that house 2 was warmer (17 C vs 13C). In addition the chickens in house 2 were much better feathered, having recently been force-molted. All three flocks were fed the same ration. Houses 1 and 2 had the same strain of birds.

Turkey breeder hens in production appear to be very sensitive to AIV infection. Affected flocks become very depressed and anorexic. Egg production is markedly affected.

We have had two flocks of very young turkeys two to four weeks of age exposed to two different low pathogenic strains of AIV. In each case the mortality exceeded 50%.

Concurrent infections are the major reason for increased morbidity and mortality associated with low pathogenic strains. Concurrent infections which have been identified included Newcastle disease virus, *Pasteurella multocida*, *E. Coli*, *Alcaligenes faecalis*, and *Aspergillus fumigatus*. These concurrent infections may be from natural infection or following the use of live vaccines. The results of three such complications are summarized in tables 1-3. The widespread use of live Newcastle and Fowl cholera vaccines in turkeys make these two agents frequent com-

plicating agents. *Pasteurella multocida* and *E. Coli* are the most common naturally occurring complicating bacterial organisms.

If more than one AIV subtype is present in a geographical area, it is not uncommon for a flock to go through a second outbreak of AI as a result of infection with a second subtype. However, isolating more than one virus subtype during a given outbreak in a flock has not occurred. Serological results support these cultural findings.

TABLE 1

INFLUENZA - NEWCASTLE DISEASE

Flock ID	79-153-1-9	Total head marketed	7674 (51.5%)
Hatched	9/20/78	Total head condemned	872
Breed	Broad White male turkeys	Percent condemned	11.4
Flock size	14,900		

<u>Age (weeks)</u>	<u>Comments</u>	<u>Weekly - Mortality Number</u>	<u>Percent</u>
5	Flock progressing well	28	0.2
6	Increased mortality-flock depressed with URI-Postmortem pericarditis, perihepatitis.	128	0.9
7	Mortality continues to increase. Serum and swabs sent to NVSL. NCD, GMT-2 AIV POS. HSW1N1.	657	4.5
8	Mortality decreasing. NCD, GMT-404, NCD virus isolated.	127	0.9
9	Increased mortality. Flock still showing signs of respiratory problem.	360	2.6
10	Flock apparently improving.	80	0.6
11	Mortality increased. Flock depressed. Postmortem-airsacculitis	230	1.8
	<u>Serology</u>	<u>10 wk-6 da.</u>	<u>11 wk-2 da.</u>
	NCD (GMT)	28	119
	Hav6N1	Neg.	Pos.
12	Medication continued	371	2.9
13	Flock returning to normal	90	0.7
	Total Mortality	2071	14.2

AVIAN INFLUENZA INFECTIONS

TABLE 2

AVIAN INFLUENZA-ORAL CHOLERA VACCINE

Flock ID 79-407-3-9
 Hatched 3/16/79
 Breed Broad White male turkeys
 Flock size 12,000

Age (weeks)	Comments	Weekly-Mortality	
		Number	Percent
6	Progressing well Received oral Cholera vaccine	27	0.2
12	Progressing well Received oral Cholera vaccine	57	0.4
18	Progressing well Received oral Cholera vaccine. Began coughing 3 days postvaccination. Postmortem findings-lesions consistent with fowl Cholera.	46	0.4
19	AIV confirmed-Hav2Neq1. <u>P. multocida</u> typed-vaccine stain.	975	8.3
20	Elevated mortality continued	1079	9.8
21	Flock beginning to improve	241	2.4
22	Flock marketed. Condemnation 8.3%.	90	0.9
Total Mortality		2385	20.0

TABLE 3

AVIAN INFLUENZA E. COLI COMBINATION

Flock ID 79-405-6-9	Total head marketed 2823 (45.5%)
Hatched 7/10/79	Total head condemned 381
Breed Broad White male turkeys	Percent condemned 13.5
Flock size 6,200	

<u>Age (weeks)</u>	<u>Comments</u>	<u>Weekly - Number</u>	<u>Mortality Percent</u>
3	Flock progressing well	20	0.3
4	Flock very depressed, coughing Postmortem-Tracheal plugs, pericarditis, perihepatitis. AIV isolated-Hav2Neq1.		
6	Medicated continued	112	1.9
7	Flock appears stunted	181	3.4
8	Mortality still increased	439	8.6
9	Flock improving	109	2.3
	Total Mortality	1481	24.3

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AVIAN INFLUENZA IN THE UNITED STATES (1964-1981)

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SUMMARY

Classical fowl plague has not been identified in the United States since 1929. Bankowski and Mikami reported the first isolation of less pathogenic strains from turkeys in 1964. Since then influenza has been recognized in turkeys in 14 states. Minnesota has reported outbreaks every year since 1966. California has had outbreaks consistently over this period. The outbreaks in other states have been sporadic and in some areas of the U.S., South Atlantic, there have been no reported outbreaks. There have been only two outbreaks reported in chickens during this period, Alabama (1975) and Minnesota (1978). A wide variety of hemagglutinin (HA) and neuraminidase (NA) antigens have been identified in isolates from domestic avian species in the U.S., HAV1, HAV2, HAV4, HAV5, HAV6, HAV9, and HSW1 in various combinations with N₁, N₂, NEQ1, NEQ2, NAV1 and NAV2.

Although HAV1 has been isolated from turkeys in three states (Oregon, 1971; Texas, 1978; Minnesota, 1980) and from pheasants (Minnesota, 1980), the pathogenicity of these isolates have been low to moderate in severity under field conditions and showed low pathogenicity under laboratory conditions. Only limited reports of influenza have been made in domestic ducks, geese, pheasants and guinea fowl.

INTRODUCTION

Classical fowl plague has not been identified in the United States since 1929. The first reported isolation in the U.S. of less pathogenic strains was made in California in 1964 by Bankowski and Mikami.¹ Olesiuk et al. reported an isolation in Massachusetts from turkeys in 1965² followed by isolations in 1965-66 from turkeys in Wisconsin.³ In 1966 the disease was identified serologically in turkeys in Minnesota and virus isolations were made in 1967.⁴ Since then, influenza has been reported in turkeys, chickens, and other domestic fowl in other states. A survey was made of the state diagnostic laboratories in the U.S. in 1980 to determine when isolations of the virus and/or serological identification of avian influenza were made. Fifteen states reported identification of avian influenza in domestic fowl. The results are given in Table 1 and the year of the first report and identification of hemagglutinin antigens since the first report.

Turkeys

Minnesota and California have reported more outbreaks than other states. Both states have large turkey populations in concentrated areas. Minnesota raised approximately 25 million market turkeys in 1979 and

had 150 breeding flocks containing approximately 659,000 birds. California marketed about 19 million turkeys and had 123 breeding flocks with approximately 462,000 birds.

In Minnesota, several million market turkeys are raised on range from May 1 to November 1. In the area where influenza has been consistently identified each year in turkeys, there are a large number of turkey flocks raised on open ranges. There are numerous lakes and ponds in the area. The Mississippi flyway is a major flyway of migratory waterfowl from Canada and northern Minnesota. Bahl has consistently isolated various influenza serotypes from ducks in their breeding grounds in northern Minnesota.⁵ There is also migration of gulls during this same period and these birds are found on turkey ranges. Breeding flocks are primarily raised in confinement and the infection may be introduced from infected market flocks, wild birds, or swine and then spread from flock to flock by artificial insemination crews and movement of personnel, equipment, feed trucks and processing trucks.

California has reported outbreaks over the years primarily in breeding flocks. Because of the climatic conditions, many breeding flocks have been maintained in open pens throughout the year, but the industry is moving toward complete confinement. The Pacific flyway is a major flyway for migration waterfowl along the Pacific Coast and influenza viruses have been isolated from wild ducks in California.⁶

Beard and Helfer reported the isolation of HAV1 NAV2 from turkey breeding flocks in Oregon (1971) and found in laboratory studies the isolate was nonpathogenic to chickens.⁷ Beard and Easterday conducted extensive studies with the isolate and found it protected chickens against the highly pathogenic Dutch strain of fowl plague virus.⁸ Texas (1979) reported the isolation of HAV1 NAV2 from turkeys.¹⁵ Four breeding flocks on two farms were involved. The isolate was found nonpathogenic to young chickens and turkeys. Minnesota (1980) reported an outbreak of HAV1 NAV2 in 16 turkey flocks belonging to one company. In the laboratory, the isolates were moderately pathogenic to young turkey poults.

It is interesting to note that outbreaks in seven states were related to HSW1N1. Mohan et al. reported an outbreak in Ohio on one farm where the breeding turkeys had close contact with swine.⁹ The outbreak in breeding flocks in South Dakota were probably related to an outbreak in Minnesota breeding flocks belonging to the same hatchery. A similar situation may have occurred in the outbreak in Missouri and Kansas. The outbreak in Colorado occurred in one large breeding operation and the initiation of the outbreak may have been related to swine. The outbreak in Iowa may also be related to swine because of the large swine population in the area. — More epidemiological studies are needed to determine if the source of the virus in future outbreaks is swine, waterfowl or man.

Except for the one report in Massachusetts (1965) and one in Penn-

sylvania (1976), avian influenza has not been identified in turkeys in other South Atlantic states where 45 million market turkeys were raised in 1979 and 778,000 breeding birds were kept. The turkey industry in that area has gone to complete confinement rearing of market and breeding flocks. The Atlantic flyway is a major flyway for migratory waterfowl and influenza viruses have been isolated from wild waterfowl.¹⁰ Texas has reported only one outbreak involving four breeder flocks.

Chickens

Only two outbreaks have been reported in chickens, one in Alabama (1975) and the other in Minnesota (1978).^{11,12} The source of the infection in Alabama was not determined. The outbreak on one chicken laying operation in Minnesota occurred on a farm that was located near turkey flocks experiencing outbreaks of influenza. Trucks and personnel from the same feed company serviced infected turkey flocks as well as chicken flocks. Commercial egg type and broiler flocks are primarily raised in total confinement in the U.S.

Other Domestic Fowl

There have been only a few reports indicating evidence of influenza viruses producing clinical disease in ducks, geese, pheasants and other fowl in the U.S.^{13,14} HAV1 NAV2 was isolated from outbreaks of two small commercial groups of pheasants in 1980 in Minnesota. These outbreaks had no direct relationship to the outbreak in turkeys. Domestic ducks, geese and game birds are usually raised in outside pens and thus may have contact with wild waterfowl and free flying birds. Very little work has been done to determine the prevalence of influenza viruses in commercially raised ducks and geese.

Table 1

AVIAN INFLUENZA SEROTYPES ISOLATED FROM TURKEYS,
CHICKENS AND OTHER DOMESTIC FOWL IN THE U.S. (1964-1981)

<u>State</u>	<u>Year First Identified</u>	<u>Hamagglutinin Antigens Identified</u>
<u>Turkeys</u>		
California	1964	HAV5, HAV6, HAV9
Massachusetts	1965	HAV6
Wisconsin	1965	HAV6, HAV9
Minnesota	1966	HAV1, HAV2, HAV4, HAV5, HAV6, HAV9, HSW1
Washington	1967	HAV6
Oregon	1970	HAV1, HAV6
Iowa	1971	HAV4, HAV5, HAV6, HSW1
Ohio	1975	HSW1
Pennsylvania	1976	NA
South Dakota	1978	HSW1
Texas	1979	HAV1
Missouri	1980	HSW1
Kansas	1980	HSW1
Colorado	1981	HSW1
<u>Chickens</u>		
Alabama	1975	HAV4
Minnesota	1978	HAV6
<u>Other Species</u>		
Pennsylvania	1969	Ducks NA
Minnesota	1974	Geese NA
	1974	Guinea Fowl NA
	1980	Pheasants HAV1, HAV7

NA - Not available

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CURRENT WORLD-WIDE SITUATION OF AVIAN INFLUENZA IN AUSTRALIA

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Influenza virus infection of commercial poultry has only been detected once in Australia. Fowl plague caused by a virus with antigenic determinants Hav1 Neq1 was detected in 2 egg layers, 1 broiler and 1 duck breeder flocks in January and February 1976. Naturally occurring disease was observed in the chickens but not in the ducks. All infected flocks were slaughtered and extensive serological surveys throughout Australia demonstrated no further occurrence of infection.

The source of fowl plague infection was not determined although serology and cloacal swab culture were used in an attempt to demonstrate infection of wild birds.

Influenza infection of sea birds was detected in the north-east region of Australia in 1972 and 1975, when virus with antigenic determinants Hav. 6 Nav. 5, Hav. 5 Nav. 2 and Hav. 3 Nav. 6 were isolated.

Since 1978 some 45 influenza viruses have been isolated from sea and freshwater birds and domestic chickens in the north-western region of Australia. These viruses have no pathogenicity for domestic chickens.

Extensive cultural and serological investigations of birds, particularly penguins, on Macquarie Island and the Australian Antarctic Continent has not resulted in the isolation of any influenza viruses. However, antibody to fowl plague virus was detected in the sera of 10.9 per cent of adult Adelie penguins sampled on Peterson Island.

AVIAN INFLUENZA IN BELGIUM

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Our laboratory is mainly concerned with the diagnosis of viral diseases of domestic poultry. Each year more than 1000 viral isolation attempts are made in SPF eggs and cell cultures. During the three last years, we have isolated 5 strains of Influenza virus.

For each strain we have determined the ICPI and IVPI as recommended by Allan et al. (1977) and all the strains were submitted to J. J. Skehel, World Influenza Center, Mill Hill, London for serological characterization. The results of these tests are given in Table 1.

Two strains were isolated in 1978. A/Duck/Belgium/314/78 and A/Chicken/Belgium/384/1978. Both strains belong to the same serotype Hav_2Nav_1 ; their ICPI and IVPI were negative. Strain 314 was isolated from a flock of 10,000 six week-old ducks affected by respiratory and nervous symptoms. At necropsy, the main lesion found was polyserositis. The mortality associated with the disease reached 10% but resulted from a mixed infection of the ducks with *Pasteurella antipestifer* and Influenza virus. Virus 384 was isolated in a flock of 7,500 laying hens, 32 weeks old. These birds experienced a mortality of 2.5% in two weeks and a drop in egg production of 15%. Lesions of tracheitis and congestion of the liver and spleen were observed in these chickens.

One influenza virus was isolated in 1979: A/Chicken/Belgium/818/79. This virus belongs to the Hav_6N_2 serotype; its ICPI and IVPI were negative. This virus was isolated from a flock of 5,918 laying hens, 47 weeks old. The affected birds showed a mucous to pseudomembranous enteritis, a blueish coloration of the comb and a drastic drop in egg production (30%). The lesions found in dead birds were distension of the crop, dehydration, enteritis, congestion of the liver, swelling of the kidneys with urate deposits, congestion of the ovaries and presence of broken follicles.

Hematological examination of sick birds revealed the existence of an elevated monocytosis. The total mortality reached 26.4% during a three weeks observation period. Oral or intravenous inoculation of the virus to SPF chickens induced enteritis and monocytosis but no mortality. As the evolution of the disease was not stopped by food change and antibiotic treatments, the flock was eradicated and the premises fully disinfected with formaldehyde. A serological survey made by the hemagglutination inhibition test on more than 1200 sera taken from 50 different laying and breeding flocks located in the infected area established the absence of spreading of the virus.

Two influenza viruses were isolated in 1980: A/Chicken/Belgium/405/80 and A/Chicken/Belgium/407/80. Their ICPI and IVPI are respectively 1.12

and 0. Strain 405 was isolated in a flock of 4000 laying hens, 36 weeks old showing a drop in egg production of 10% without excessive mortality. Lesions of tracheitis and liver congestion were observed in the affected chickens. Strain 407 was isolated in a flock of 36,542 three week old broilers, having respiratory disorders. The main lesion in these birds was also tracheitis. Both strains belong to the H₇N₇ serotype.

As a conclusion we may note that different serotypes of Influenza viruses are not frequently but however regularly found in domestic poultry affected by different clinical conditions. All the isolated viruses may be classified in the lentogenic or mesogenic types but some discrepancy may be observed between field pathogenicity and laboratory tests.

Table 1 : Avian Influenza virus isolations in Belgium

Year	Strain	Serotype	Infected species	ICPI	IVPI
1978	314	Hav ₃ Nav ₁	Duck	0	0
	384	Hav ₃ Nav ₁	Laying chickens	0	0
1979	818	Hav ₆ N ₂	Laying chickens	0	0
1980	405	H ₇ N ₇	Laying chickens	1.12	0
	407	H ₇ N ₇	Broilers	1.16	0

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1980	405	H ₇ N ₇	Laying chickens	1.12	0
	407	H ₇ N ₇	Broilers	1.16	0

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A REVIEW OF INFLUENZA IN CANADIAN DOMESTIC AND WILD BIRDS

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Avian Influenza was not on official records in Canada until the end of the year 1962; up to then Newcastle disease was the most serious recognized virus problem in poultry. The emergence of avian influenza as a veterinary problem can be attributed to two impulses. Firstly, the diagnostic methodology for influenza viruses became simplified in the 30's and 40's by the use of embryonated hen's eggs for virus isolation and propagation, and the hemagglutination (HA) and HA-inhibition (HI) tests introduced practical means for specific serodiagnosis. These methods, pioneered by medical laboratories, found widespread adoption in veterinary laboratories during the 50's. The other contributing influence on the disease situation was the drastic change in poultry rearing from the small diversified family farm with a few backyard fowl to the specialized large scale and very competitive agribusiness of today's poultry industry. The immense concentration and confinement of young susceptible birds created a new and very favourable situation for the spread of viral infections. In addition, diseases of relative mildness to the individual bird, often overlooked on the family farm, became serious problems on industrial farms when thousands of birds failed to grow, or to lay eggs, in accordance with very narrow production performance requirements. The tendency of such problems to spread to other flocks added to the poultrymen's alarm, and they sought assistance from the by now better equipped poultry diagnostic services.

The interplay of these factors led to the recognition in Canada of a steadily increasing number of influenza outbreaks since the winter of 1962/63 (Table 1). Turkeys were mostly affected, and very rarely ducklings, but never chickens. This observation is surprising, since not only were chickens the principal victims of the historical episodes of fowl plague in Europe and North America, but the chicken population is at least ten times more numerous than the turkey population in Canada, and in Ontario in particular. The data presented originate mainly from Ontario, where over 40% of the Canadian poultry industry is located. Disease statistics at the Ontario Veterinary College list 69 influenza outbreaks in poultry from the early 60's to the early 80's. Outbreaks occurred annually during the first decade, and declined progressively during the second decade to a level of near insignificance, although farmers were by then well acquainted with the influenza problem. The reduction of influenza cases is essentially the result of the recognition of wildlife influenza as a permanent and dominant source of turkey influenza. Initial assumptions that the influenza viruses were circulating in the turkey

population, as influenza viruses do in humans, horses and swine, could not be substantiated. While influenza did break out recurrently on certain large turkey farms, the infecting viruses were of different antigenic types (Table 4) thus must have been introduced on the premises anew from outside sources. Our experience indicates, that simple sanitation after an outbreak suffices to eliminate the viruses effectively, and never did we encounter in Ontario a situation where turkey influenza became enzootic. Influenza seems, however, enzootic on duck farms, but complaints from farmers were few during the two decades of poultry diagnostic activities covered by this report; only two instances of duckling mortality investigated yielded influenza viruses, but in both cases pathogenic bacteria were also found in the carcasses, and no definite pathogenic effect to ducklings could be demonstrated experimentally with the influenza isolates (unpublished data). A third avian species affected by influenza was brought to our attention from Quebec, where pheasants reared on a game farm were stricken by the virus.

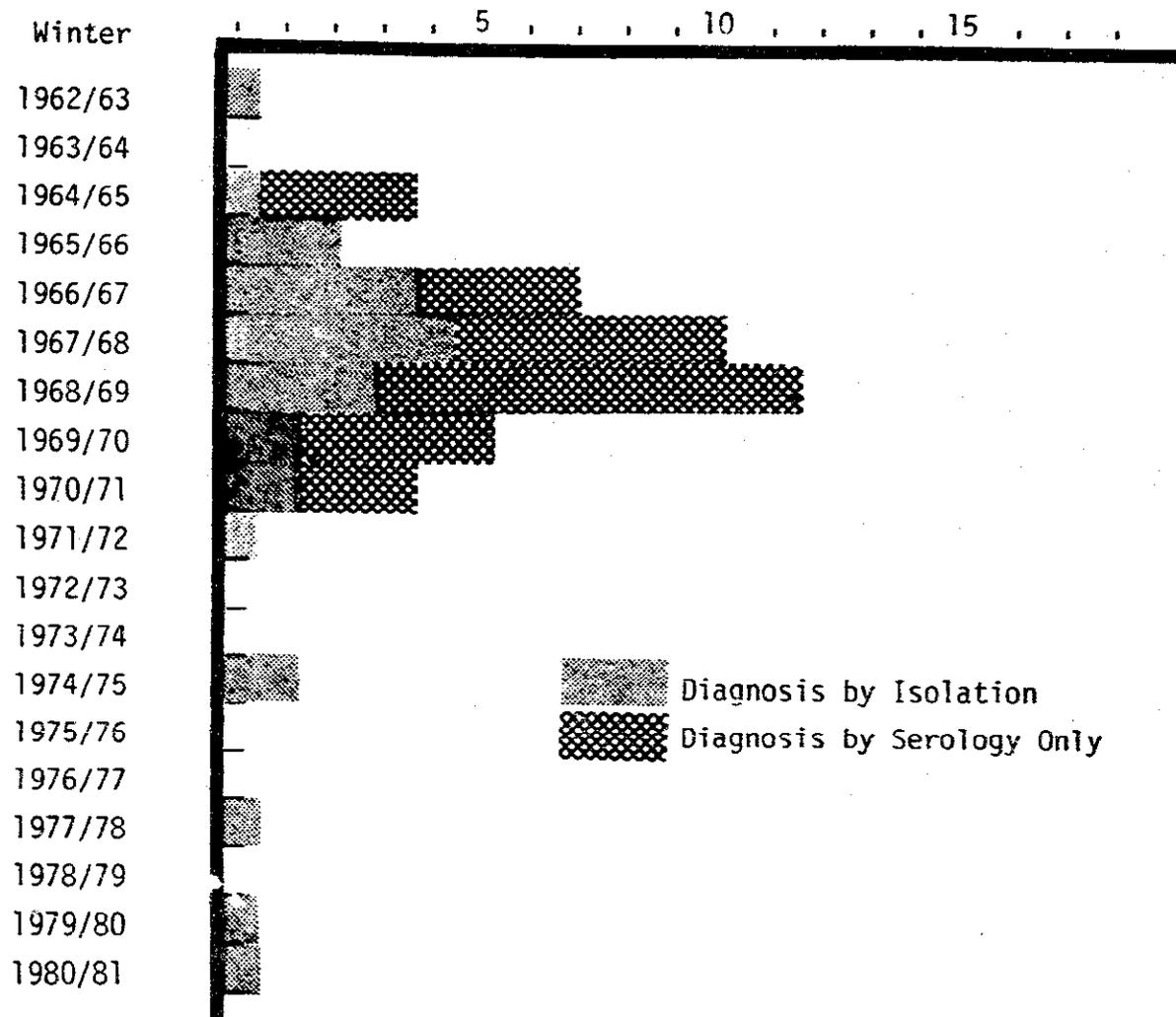
Recent investigations on influenza in Canada's wild bird fauna (Hinchshaw et al., 1978/79; Thorsen et al., 1980; Boudreault et al., 1980) have brought out new features of the influenza epidemiology, in particular the rich variety of virus antigenic types circulating in this ecosystem. Table 2 attempts a consolidation of the data from these studies. Although not an exhaustive reflection of wildlife influenza in Canada, the table projects a trend of prevailing serotypes on the basis of the frequency with which individual serological combinations were detected in isolates. But above all, the table indicates that every hemagglutinin type of the latest influenza classification system (Schild et al., 1980) has been found in the country. Superimposed on the tabulation of wildlife serotypes are the serotypes identified in Canadian poultry (dark framings). Since we can safely assume that every influenza antigenic type can infect turkeys a hypothetical vaccination program for domestic turkeys would require a vaccine encompassing most or all hemagglutinin types, a difficult and costly proposition. It is common knowledge that, despite numberless attempts, vaccination has not achieved a lasting reduction of influenza in humans, horses or swine. The main reason for the poor influenza immunity is the short lifespan of humoral antibodies, which is variable from one species to another, but particularly short in turkeys immunized with inactivated or low-virulent live virus preparations (Narayan et al., 1970; Rouse et al., 1971).

The discovery of the permanent influenza virus reservoir in wild birds and its quasi exclusive role as source of infection for domestic turkeys suggests a more promising method of influenza control on turkey farms. This method is based on the strict separation of domestic turkeys and chickens from all wild and feral birds. The concept is but a corollary of the 'all in - all out' rule practiced since the beginning of the poultry industry. Our control method is being put to the test in a research project involving the two largest turkey breeders in Ontario. The two organizations main-

tain about half a million turkeys on several farms at any one time and account for about 75% of the yearly hatchery output in the Province. Both organizations have repeatedly experienced influenza during the past. The program calls for HI spot tests with the six prevailing HA types (H4,5,6,7,8,9) of every flock at the age of 22 weeks, and again during the laying season whenever the egg yield declines. The supervised premises have stayed free of influenza since the beginning of the program in early 1978 until this spring, when influenza broke out at one farm managed by a new and inexperienced employee. This break underlines the importance of proper training of the personnel in the quarantine strategy since these persons must fully cooperate and play a crucial role in the identifying loopholes in the system through which the domestic birds can be exposed to contamination from wild birds. The contamination can not only take place by direct contact with wild birds, but also by contamination of feed bins or straw and other bedding material stored in sheds and barns accessible to free-flying birds. The marked reduction in turkey influenza has been the most persuasive argument in convincing turkeymen of the validity and practicability of this method.

TABLE 1
ANNUAL FREQUENCY OF AVIAN INFLUENZA OUTBREAKS DIAGNOSED
AT THE ONTARIO VETERINARY COLLEGE

Number of Influenza Outbreaks



INFLUENZA IN CANADA

TABLE 2

INFLUENZA A SEROTYPES IN CANADIAN BIRDS

NEURAMINIDASE TYPES

HA TYPES		N1	N2	N3 av2/3	N4 av4	N5 av5	N6 av1	N7 eq1	N8 eq2	N9 av6
H1	H0	E 144				1				
	H1	O								
	HSW	W 52							1	
H2	H2	E	2							
		O								
		W		2						1
H3	H3 eq2 av7	E 6	26				25	2	59	2
		O 1	1						4	1
		W 1	8	1			35	1	35	1
H4	av4	E	34				60		30	
		O	2				5		1	
		W 5	8	1	1		228		28	1
H5	av5	E	1							
		O	1							
		W	4							
H6	av6	E 17	7			5	4		78	1
		O	2				1		6	
		W	523			1			5	
H7	av1 eq1	E								
		O								
		W		10			1			
H8	av8	E								
		O								
		W				1				
H9	av9	E								
		O								
		W								
H10	av2	E								1
		O								
		W			1			3		
H11	av3	E								
		O								
		W	2							4
H12	av10	E								
		O								
		W					1			

LEGEND = E: EASTERN CANADA, O: ONTARIO, W: WESTERN CANADA, DARK CASES: SEROTYPES FOUND IN DOMESTIC BIRDS.

TABLE 3

Frequency of Antigenic Types Identified in Influenza Outbreaks
in Canadian Poultry Diagnosed at the Ontario Veterinary College

Antigenic Types	Number of Outbreaks Diagnosed by:	
	Virus Isolation	Serology Only
H4 N1	1 (Duck)	
H4 N2	1 (Duck)	
H4 N6	2 (Turkey)	
H5 N1	1 (Turkey)	
H5 N2	4 (3 Turkey; 1 Pheasant)	H5 7 (Turkey)
H5 N9	6 (Turkey)	
H6 N1	7 (Turkey)	
H6 N2	8 (Turkey)	H6 27 (Turkey)
H6 N8	6 (Turkey)	
H8 N4	1 (Turkey)	
H9 N2	1 (Turkey)	
	Number of Outbreaks 35	34 Total 69

TABLE 4

SEROTYPES OF INFLUENZA VIRUSES ISOLATED DURING SUCCESSIVE OUTBREAKS
AT THE SAME BREEDING ORGANIZATIONS

I INFLUENZA OUTBREAKS AT TURKEY BREEDER WG-WM			
20/ 01/ 1967	T/Ontario 6118/67		H8N4
03/ 03/ 1967	T/Ontario 6828/67		H5N9
11/ 12/ 1967	T/Ontario 4845/67		H6N1
II INFLUENZA OUTBREAKS AT TURKEY BREEDER CY-ST			
06/ 01/ 1966	T/Ontario 6213/66		H5N1
28/ 11/ 1967	T/Ontario 4689/67		H6N1
29/ 12/ 1969	T/Ontario 3849/69		H6N8
26/ 09/ 1975	T/Ontario 9313/75		H6N2
15/ 10/ 1975	T/Ontario 9365/75		H6N2
III INFLUENZA OUTBREAKS AT TURKEY BREEDER CO-TH			
20/ 03/ 1966	T/Ontario 7732/66		H5N9
12/ 12/ 1966	T/Ontario 5379/66		H6N2
28/ 11/ 1969	T/Ontario 3575/66		H6N8
18/ 11/ 1970	T/Ontario 3348/66		H6N1
21/ 10/ 1974	T/Ontario 8009/74		H6N1

CURRENT SITUATION OF AVIAN INFLUENZA IN FRANCE

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It seems that the current epidemiological situation in France concerning avian influenza is quite clear as far as the information given by the diagnostic laboratories is complete.

In domestic birds, especially chickens and turkeys, no outbreak was observed. During the past years two serological surveys were done, the first during 1975-76 in poultry flocks by Dr. Fontaine (Lyon Veterinary School) and more recently during 1980-81 in broiler-breeders, turkey breeders and broilers by our laboratory. All AGP serological reactions were negative except a very few positive reactions in turkey breeder flocks. These sera don't show inhibition with HAV antigens and were actually checked for other specie hemagglutinins. In 1980 an avian influenza virus (AIV) (HAV6N2) was isolated in the Northern part of France in a broiler-breeder flock where sanitary problems occurred. This strain was examined in our laboratory and was found in fact to be of low virulence (IVPI = 0, ICPI = 0).

In wild birds, especially feral ducks, between 1976 and 1979 AVI has been isolated from cloacal swabs by Hannoun in the North East of France. Four types of hemagglutinin HAV1, HAV6, HAV7, (H3) HSW1 and five types of neuraminidase N2, NAV2, NAV4, (N1), NAV5 et Neq2 were identified.

This epidemiological situation showed evidence of circulation of AIV in wild birds in France and consequently the risk of infection to domestic birds exists.

Because in our country, all domestic birds are kept in closed poultry houses, and no influenza outbreaks have occurred, the infection rate by AIV in poultry flocks is very low. Therefore it is suggested that keeping domestic birds in closed houses to prevent AIV contamination by wild birds is very important.

AVIAN INFLUENZA IN HONG KONG

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Approximately 36 million domestic poultry were raised in Hong Kong for local consumption in 1970. This comprised only 60% of requirements, the balance being supplied mainly as live birds from neighbouring Guangdong Province and also from Guangxi Province, China.

Since November 1975, continuous market surveillance of ducks, geese and chickens and local duck farm studies have resulted in the isolation of 41 different antigenic combinations of influenza viruses from Chinese poultry and 21 from Hong Kong poultry. Whereas swabs taken from Chinese poultry comprised only 44% of the total samples, they yielded 85% of the isolates, the most common isolate being H4N6 (Hav4Nav1). Although ducks constituted about 20% of the poultry sold in Hong Kong, because of their considerably higher virus isolation rate, they yielded 96% of the influenza isolates. The isolation of these viruses showed a cyclical and seasonal trend, being greater in the warm, humid summer months.

All birds sampled were apparently healthy including those from which H7N1 (Hav1N1) and H7N2 (Hav1N2) were isolated; H7N2 infection of experimental poultry was asymptomatic. Surveillance of local duck farms on which H3N2 (Hav7N2), H7N1, H7N2 and other antigenic combinations occurred confirmed the asymptomatic nature of infection and indicated (1) faecal-water-oral transmission of virus (2) maintenance of virus by regular (monthly) introduction of ducklings onto the virus-contaminated pond and (3) birds >70/80-days-old were essentially free of detectable virus.

Whilst only limited data are available for isolates from domestic quail and pigeon (and even less from feral and migratory birds), it seems likely that, as no outbreaks of disease attributable to influenza have been recorded in Hong Kong during the period of surveillance, avian influenza is of limited pathogenicity in the local poultry.

STUDIES ON THE ECOLOGY OF AVIAN INFLUENZA VIRUSES IN ISRAEL

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Israel is a unique place for the studies on the ecology of avian influenza viruses because of its geographical, ecological and economical peculiarities; i.e., its location along the main flyway of feral birds migrating from Eastern Europe to Africa and highly developed poultry industry. Systematic studies in this field were established in Israel in 1978. Since then tracheal and cloacal swabs were taken from 1409 birds including 473 domestic poultry: turkeys (332), chickens (56) and ducks (85); and 936 feral birds: mallard ducks (47), teals (31), pintail ducks (4), coots (64), moorhens (12), rock partridges (126), cattle egrets (99), pigeons and doves (137), starlings (282), quails (35), larks (20) and other various species of migrating birds (79). A total of 29 influenza viruses has been isolated. The majority (24) of the isolates were derived from the feral birds: mallard ducks (16), pintail ducks (1), coots (4), rock partridges (2) and starlings (1). Five isolates were derived from the domestic poultry: turkeys (2), (2), chickens (1) and ducks (2). Besides, three unidentified hemagglutinating agents (isolated from turkeys in the past — 1971, 1973 and 1978 — and preserved in a viable form up to now) were identified retroactively as influenza viruses. Five serologically different influenza A viruses have been identified: H7N7, H7N2, H10N4, H11N3 and H5N2. Of these combinations, H5N2 was found in all the three retroactively identified isolates from turkeys and was not found in the recent isolations. H7N2 combination being more frequent was isolated from the turkeys, chickens, mallard ducks and rock partridges and H7N7 combination was isolated from the starlings. The latter isolates which are serologically similar by hemagglutinin antigen to fowl plague viruses (the isolate from the starlings is serologically identical to A/FPV/Dutch/27(H7N7) prototype strain by both hemagglutinin and neuraminidase antigens) appeared to be avirulent to chickens, turkeys and ducklings (the studies on pathogenicity were performed by Dr. D. J. Alexander).

One of the studied cases has offered evidence suggesting immediate epizootiological connection between occurrence of influenza among the mallard ducks and influenza outbreak in a turkey farm. The case is that about 200 ducks were found dead in fields located about 1.5 km from the turkey farm in which a month later the influenza outbreak was observed. From each of the 15 ducks taken at random for investigation, influenza viruses were isolated which were serologically identical to the isolate from turkeys in hemagglutination inhibition, neuraminidase inhibition and double immunodiffusion tests.

CURRENT SITUATION IN ITALY

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INTRODUCTION (1872-1972)

Early studies by Perroncito and Rivolta, Dal Prato (1878-1880) described a new disease entity in chicken, not caused by bacteria, called *typhus exudativus gallinarum* (from the gelatinous or fibrinous exudate lining the serous cavities), later called peste aviare (fowl plague). In 1901-05 another epizootic wave of the disease was observed in Italy. The disease spread also to other European countries. In 1901 — three years after the discovery by Loeffler and Frosch of the filterability of foot and mouth disease virus — Centanni and Savonuzzi showed that the agent of fowl plague passes Chamberland filters. Transmission was accomplished by subcutaneous inoculation of organ suspensions and by the oral route. The faeces were shown to be infectious.¹

The virus was highly pathogenic for chickens, pheasants, guinea fowl and turkeys. The disease was very frequent in Northern Italy but outbreaks were observed in the South: 800 pheasants of the Royal Reservation in Capodimonte near Naples showed a 96% mortality. Water fowls usually appeared resistant to the virus, but there were reports of the susceptibility of mallard ducks and geese. Thrushes were very susceptible as were also budgerigars and cockatoos. Already in the first years of the century the observation was made of natural "host range mutants."

During the following years until the early 30's the disease continued to ravage the country, until it extinguished itself by 1937, when the new epizootic of Newcastle disease took over. During one of the last outbreaks of the fowl plague in 1935, Cominotti² isolated from chickens the strain presently known as strain Brescia, recently identified as Hav1 N1. This strain, sometimes erroneously called 1902, should be correctly labelled chicken/Italy/Brescia/1935. No further outbreaks of influenza A were reported in Italy for about 30 years, until the late Dr. Rinaldi and his group at Pavia, in collaboration with Dr. Pereira of the WHO Influenza Center in London started a series of studies (1965-68) isolating in Lombardy (Pavia, Milano) many strains from ducks, quails, turkeys, pheasants and chickens.⁹

Most strains were related to the serotype quail/Italy/1117/65, later shown to be Hav2 Neq2. A few strains were serologically different, possibly related to turkey/Massachusetts/65 (Hav6 N2). In the same period in Veneto (Vicenza, 1967) we isolated two strains of the Hav2 Neq2 serotype in Japanese quail.¹⁰ Common characteristics of the disease in the 1965-68 in turkeys and quail were respiratory symptoms with variable mortality, up to 80% in turkeys and up to 75% in quail. The laboratory tests showed that the Hav2 Neq2 strains isolated were of in-

intermediate virulence. In Veneto (Vicenza) during the years 1970-71 we isolated from quail 5 additional strains, probably related to Hav2 Neq2.¹⁰ In Southern Italy (Naples) a strain of influenza A (H? N?) was isolated in 1969 from a respiratory disease of turkeys.⁷

RECENT OUTBREAKS (1973-1980)

Epidemiology.

The first isolation of influenza A virus from affected turkeys in NE Italy (Veneto) was made in 1973, but until 1975 the outbreaks of the disease were sporadic and limited to a very narrow area. During the month of December 1976⁴ episodes in broiler turkeys were observed with an enhanced frequency in animals older than three months, in farms situated in the southern part of the province of Verona. From 1977 to 1979 outbreaks of the disease spread to a much wider area covering most of the province and probably nearby provinces.⁵

The number of the disease outbreaks and the serologically positive groups of turkeys in the province of Verona showed a steady increase between 1st January 1977 and 1st September 1979. The seasonal incidence of the disease was quite marked and the outbreaks of influenza were observed as a rule during the autumn and winter months, except in 1979, when the disease lasted from January to September. In these 9 months 44/200 (22%) of the groups of turkeys examined had shown serological evidence of infection with the influenza virus Hav6 N2 subtype.

The sudden disappearance of both disease outbreaks and serological evidence of infection after 1st September 1979 coincided with the introduction of much stricter hygiene and control measures.

Clinical picture.

In affected flocks all turkeys showed signs of sneezing and lacrimation with anorexia, prostration and fever. Some birds showed swelling of infraorbital sinuses and nasal mucous (or purulent) discharge. The disease signs usually lasted about 10 days after which the birds returned to normal. Mortality varied from one to six per cent in different groups. In rare cases where the animals were less than 3 months old (two outbreaks in five and seven-week-old birds) about 40% of the birds showed swelling of the infraorbital sinuses with a fibrinous or caseous plug. In these birds up to 20% mortality occurred during the acute stage of the disease.⁵

Post mortem examinations revealed mainly infraorbital sinusitis and seromucous rhinitis. Tracheas were usually congested, sometimes with mucofibrinous plaques. Edema of the lungs was seen and sometimes bronchopneumonia. In some outbreaks secondary infections from *E. coli* and *P. multocida* were observed: in these cases mortality reached higher levels.

Pathogenicity tests.

Intravenous pathogenicity tests on 6 week-old-chickens were performed on 7 isolates of Hav6 N2. Six strains, each produced an index of 0.00. For strain A 303/78 an index of 0.15 was calculated, one bird showing slight symptoms.

With the intranasal and contact pathogenicity tests on 2 week-old-turkeys, a pathogenicity index of 0.7 was obtained for directly infected birds and of 0.5 in contact turkeys, for the isolate A 21/76. On the whole, the strains should be considered of low virulence. It is interesting that most isolates were very resistant to pH 4.¹¹ The virus could be isolated from the faeces 60 days after the beginning of the symptoms.

With the exception of a Hav2 N2 isolated in 1977, the numerous isolates of the Hav6 N2 from turkeys in Veneto, 1973 to 1979, were identical with very little evidence of antigenic drift.⁸ This may be indicative of an endemic infection among these birds.

In 1974, in Lombardy (Pavia) a Hav6 N2 was isolated from turkeys, probably introduced from Veneto.

In Lombardy in this period a strain of Hav2 Neq2 from a swallow was isolated in 1977, during a survey of migratory birds.² The infection probably came from domestic fowls which were antibody positive. In the same survey performed in 1976 and 1977 all isolation attempts from numerous migratory birds (Anatidae and a rook) were negative.²

In Emilia (Forli) a Hav6 N2 was isolated in 1979 from guinea fowls.⁸ In the year 1980, the outbreaks were sporadic.

A single strain of a new serotype Hav6 Nav1 was isolated in turkeys in Veneto (Verona)⁶ where Hav6 N2 was previously prevalent.

During this year one more outbreak was diagnosed in turkeys in Lombardy (Bergamo) due to Hav5 N2.⁶ This same strain was also isolated from hens with a drop in egg-production, in the same area.

The strain prevalent in quails from 1965 (Hav2 Neq2) was again isolated in this species in Veneto³ in 1980.

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CURRENT SITUATION OF AVIAN INFLUENZA IN POULTRY IN GREAT BRITAIN

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INFLUENZA IN FOWLS

After the first description of "fowl plague" as a disease of fowls in Italy in 1878 (Perroncito cited by Stubbs, 1965), the disease appears to have become widespread throughout Europe and persisted there during the end of the 19th and beginning of the 20th centuries. Outbreaks of "fowl plague" occurred in Great Britain in 1922 and probably in 1929 (Advisory Committee on Poultry Disease, 1951). However, in the era following the identification of "fowl plague" virus and other virus pathogens of fowls as influenza viruses, which began in 1955 (Schafer, 1955), only one report of influenza virus infection of chickens has been made in Great Britain. This was an outbreak of virulent disease of chickens in Scotland which occurred in 1959 (Wilson, cited by Pereira *et al.*, 1965). The causative virus was later typed as A/Chicken/Scotland/59 (H5 N1).

Since 1959 no reports of disease in fowls in Great Britain attributable to influenza virus infection have been made. In view of the susceptibility of chickens to influenza viruses in laboratory experiments (Narayan *et al.*, 1969; Alexander *et al.*, 1978; Westbury *et al.*, 1979), lack of clinical signs in the field may, in the absence of a systematic survey, be regarded as evidence that commercial fowls in Great Britain are free of influenza virus infection.

INFLUENZA IN TURKEYS

Influenza virus infection of turkeys was first reported in Great Britain in 1963 by Wells (1963) when a fully virulent influenza virus was isolated from turkeys in Norfolk showing severe disease. Between 1964-1978 isolations of influenza viruses of low virulence from outbreaks in turkeys were made in 1966, 1969, 1970, 1973, February 1977 and October 1977 (Madeley *et al.*, 1971; Allan *et al.*, 1970; Alexander *et al.*, 1978). The antigenic characterization and pathogenicity indices of these viruses are shown in Table 1. Generally these viruses were associated with mild respiratory disease and egg laying problems in the field outbreaks. Ty/Eng/69 produced the most virulent clinical signs of the six viruses and over a 14-day period 6% mortality was seen in the turkeys in which the outbreak occurred (Allan *et al.*, 1970). Ty/Eng/647/77 was of considerable economic importance to the turkey producer concerned in the outbreak as it caused complete cessation of egg laying in the breeder flock from which it was isolated. All the outbreaks in turkeys up to 1979 appeared to be distinct, isolated occurrences and on no occasion was evidence of spread detected.

Between March and May 1979 a series of influenza A virus infections were detected on turkey farms in England and eight influenza viruses were isolated from turkeys on different affected farms (Alexander and Spackman, 1981). Altogether 16 farms were shown to be affected by influenza virus infections. Fourteen of these farms were situated in Norfolk, one was in Suffolk (but under the same ownership as affected farms in Norfolk) and one farm, apparently unassociated with those in Norfolk, was in Hertfordshire. The eight viruses isolated on different sites were of H7N3 (two), H7N2, H7N7 (three), H1N1 and H10N4 subtypes; while serological evidence of H7 infections were detected on 8 sites and H10 infection on one other site. The H7N7 viruses isolated from two sites (three farms) under joint ownership were extremely virulent viruses and birds were slaughtered under Fowl pest (fowl plague) legislation (Alexander and Spackman, 1981). The clinical signs seen on the affected farms and the characterization of the viruses are summarized in Table 2.

The overall pattern of the 1979 outbreaks suggests that several subtypes of influenza virus were introduced to a number of unrelated foci. Some evidence of spread amongst closely situated farms under the same ownership was evident, presumably due to the agency of man, but no further spread was apparent.

As a result of the unprecedented number of outbreaks seen in 1979, two surveys of turkey flocks were undertaken. Both surveys were restricted to Norfolk and the area of eastern England considered to be most at risk and where turkey farming is most intensive. The surveys were done in conjunction with two multisite producers. In Survey I, which covered a six month period, ten serum and ten cloacal swab samples were submitted from each flock, both breeders and fatteners, of one of the producers. In Survey II 20 serum samples, and occasionally cloacal swabs, were taken from each flock only at end of lay (48-50 weeks of age). Serum samples were tested by agar gel precipitin tests to Influenza A ribonucleoprotein (Beard, 1970) and by haemagglutination inhibition tests. Cloacal swabs were passaged two or three times in 9-day-old embryonated chicken eggs. The overall results of the two surveys are summarized in Table 3. The four sites on which positive sera were detected were known to have been affected during the spring of 1979 (sites Norfolk - 3, Norfolk - 4, Norfolk - 6 and Norfolk - 10) and haemagglutination inhibition (HI) antibodies were against the same haemagglutinin subtypes as previously seen on these farms. No new sites showing evidence of influenza virus infection were detected. No viruses were isolated from birds on any of the sites tested, as part of the surveys, including those with serologically positive birds.

INFLUENZA IN COMMERCIAL DUCKS

Influenza viruses A/duck/England/56 (H11 N6) and A/duck/England/62 (H4N8) were isolated from the same duck farm as a result of investigations of outbreaks of chronic respiratory disease (Simmins and Asplin

cited by Roberts, 1964, Roberts, 1964). During 1963-1978 there were no reported isolations of influenza virus from commercial ducks in Great Britain.

In August 1979 influenza A viruses were isolated from commercial ducks showing respiratory disease on a fattening farm in Norfolk (Alexander *et al.*, 1981). The disease continued to reappear in birds introduced onto the farm and, over a six month period, further isolations of influenza viruses were made (Table 4). Serum samples were also taken at each of the occasions listed in Table 4 but none of these showed positive precipitin lines in agar gel precipitin tests to influenza. A ribonucleoprotein or gave positive titres in haemagglutination inhibition tests to the viruses isolated (Alexander *et al.* 1981).

During the summer of 1980 a survey was undertaken to examine duck carcasses at several Norfolk slaughter houses for influenza viruses. Cloacal swabs were taken from the dead birds and pooled in batches of ten. Thirty-two influenza viruses were isolated from the first 60 pools of swabs examined. The details of these isolations antigenic characterization of the viruses are given in Table 5. Viruses were isolated from birds sampled at three slaughter houses and implicated several farms including that farm from which viruses had been isolated earlier in 1980.

DISCUSSION

Migratory water fowl have been shown to be carriers of influenza viruses (Easterday, 1975; Lvov, 1978; Hinshaw *et al.*, 1980; Hannoun and Devaux, 1980) and it has been speculated that outbreaks of influenza in turkeys, both in Great Britain and the United States of America, have occurred as a result of introduction into the area by such birds (Wells, 1963; Alexander *et al.*, 1979; Alexander and Spackman, 1981; Bahl *et al.*, 1979). Whether transmission from infected waterfowl to the turkeys occurred directly, by an intermediate host, such as small wild birds, or mechanically is not known. Turkeys are generally kept in confinement in Great Britain and this may, in part, account for the relative freedom from disease compared to turkeys in the U.S.A. (Bahl *et al.*, 1979). However, the buildings used to house turkeys are not usually as substantial as those in which chickens are housed and are certainly not proofed against invasion of small birds. This may account for differences in the frequency of outbreaks of influenza in turkeys and fowls. In addition, at the time of the outbreaks in turkeys in 1979, which correlated with the passage of several species of water fowl through Norfolk, damage had occurred to many of the turkey houses as a result of the harsh winter and high winds, so that access was particularly available. Although some ducks were kept in confinement, fatteners are generally kept in open fields and would seem even more at risk to influenza infection from wild birds than turkeys. This could account for the ease and frequency with which viruses have been isolated from commercial ducks since 1979. Ducks are particularly refractory to infection with even the most virulent influenza

viruses for other birds (Alexander *et al.*, 1978; Westbury *et al.*, 1979; Slemons and Easterday, 1972) and it may be that the lack of reports of influenza in commercial ducks between 1962-1979 is because such infections are normally inapparent.

In conclusion, it would seem that the current situation of influenza virus infections of domestic poultry falls into three categories, each relating to a different species of bird. In chickens there is no evidence that any influenza outbreak has occurred since 1959. In turkeys isolated outbreaks of influenza virus infections have been seen fairly regularly since 1963, but there is no evidence that the influenza viruses have remained endemic in the national turkey flock. Evidence from commercial ducks since 1979 suggests that influenza viruses may be continually present on some duck farms. Whether this represents repeated re-introduction to the ducks or spread of virus from one batch of birds to the next is not clear.

Table 1: Influenza viruses isolated from turkeys in Great Britain during 1963-1978

Viruses	Location of outbreak	Intravenous pathogenicity index in six-week-old:-	
		Chickens	Turkeys
A/ty/England/63 (H7N3)	Norfolk	2.97	2.78
A/ty/England/66 (H6N2)	Norfolk	0.00	0.04
A/ty/England/69 (H3N2)	Norfolk	0.81	0.15
A/ty/Scotland/70 (H?N7)	Scotland	0.00	0.00
A/ty/England/N28/73 (H5N2)	Norfolk	0.00	0.00
A/ty/England/110/77 (H6N2)	Norfolk	0.00	0.00
A/ty/England/647/77 (H7N7)	Herefordshire	0.00	0.00

Table 2. Summary of clinical signs and characterization of viruses involved in influenza outbreaks in turkeys in 1979.

Site	Age of turkeys in weeks	Clinical signs	Virus isolated/serology	IVPI ^{**} in chickens
Norfolk - 1*	48-52	Mild respiratory signs	None - serologically - H7	-
Norfolk - 2	10-25	Respiratory signs 1% mortality in one week	None - serologically - H10	-
Norfolk - 3	9-27	Severe respiratory signs	A/ty/Eng/192-328/79 (H7N3)	0.00
Norfolk - 4	30-45	Fall in egg production, 1% mortality	A/ty/Eng/192-329/79 (H7N2)	0.16
Norfolk - 5a	9	High mortality - sudden deaths	A/ty/Eng/199/79 (H7N7)	3.00
Norfolk - 5b	18	High mortality - sudden deaths	A/ty/Eng/214/79 (H7N7)	2.80
Norfolk - 6	33	Respiratory signs, rise in mortality	A/ty/Eng/250/79 (H1N1)	0.00
Norfolk - 7	48	40% drop in egg production, sick birds, some deaths.	A/ty/Eng/262/79 (H7N3)	0.12
Norfolk - 8	6	Sudden deaths	A/ty/Eng/272/79 (H7N7)	2.47
Norfolk - 9	40	Fall in egg production	None-serologically - H7	-
Hertfordshire	36	Unusual deaths amongst stags	A/ty/Eng/384/79 (H10N4)	1.51
Suffolk	48	No signs	None-serologically-H7	-
Norfolk - 10	40	2% "white misshapen" eggs	None-serologically-H7	-

* Site Norfolk-1 consisted of four, jointly owned, very closely situated farms all of which were serologically positive.

** IVPI - intravenous pathogenicity index in six-week-old chickens.

Sites are listed in chronological order of virus isolation and serological detection in the laboratory and do not necessarily relate to onset or detection of clinical signs. For the Norfolk and Suffolk sites the outbreaks covered a period from the first week in March to the first week in April. A/ty/Eng/384/79 was isolated in early May but clinical signs had been seen in the infected flock in April.

Table 3. Summary of serological surveys of commercial turkeys 1979-1981

Survey	Dates	Number of sites	Number of sites positive by AGP test*	Number of sites on which virus isolated	Total sera examined	Number of sera positive by AGP test*
I	21.3.79 - 20.11.79	45	2	0	1902	67
II	9.4.79 - 10.2.81	15	2	0	1090	18

* Agar gel precipitin test to influenza A ribonucleoprotein (Beard, 1970).

Table 4. Isolation of influenza viruses from a commercial duck fattening farm in Norfolk.

Date	Sample	Age of ducks (weeks)	Virus isolated	IVPI*	Comment
13.8.79	Pooled lung spleen	3-6	713/79 (H6N2)	0.00	two similar viruses isolated
13.8.79	Pooled lung spleen	3-6	739/79 (H4N6)	0.00	-
29.12.79	Pooled organs	8	1149/79 (H4N6)	0.34	-
23.1.80	Lung samples	8	96L/80 (H4N1)	0.78	¹ / ₁₅ samples positive
23.1.80	Cloacal swabs	8	96F/80 (H4N1)	0.45	7 similar viruses from 29 swabs
4.3.80	Cloacal swabs	8	329/80 (H4N6)	0.00	² / ₁₆ swabs positive
4.3.80	Intestinal contents of dead duck	6	332/80 (H3N8)	0.00	-

* IVPI - intravenous pathogenicity index in six-week-old chickens.

Table 5
Isolation of influenza viruses from duck
carcasses at Norfolk slaughterhouses
during June-July 1980

Slaughterhouse	Date	Viruses isolated subtype ^a	number
A	12.6.80	H3N6	5
B	16.6.80	H3N6	1
		H3N8	1
		H3N1	1
C	19.6.80	H4N8	5
	14.7.80	H4N8 ^b	5
D	23.6.80	H3N8	3
E	26.6.80	H4N2	1
		H4N8	1
		H9N8	1
		H3N?	1
		not typed	1
	3.7.80	H3N2	5

a: all isolates were from pools of ten cloacal swabs except:-

b: two isolates from nasal swab pools
two isolates from eye swab pools

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OCCURRENCE OF AVIAN INFLUENZA VIRUS TYPE A IN GERMANY

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In Germany, as in many parts of the world, the size of poultry farms and the methods of animal husbandry used are such that they would further the spread and persistence of highly contagious virus infections like influenza A. This agent, however, has apparently not played a significant role in the etiology of the various clinical diseases occurring in domestic poultry. Outbreaks of clinical influenza have not been recorded for domestic chickens, turkeys or ducks in Germany, with one exception. SCHETTLER (1) isolated influenzavirus type A (A/duck/Germany/1868/68; Hav6N1; H6N1) from domestic ducks during an outbreak of respiratory disease in 1968. Whether this isolate was the only agent involved in the etiology of the disease remains doubtful, since an attempted experimental reproduction of the respiratory symptoms in ducklings proved unsuccessful.¹

We can only speculate upon this somewhat puzzling situation. In Germany, poultry farms are generally located in isolated areas, and there is little traffic or contact between them. Furthermore, chickens and turkeys are kept indoors so that feral fowl can be ruled out as transmitters of influenza, although they are a reservoir for many antigenic subtypes.²

Another possible source of infection in Germany, namely, imported psittacine birds do not seem to be of importance as a reservoir from which influenza A could spread to domestic fowl. Attempts in our laboratory to isolate influenzaviruses from psittacines imported from Africa (Senegal, Tanzania), Asia (Indonesia, Singapore) and South America (Peru, Bolivia, Ecuador) shortly after their arrival in Germany, and during quarantine, were also unsuccessful. During the period 1978-80, a total of 357 samples from various species were investigated. Newcastle disease virus was isolated 10 times from the imports.

These findings are surprising and do not correlate with data published by others.^{3,4,5} In England, influenza A viruses were isolated from dead birds arriving at the airport in about 25% of all shipments, and in Japan similar results were obtained. Hav7Neq2 (H3N8), which resembles strain A/duck/Ukraine/1/63, and Hav4Neq2 (H4N8) strains were the main isolates. The significance of these findings is difficult to assess, especially when one keeps in mind the numerous influenza A virus isolates that have been isolated from migrating feral birds.

We have collected 3421 swabs during 1978-1980 from a number of dif-

ferent wild avian species, and have investigated them for influenzaviruses. The results clearly show that different antigenic subtypes of influenza A viruses are present in various parts of Germany.

Table 1 shows a list of species from which tracheal or cloacal samples were obtained, the number of birds investigated from each species, and the number of isolates obtained from the different groups of birds. They mainly comprise songbirds, geese, ducks, and other waterfowl.

Sixty-four influenza A virus strains were isolated from these birds. In addition, 31 Newcastle disease viruses and 43 avian paramyxoviruses were isolated from the same group of birds.

The different combinations of hemagglutinin and neuraminidase antigens of the influenza A isolates obtained are listed in Table 2. All hemagglutinin subtypes, except H5 (Hav5), H8 (Hav8), H9 (Hav9) and H10 (Hav2), H12 (Hav10), and all neuraminidase subtypes, except N4 (Nav4), were demonstrated in different combinations. The most frequent combinations were H3N8 (Hav7Neq2) and H1N3 (Hav3Nav2-3). These results show that all influenzavirus A isolates were made from waterfowl (ducks and coots). Songbirds and geese did not yield influenza A viruses. There was a marked variation of isolations by years: in 1977-78, influenza A virus isolates were made from 6/531 ducks; in 1979, 41/949 ducks and coots; and in 1980, 17/829 ducks and coots. These results correlate with observations made by others.^{6,7} and Easterday, personal communication 1978

Especially noteworthy are two isolates containing H2 antigens which are found mainly in human influenza strains. One isolate had the antigenic configuration H2N2 (prototype strain A/Singapore/1/57). Another isolate was an H2N3 (H2Nav2-3; prototype strain A/duck/Germany/73). Serological comparisons of the hemagglutinin of the isolated strains show a close antigenic relationship with the pandemic human strain A/Singapore/1/57.

Similar strains were isolated previously,⁸ and further work is required to demonstrate more precisely how close the relations are between these viruses.

Of great interest was the finding that ducks harbor influenza — viruses that are antigenically related to swine influenza virus strains. The first isolation was made in Canada by HINSHAW et al.⁶ in 1976, and only a short time later similar viruses were isolated in the U.S., in Hong Kong and by us in Germany.^{2,8}

Hemagglutinin and neuraminidase antigens of these isolates were antigenically very similar to the classical swine influenza viruses, although not identical. The base sequence homology of A/duck/Bavaria/77 to segment 6 (NA gene) is 86%, which is in accordance with our findings that these strains carry a N1 neuraminidase.¹⁰ SCHOLTISSEK and VON HOYNIGEN-HUENE¹⁰ further investigated the genetic relatedness of 2 A/duck/Bavaria/77 (H1N1) isolates to other avian strains using homology hybridization techniques with the RNA of segment 8 (NS gene). They

found a base sequence homology of 45% with fowl plague virus RNA, and 81% with the RNA of virus N, indicating that the duck/Bavaria/77 isolates are not directly derived from swine virus. One duck/Bavaria/77 strain infected 6-8 week old pigs after experimental application, and there was evidence (virus isolations) for natural transmission to contacts, although there were no or only minimal clinical symptoms and no antibody production to the agent.² These experiments clearly indicate that avian-derived viruses can infect and spread in a mammalian population.

While we were still considering the importance of these findings for the epidemiology of influenza in animals and man, an outbreak of naturally occurring influenza was reported¹¹ in Belgium, from which were isolated strains of Hsw1N1 (H1N1) closely related to the strains from wild ducks in North America and West Germany referred to previously. Several outbreaks of influenza were observed in Belgian swine farms starting in January 1979. The outbreaks were characterized by fever, dry cough, and anorexia. The majority of the sows and weaned pigs became sick, whereas suckling pigs were exempt or only slightly affected. Mortality was low, and recovery was uneventful. Altogether 6 identical influenza A viruses were isolated from pigs during these outbreaks, and infection with the isolate A/swine/Belgium/1/79 was confirmed by demonstration of a rise of specific HA antibodies in animals on two farms.

The antigenic characterization of the A/swine/Belgium/1/79 isolate revealed a N1 neuraminidase type, and in hemagglutination-inhibition tests, the only significant reactions were obtained with chicken antisera to the two duck strains, Alberta/76 and Bavaria/77.¹²

Table 3 shows the results of HI tests with these isolates in comparison with a number of other influenza A viruses. Whether the strain contains an A/NJ/76 component for which we and others have some indications, remains to be resolved. Limited genetic investigations of the Belgian isolates revealed, however, that these viruses differ in their base sequence homology of segment 8 (NS gene) from the duck/Bavaria/1/77 isolate. The Belgian isolates show a homology of about 90% with the fowl plague virus. Identical results were also obtained with more recent (1980) duck isolates with the antigenic configuration (H1N1; Hsw1N1), i.e., the isolate A/duck/Schleswig/4/80 (SCHOLTISSEK, personal communication 1981). The results show very clearly that more than one subtype occurs of the avian duck-derived Hsw1N1 viruses, and that genetic material from either fowl plague virus or virus N is probably involved to a high degree in the NS gene of the duck type HswN1 strains.

We might add at this point that experimental infection of adult swine with two isolates (A/swine/Belgium/79/1 and 2) did not result in overt disease. Infected animals, however, showed low titer antibody production to the homologous strain. We postulate, therefore, that Hsw1N1-like influenza strains which infect ducks under natural conditions can cross an

assumed avian to mammalian species barrier. Such strains thus far show genetic material of fowl plague virus origin. What role these strains may play in the ecology of influenza is difficult to assess at the present time.

Table 1 Total no. of birds by species and no. of virus isolates obtained over three years of surveillance (1978-1980)

Bird species	Total no. of samples collected	no. of influenza A-virus isolates	no. of paramyxovirus isolates incl. NDV*
Wild ducks	1446	52	51 (21)
Other water fowl (geese, rails, gulls)	1101	12	13 (--)
Domestic poultry	29	--	-- (--)
Song birds	488	--	-- (--)
Psittacines (imported)	357	--	10 (10)
Total	3421	64	74 (31)

*()= NDV

Table 2 Combinations of hemagglutinin and neuraminidase antigens of avian influenza A virus isolates obtained over three years of surveillance (1978-1980)

		H e m a g g l u t i n i n							
		H1 (Hsw1)	H2 (H2)	H3 (Hav7)	H4 (Hav4)	H6 (Hav6)	H7 (Hav1)	H11 (Hav3)	H?
Neuraminidase									
N1	(N1)	★							
N2	(N2)		★	★	★	★			
N3	(Nav2-3)		★					★	
N5	(Nav5)					★			
N6	(Nav1)			★	★				
N8	(Neq2)			★		★	★		★
N9	(Nav6)							★	

(in brackets): previous influenza nomenclature (1971 system)

Table 3. Results of haemagglutination-inhibition (HI) tests with Belgian swine strains and a variety of selected human, swine and avian strains

ANTISERA ^a TO:	A N T I G E N S							
	Swine/ Belg/ 1/79	Swine/ Belg/ 2/79	Duck/ Alb/ 35/76	Duck/ Bav/ 1/77	Human/ N.J./ 8/76	Swine/ Wis/ 1/67	Swine/ Camb/ 39	Swine/ Iowa 15/30
A/swine/Belg/1/79(Hsw1) ^{bx}	160 ^f	nd ^g	40	20	80	80	20	80
A/duck/Alb/35/76 (Hsw1) ^c	2650	nd	2650	640	2650	2050	1280	2650
A/duck/Bav/1/77 (Hsw1) ^b	160	320	160	160	160	80	<20	160
A/human/N.J./8/76(Hsw1) ^d	<20	<20	20	20	640	<20	320	<20
A/swine/Wis/1/67 (Hsw1) ^d	<20	<20	<20	<20	40	160	40	<20
A/swine/Camb/39 (Hsw1) ^d	<20	20	<20	<20	80	<20	1280	<20
A/swine/Iowa/15/30(Hsw1) ^d	<20	20	<20	<20	320	160	80	640
A/human/PR/8/34/ (H0) ^e	20	<20	nd	nd	nd	nd	nd	nd
A/human/FM/1/47 (H1) ^e	40	40	nd	nd	nd	nd	nd	nd
A/human/Sing/1/57 (H2) ^e	<20	<20	nd	nd	nd	nd	nd	nd
A/human/HK/1/68 (H3) ^e	<20	<20	nd	nd	nd	nd	nd	nd

^aAnimal origin of antisera: b=chicken; c=rabbit; d=ferret; e=goat. The H component is given in brackets. bx= chicken antiserum prepared after absorbing the Belgian strain with N.J. antiserum*
^fReciprocal of serum dilution inhibiting 4 haemagglutinating (HA) units of antigen. ^gnd = not done

* (see Results)

(From Pensaert et al., 12)

DR. ROSENWALD: The question is to Dr. Bachman. Were the isolates from swine that you described at the end of your talk examined for virulence?

DR. BACHMAN: Virulence studies for chickens just one, and for ducklings we haven't done them yet. I can't tell you anything—No. The one virus was not pathogenic for chickens.

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STUDIES ON AVIAN INFLUENZA OF DUCK IN REPUBLIC OF CHINA

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A severe respiratory symptoms with high mortality (about 75%, 600/800) was noted in a flock ducklings of 4-week-old at Tansui in May, 1972.

A hemagglutinating virus was isolated from trachea and lung of moribund ducklings in duck kidney cell cultures or embryonated chicken eggs. It could agglutinate erythrocytes of many mammalian or avian species. The physical and chemical property tests revealed that the isolate belonged to influenza virus. The hemagglutination-inhibition test and neuraminidase-inhibition test indicated that the isolate was composed of the Hav6 N1 antigens. The virus was designated as A/Duck/Tansui/72 (Hav6 N1).

Sera from 89 duck farms in 12 counties throughout the country were subjected to HI tests against four different strains of duck influenza virus. (Table 1) Results indicated that there were positive farms in many counties. However, no other clinical case was noted, and no virus was isolated from the serologically positive farms.

Table 1.

Survey on HI Antibody of Avian Influenza
in Republic of China

	A / duck / Tamsui / 72 (Hav6 N1)	A / duck / Czech / 56 (Hav4 Nav1)	A / duck / Eng / 56 (Hav3 Nav1)	A / duck / Eng / 62
I lan	1 / 3	0 / 3	0 / 3	0 / 3
Taipei	1 / 6	0 / 5	0 / 5	0 / 5
Tauyuan	0 / 2	0 / 2	0 / 2	0 / 2
Hsinchu	1 / 5	0 / 5	0 / 5	0 / 5
Miauli	1 / 4	0 / 4	0 / 4	0 / 4
Taichung	0 / 7	0 / 7	1 / 7	0 / 7
Tainan	1 / 29	0 / 29	1 / 29	0 / 29
Kaoshiung	0 / 15	0 / 10	0 / 10	0 / 10
Pintung	1 / 5	0 / 4	0 / 4	1 / 4
Taitung	0 / 6	0 / 6	0 / 6	2 / 6
Hwalien	0 / 6	0 / 6	0 / 6	1 / 6
Ponfu	1 / 8	0 / 8	0 / 8	0 / 8
Total	7 / 96	0 / 89	2 / 89	4 / 89
(%)	(7.29)	(0)	(2.25)	(4.49)

Denominator: No. of farms tested. Numerator: No. of positive farms.

EPIDEMIOLOGY OF AVIAN INFLUENZA AND SOURCES OF INFECTION IN DOMESTIC SPECIES

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PERSPECTIVE

The term "fowl plague" was ascribed to early disease outbreaks amongst domestic chickens and was subsequently associated with isolations of influenza A viruses later identified as of the antigenic combination H7N7* (formerly Hav1Neq1). In more recent years, largely as a result of extensive surveillance of avian species, domestic and wild, in the quest to obtain background information on sources of human influenza, there have been numerous isolations of influenza viruses, the majority of which were from apparently healthy birds. The disease causing potential of these isolates is unclear. In some instances, antigenically identical viruses may be pathogenic or apathogenic for different species (Allen et al 1977; Slemons and Easterday 1972).

The term influenza for many implies respiratory infection but in avian species it tends to be of an intestinal nature. A significant factor in the large number of influenza virus isolations made in recent years was the finding that many more viruses could be isolated from the cloaca than the trachea (Rosenberger et al 1974; Slemons and Easterday 1975). Multiplication of influenza viruses in the duck intestinal tract was subsequently confirmed by Webster et al (1978). The demonstration that the cloaca was a plentiful source of influenza viruses was, I believe, the starting point of a period of heightened interest in influenza natural history which led to the recognition of a vast reservoir of influenza viruses in avian species in the latter part of the last decade.

In respect of domestic species, commercial pressures arising from food preferences have resulted in a bias in the availability of data on species susceptibility. Outbreaks attributed to avian influenza which have affected turkey raising farms particularly in North America, have resulted in considerable data being available for this type of bird whilst in Southern China, where the turkey is not raised on a commercial basis, no comparable data are available and the susceptibility of the domestic poultry of this region was hitherto unknown. The studies on domestic ducks, geese and chickens conducted by the author in Hong King (Shortridge et al, 1977 and 1979; Shortridge 1980, submitted for publication) represent the only long term continuous study of such birds and are used

*Subtype designations are in accordance with the revised system of nomenclature for influenza viruses (WHO 1980).

here as the basis from which to extrapolate the possible overall incidence and implications of avian influenza for domestic species.

VIRUS ISOLATIONS

A long term surveillance study at a local dressing plant of ducks, geese and chickens originating from Southern China including Hong Kong was commenced in November 1975. The isolation frequencies from apparently healthy ducks, geese and chickens, respectively, were 16:3:1 (Table 1).

The classic fowl plague antigenic combination H7N7 has so far proved to be a conspicuous absentee from the range of Hong Kong isolates with the H7 subtype present in only two combinations in duck isolates (Table 2). Whilst the observed antigenic combinations essentially cover those resulting from disease outbreaks in turkeys in North America and the United Kingdom, especially the H6 (Hav6) subtype (Alexander 1980; Bahl et al 1979; Pomeroy et al 1980), the H8 (Hav8) haemagglutinin has still only once been recorded in domestic poultry during an epizootic in a Canadian turkey hatchery (Lang et al 1972). The most frequently recorded combination was H4N6 (Hav4Nav1) which comprised approximately one fifth of the Hong Kong isolates.

Range of antigenic combinations

To date, WHO (1980) recognizes 12 haemagglutinin and 9 neuraminidase subtypes giving rise to a theoretical pool of 108 distinct antigenic combinations, 47 of which have been recorded amongst the Hong Kong isolates.

Statistical analyses of these isolates over five years' surveillance have suggested that some combinations may not occur for molecular reasons or if they do they are selected against in nature (Gardner and Shortridge 1979; Shortridge, submitted for publication). As recombination (genetic reassortment) has been shown to occur in the intestinal tract of ducks during mixed infection (Hinshaw et al 1980a), it is possible that some less commonly recorded antigenic combinations, for example H4N1 (Hav4N1) and H3N1 (Hav7N1), may be a consequence of such an event and represent less stable recombinants derived from more stable parents prevailing in the duck populations.

The marshalling of migratory birds prior to winter migration may provide the best situation for recombination to occur in nature. Hinshaw et al (1980b) recorded 27 different antigenic combinations over a three year period of study of wild waterfowl in Alberta, Canada contrasting with the 47 recorded in the "static" domestic ducks examined in Hong Kong (Table 2). This was in spite of the fact that the frequency of virus isolation from Canadian waterfowl (up to 60%) was much greater than the 6.5% observed in Hong Kong ducks (Table 1). These very different bird populations, one domestic and one wild, provide pools of virus wherein intensive multiplication and interchange may occur; the combinations recorded in Hong Kong possibly approach the limit of the range of viable viruses within nature.

Do the observed isolation frequencies and antigenic combinations represent actual occurrence?

The work of Hannoun and Devaux (1980) and Hinshaw et al (1980a) has recognized the existence of mixed infections in wild ducks. Similarly, two and even three antigenic combinations have been recorded in domestic poultry (Shortridge et al 1977 and 1979; Markwell and Shortridge, submitted for publication). In infection experiments on Hong Kong varieties of domestic ducks, geese and chickens using a duck isolate H7N2 (Hav1N2), a seemingly silent virus H6N2 (Hav6N2) was detected only in geese after H7N2 excretion had ceased. When the original isolate was treated with specific antiserum and reinoculated into embryonated eggs, the H6N2 virus was expressed implying that it was originally present in a totally non-avid form (King and Shortridge, unpublished data). Species specificity was apparent and this may be a contributing factor to the different isolation frequencies and observed antigenic combinations in geese and chickens compared with ducks, considerations that might also apply to turkeys. Apart from the inherent problems of the production of *in ovo* recombinants, these findings question the sensitivity of the embryonated hen egg as an initial isolation system.

Causative agent?

Newcastle disease virus (NDV) is well documented in its disease producing capacity, infection ranging from subclinical to fatal. On the other hand, although influenza viruses have been isolated from diseased poultry, the question arises whether or not they are the causative agents. NDV may be endemic or may be introduced onto farms in which influenza virus is asymptotically present. In view of the different biophysical and biochemical characteristics of these two virus groups, is it safe to assume that the observed isolation of either one is a true indication of its actual occurrence?

To test this possibility, embryonated eggs, the conventional isolation system for both categories of virus, were mixedly infected with H9N2 (Hav9N2) and NDV (Table 3). H9N2 was preferentially isolated over NDV even when NDV was in excess in the inoculum. Similar experiments were done with H4N6/NDV and H5N3 (Hav5Nav2)/NDV mixtures which resulted in the preferential isolation of influenza viruses. Hence, the ascribing of an influenza aetiology to certain disease outbreaks may be in doubt. Studies with the avian paramyxoviruses also indicate that their isolation rates may not represent their true incidence in nature (Shortridge, Unpublished data). The role of these viruses as possible causative agents of disease should not be overlooked.

If it is accepted that there may be doubt as to the absence of NDV in diseased poultry from which influenza viruses have been isolated, then we must also consider the possibility of concurrent infection with other organisms whose presence may or may not interfere with the detection of the causative agent. Such organisms as *Mycoplasma*, *Salmonella*,

Pasteurella and *Coccidia* may be carried by poultry but to date it is not known whether or not they have a synergistic effect when present with influenza.

Serology as a guide to occurrence of virus

One fifth of the influenza viruses isolated from domestic ducks sampled in Hong Kong were H4N6 with an overall isolation rate of 1.4% (Table 4). Serological evidence of infection by this virus was nil whereas in the case of H9N2, an infrequent isolate with an isolation rate of 0.2%, 6% of the sera tested were positive. This suggests that the more commonly encountered viruses may be better adapted to the duck and are less likely to produce an immune response. Experimental infection of ducks with these antigenically distinct viruses are in accord with the surveillance findings (Cheung and Shortridge, unpublished data). Serological surveillance may be of some value in limited situations perhaps showing up species specificity for particular haemagglutinin subtypes or antigenic combinations.

SEASONAL VARIATION

The observed isolation rates of influenza and paramyxoviruses over five years' surveillance of domestic ducks in Hong Kong, when expressed in four monthly intervals, exhibited a trend related to seasonal variations (Figure 1). In the warm/hot, humid months from March to October, influenza viruses predominated whereas in the cool/cold, dry months from November to February, the converse was true. There are two deviations from the idealised pattern; in November 1977/February 1978 when there were more influenza viruses isolated than expected and in November 1978/February 1979 when there were fewer paramyxoviruses isolated than expected, differences that may be due to sampling phenomena.

These seasonal variations may represent the true incidence of these viruses in nature reflecting basic differences in their stability or some other physical parameter. Alternatively, because of the possible insufficiency of the isolating system referred to earlier, the influenza isolates may be masking the true incidence of the paramyxoviruses.

SOURCE AND SPREAD

Routes of transmission

There is strong evidence that influenza infection in ducks is mediated by the faecal-water-oral route in that these viruses can be frequently isolated from pond water and faeces (Markwell and Shortridge, submitted for publication) and that in Hong Kong, cloacal isolates predominate over tracheal (Table 5). In contrast, isolates from chickens show no difference in the tracheal and cloacal isolation rates. It might be reasonable to infer, bearing in mind the coprophagous habits of ducks, that the less aquatic a bird is, the more likely it is to be susceptible to transmission by the respiratory route. Influenza isolates from turkeys have come mainly from the trachea and these birds are readily infected by virus aerosols (Easterday, 1975).

The isolation of influenza viruses from cloacal swabs and the results of investigations into the sites of replication of these viruses in avian species (Webster et al 1978), have reinforced the view that they replicate largely in the intestinal tract. However, both the reproductive and intestinal tracts open into a common site and the possibility of replication in the ciliated epithelium of the magnum should be considered. Circumstantial evidence is available to show that NDV may be transmitted vertically (Lancaster and Alexander 1975). The similarity of epithelial linings in both respiratory and reproductive tracts invites closer investigation to establish whether or not influenza viruses may be maintained or transmitted by this route.

Regional factors

While migratory birds may contribute to a pool of viruses in nature, the agricultural economics of Southern China seems to have unwittingly provided a huge reservoir in which a great diversity of viruses occur. Contributory factors to this include —

1. The Pearl River delta is a rapidly prograding delta ideal for raising ducks. Large numbers are to be found in the lower delta in "open farms" where water comprises approximately one-quarter of the surface area.
2. Ducks are an important food item in the diet of the Southern Chinese and are intensively raised in the region.
3. Virus is transmitted in the countless duck ponds of the region by the faecal-water-oral route and is maintained by the regular introduction of susceptible ducklings onto infected ponds.
4. The delta area, particularly the marshes and mud flats, supports considerable bird life and is attractive to overwintering and migratory birds especially waders which move along the east Asian coast, are conditions favourable for interaction between domestic and other species.
5. Virus appears to be apathogenic for ducks (and other poultry) in the region.

Dissemination of virus

The interaction between the vast domestic duck population of Southern China and wild birds which might be considered carriers of influenza virus provides an ideal milieu for the dissemination of viruses over long distances. Migratory birds pass through the region at times when the influenza isolation rates from the ducks are still high. Known flight paths of migratory and other birds indicate that there is the opportunity for birds from different regions to interact directly, or indirectly through intermediate species, resulting in widespread exchange and spread of viruses.

While domestic ducks of southern China represent an important focus or gene pool of influenza viruses, a great diversity of viruses has also been isolated from wild birds. Possible reasons for this include (1) wild

birds comprise a far greater range of species than the few domestic ones (2) the birds studied have derived from a vast geographical area, almost global (3) the concerted effort made in recent years to study the influenza viruses of these birds. Apart from the isolation of H5N3 (Hav5Nav2) from a colony of dead terns in South Africa (Becker 1966), most isolations have been made from healthy wild birds and it is not known to what extent they suffer from disease and thus their ability to disseminate virus. The apparent susceptibility of non-domestic species to influenza infection may be adduced from the frequent isolation from dead caged birds at international airports of viruses of the H4N6 (Hav4Nav1) and H3N8 (Hav7Neq2) antigenic combinations, the same as those most frequently in domestic ducks in Hong Kong (Alexander et al 1977; Matsuoka et al 1979; Nerome et al 1978).

A pertinent factor in the spread of virus is the duration of virus shedding in the faeces of wild birds. Hinshaw et al (1980) observed that H1N1 (Hsw1N1) virus isolated from a mallard duck was shed for one month from experimentally infected ducks, considerably longer than the one to two weeks usually observed for isolates from domestic species.

A finite number of haemagglutinin and neuraminidase subtypes may exist in nature. Because wild birds comprise a greater range of species, it is likely that any hitherto recognized subtypes will derive from these birds rather than domestic species. It is of interest that the newly recorded H12 subtype was first recognized as H12N5 (Hav10Nav5) amongst a large number of isolates obtained from mallard ducks assembled in Alberta before the 1976 winter migration (Hinshaw and Webster, 1979; Hinshaw et al 1980b). The H12N5 virus was subsequently isolated four years later on a single sampling occasion from southern Chinese domestic ducks. Notwithstanding sampling factors, it might be reasonable to infer that (1) this virus may have been introduced by migratory birds and (2) failure to isolate the viruses is indicative of the domestic duck's inability to maintain the virus. Experimental infection of domestic ducks with H12N5 virus did not lead to significant virus shedding (Cure and Shortridge (Unpublished data). Further, the H8 subtype was first recognized in a virus (H8N4 (Hav8Nav4)) isolated from fatalities in an episode in 1967 at an Ontario turkey hatchery (Lang et al 1972). Subsequent failure to detect the H8 subtype in domestic species suggests that it is a rare subtype probably limited to wild birds in certain ecological settings as indicated in recent studies (Hannoun and Devaux, 1980; Hinshaw et al 1980b).

COMMENT

Continuous exposure of aquatic birds to waterborne influenza infections as in the case of domestic ducks on the duck ponds of Southern China, has perhaps rendered them less susceptible to the disease causing potential of the virus. The possibility exists that these viruses survive as normal flora, particularly in the intestinal tract. Thus, it may follow that

the more land based the poultry the greater is its susceptibility to disease following exposure to the virus, hence the economic significance of turkey infections. As the tendency develops towards farming these birds in more intensive, yet more protected environments, the less chance there is of their being exposed to the introduction of influenza from wild species. Early reports of "fowl plague" were at times when chickens were not intensively raised under cover and there is little evidence today that influenza is of significance to the chicken industry. However, NDV is still a problem and it is possible that the reduction in influenza infections in chickens may be relevant to the expression of Newcastle disease.*

Alterations to farming practices which minimise contact with wild species may lead to a situation wherein certain influenza viruses may become endemic. This becomes particularly relevant as turkey raising tends towards a year-round operation. It should also be noted that if the raising of ducks becomes more economically attractive, changes in husbandry may result in their being farmed in more land based environment. A recent study on diseased birds grown on stubble/grass as opposed to ponds implicated influenza as the causative agent (Alexander et al 1981).

*Limitations of the embryonated egg may lead to the preferential isolation of influenza viruses over NDV and other avian paramyxoviruses; the possibility that the latter group of viruses may be causally associated with disease in poultry in general should not be excluded.

Table 1. Isolation of influenza A viruses from domestic poultry originating from southern China including Hong Kong at a Hong Kong dressing plant, November 1975 to October 1980

Type of poultry	No. of swabs ^a	Virus isolations		
		Number	Percent	Ratio
Duck	8737	564	6.5	16
Goose	1353	15	1.1	3
Chicken	1708	7	0.4	1

^a Total of swabs from trachea and cloaca

Table 2. Antigenic combinations^a of influenza A viruses isolated in Hong Kong from domestic poultry November 1975 to October 1980^b

	N1	N2	N3	N4	N5	N6	N7	N8	N9
H1	+	+	+						
H2		+	+						+
H3	+	+	+			+		+	+
H4	+	+	+	+	+	+	+	+	
H5		+	+						
H6	+	+	+	+	+	+		+	+
H7	+	+							
H8									
H9		+	+			+			
H10	+	+	+	+	+			+	+
H11		+	+						+
H12					+				

^a Subtype designations are in accord with the revised system of nomenclature for influenza viruses (WHO, 1980)

^b Studies at a Hong Kong poultry dressing plant (Table 1) and on local duck farms

Table 3. Identity of isolates obtained from embryonated eggs mixedly infected with influenza and Newcastle disease viruses in differing ratios

		Influenza virus (H9N2) dilutions →					
		10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
← Newcastle disease virus (NDV) dilutions	10 ⁰	H9N2	H9N2	H9N2	NDV	NDV	NDV
	10 ⁻¹	H9N2	H9N2	H9N2	NDV	NDV	NDV
	10 ⁻²	H9N2	H9N2	H9N2	NDV	NDV	NDV
	10 ⁻³	H9N2	H9N2	H9N2	-	-	-
	10 ⁻⁴	H9N2	H9N2	H9N2	-	-	-
	10 ⁻⁵	H9N2	H9N2	H9N2			

- denotes virus not isolated.

The titre of each virus was adjusted to 10² EID₅₀/0.1 ml. Equal volumes of each virus over the 10⁰ through 10⁻⁶ range were mixed in chequerboard fashion and 0.1 ml aliquots inoculated into two embryonated eggs per mixture and incubated at 37°C for 48 hrs. Allantoic fluids with haemagglutination activity were examined in haemagglutination inhibition tests using H9 and NDV antisera.

Table 4. Comparison of virus isolation and serology as indices of virus infection in domestic ducks sampled at a Hong Kong dressing plant

Virus	Virus isolation	Serology
	Per cent isolation from swabs ^a	Per cent samples positive ^b
H4N6	1.4	0
H9N2	0.2	6.0

^a Tracheal and cloacal sources

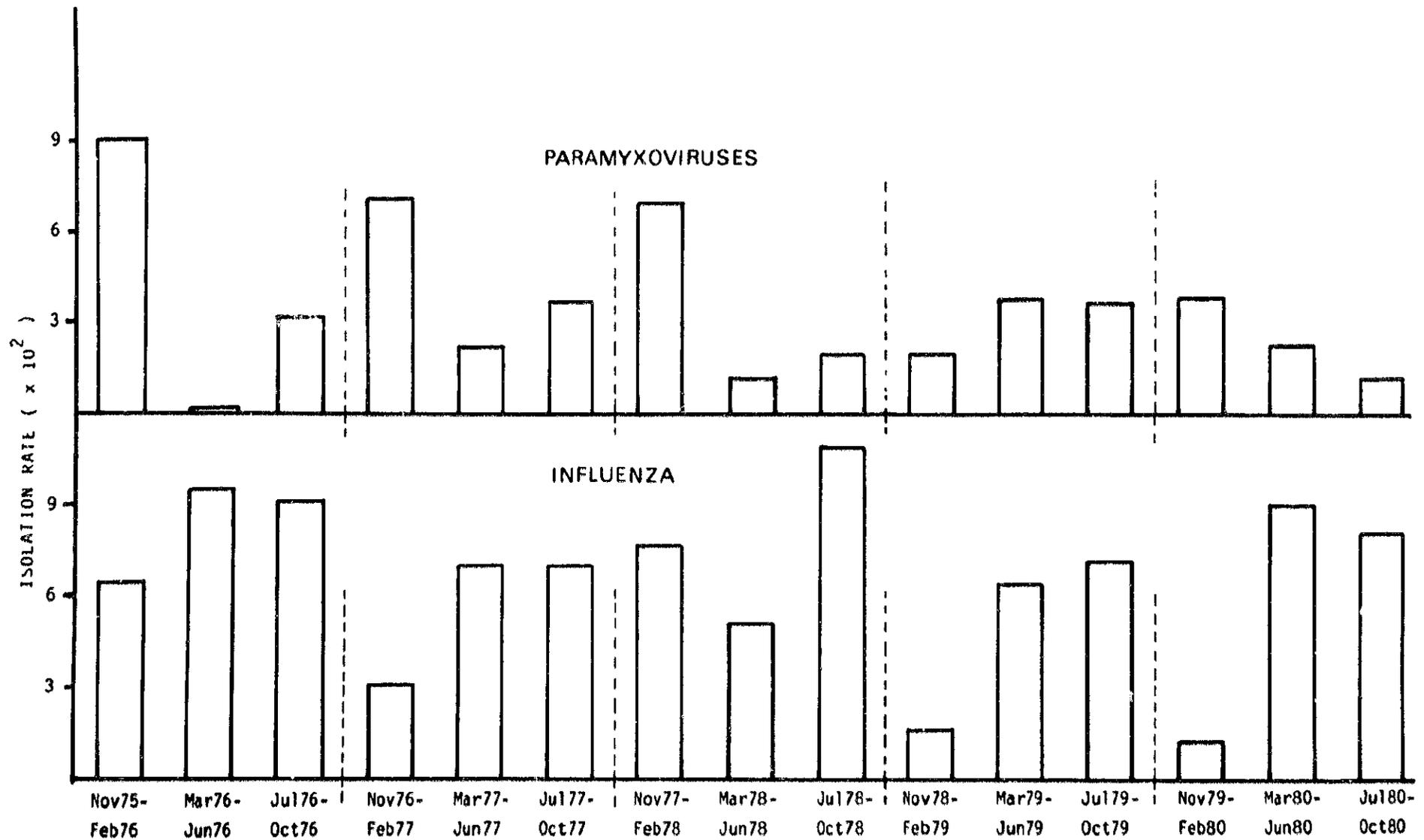
^b Sera examined in haemagglutination inhibition (HI) and neuraminidase inhibition test, HI titres of positive sera ranged from 10 to 60.

Table 5. Site of isolation of influenza A viruses from domestic poultry at a Hong Kong dressing plant

Type of poultry	Site of sample	No. of swabs	Virus isolations		
			Number	Percent	Ratio Cloaca/Trachea
Duck	Cloaca	4168	350	8.4	1.8
	Trachea	4569	214	4.7	
Goose	Cloaca	661	9	1.4	1.6
	Trachea	692	6	0.9	
Chicken	Cloaca	1014	4	0.4	-
	Trachea	694	3	0.4	

Figure 1.

Isolation rates of influenza viruses and paramyxoviruses assessed by four monthly intervals from November 1975 to October 1980. The paramyxoviruses comprised mainly Newcastle disease virus with limited numbers of recently described avian paramyxoviruses (Shortridge et al 1980).



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REVIEW OF THE THREE-YEAR STUDIES ON THE ECOLOGY OF AVIAN INFLUENZA VIRUSES IN ISRAEL

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Some geographical, ecological and economical peculiarities of Israel as a Middle East country make it a unique place for studies on the ecology of avian influenza. First, Israel is located along the main flyway of feral birds migrating from Eastern Europe (mainly European part of USSR) to North-East Africa (Fig. 1). Secondly, Israel has a highly developed poultry industry which is run throughout the country in farm villages of two kinds: *moshavs* with individual poultry farms containing 1,000 to 5,000 birds and *kibbutzes* which are communal farms with large poultry houses containing tens of thousands of birds. The third peculiarity is a relative isolation of Israel from neighboring countries, resulting in the absence of any poultry trade between them; this situation presents artificial control conditions for a natural experiment on elucidation of the possibility of interspecies transmission of influenza viruses from wild birds to poultry

Systematic studies on avian influenza were established in Israel in 1978, both feral and domestic birds being covered by the research. The domestic birds, including turkeys, chickens and ducks, were surveyed in the case of information sent by local veterinarians about any respiratory disease occurring in kibbutz or moshav poultry farms. As to the feral birds, their catching was carried out in cooperation with the Department of Zoology of Tel Aviv University, Israel Reservation Committee and Israel Hunter Association.

MATERIALS AND METHODS

Cloacal and tracheal swabs were used as the main field source for virus isolation (Lipkind *et al.*, 1979a,b,c; 1980a). Sometimes, in the case of domestic birds, organ materials such as trachea, lungs and brain were used. The propagation of the field materials through embryonated eggs, titration of hemagglutinating (HA) agents, their passaging, performance of neuraminidase (Nase) reaction, HA inhibition (HI) and Nase inhibition (NI) tests were performed according to the "Advanced Laboratory Techniques for Influenza Diagnosis" (Palmer *et al.*, 1975). Serological identification was carried out using goat monospecific antisera from Influenza Reference Center kindly provided by Dr. R. G. Webster (St. Jude Children's Research Hospital, Memphis, USA), as well as complete

*A revised system of nomenclature for influenza viruses (Bull. WHO 1980, 58,585-591) is used for designation of influenza virus strains.

rabbit and ferret reference antisera from the World Influenza Center kindly provided by Dr. J. J. Skehel (National Institute for Medical Research, London, England). Double immunodiffusion tests (DID) were performed as described previously (Lipkind *et al.*, 1980a), using preformed gel plates (Meloy, Springfield, VA, USA).

RESULTS

Table 1 shows the scope of avian species which were surveyed and the number of influenza virus isolations. As it can be seen, most of the work was done with feral birds, including various species of waterfowl (mallard, teal and pintail ducks, as well as coots and moorhens), starlings, rock partridges, quails, cattle egrets, larks, pigeons, turtle doves and some other species. A total of 30 influenza viruses was isolated. Most of the isolates (24) were derived from the feral birds, mainly from waterfowl (mallards, pintail ducks and coots), and also from rock partridges and starlings. Five viruses were isolated from each kind of poultry raised in Israel; turkeys (3), chickens (1) and ducks (2).

In addition to the viruses isolated within the last 3-year period, a number of unidentified HA agents isolated in Israel in the past (by veterinarians dealing with Newcastle disease virus) and luckily preserved in a viable form up to now were included into the studies. From this source 3 HA agents isolated from turkeys in 1971, 1973 and 1978 were identified retroactively as influenza viruses (Lipkind *et al.*, 1980b).

Table 2 presents characterization of the outbreaks of influenza registered in poultry farms, including those "old" outbreaks from which the unidentified HA agents were isolated and identified as influenza viruses retroactively. All the outbreaks were characterized by mild respiratory syndrome with low to moderate mortality. The outbreak in the breeder turkey farm in moshav Ramon was characterized by sharp drop in egg production and by some other symptoms of affection of reproductive tract.

Table 3 presents the list of the influenza virus strains isolated in Israel up to now. All the "old" strains isolated from turkeys in 1971-1978 are of the same antigenic composition which did not occur among the more recently isolated strains. The prevalent subtype among the "new" strains is H7* and the prevalent HA-Nase combination is H7N2, which was isolated from turkeys, chickens, mallards and rock partridges. These strains being serologically identical to fowl plague viruses (the isolate from starlings is serologically identical to the A/FPV/Dutch/27 (H7N7) prototype strain by both HA and Nase antigens) appeared to be avirulent to chickens, turkeys and ducklings. The studies on pathogenicity were performed by Dr. D. J. Alexander (Central Veterinary Laboratory, New Haw, Weybridge, England).

DISCUSSION

The studies on the ecology of animal influenza viruses in Israel have shown that influenza viruses circulate in avian populations in Israel, including both feral birds and all kinds of poultry raised in Israel. The influenza outbreaks in poultry farms were characterized by mild respiratory syndrome with moderate losses, the impact being more expressed in the case of a breeder farm showing sharp drop in egg production (Table 2). There is evidence on considerable influence of concomitant infections on severity of the disease, this influence being confirmed by experiments on artificial infection of turkeys in laboratory conditions (Weisman, unpublished data). As to antigenic composition of the isolates from poultry, it is of interest that all of them isolated within 1971-1981 period had the same N2 subtype of Nucleoprotein which was combined in consecutive order with H5, H7 and H11 subtypes of HA.

The usual question when a new influenza virus strain appears on poultry farms is whether feral, especially migrating birds, were involved in the introduction of the "new" influenza virus to domestic birds. In this respect, analysis of antigenic composition of our isolates determined by HI, NI and DID tests permits some tentative conclusions about possible epizootiological connections. It can be suggested that H5N2 strains circulated in poultry in Israel during at least 8 years but was replaced by H7N2 and H11N2 strains. Among the influenza outbreaks registered in poultry in Israel, there are two cases when the outbreaks occurred twice on the same place: (a) moshav Ramon in which the first outbreak was in 1973 and the second one in 1979, both having occurred on the same turkey farms and (b) kibbutz Gan Shmuel in which the first outbreak was observed in 1978 on the turkey farm and the second one in 1980 on the duck farm. In both cases, the first outbreak was caused by H5N2 strain, while the second one was caused by H7N2 strain in Ramon and by H11N2 strain in Gan Shmuel. In Ramon, the H7N2 strain was also isolated from migrating mallard ducks found dead in fields located in about one kilometer from the turkey farms on which a month later, the H7N2 strain-caused an influenza outbreak (Lipkind *et al.*, 1979a,b; 1980a). Such ecological circumstances offer strong evidence on interspecies transfer of influenza virus from wild migrating ducks to domestic turkeys. However, antigenic similarity between the isolates from mallards and turkeys shown by serological methods using non-clonal sera is insufficient for the conclusion about the identity of the two strains and, hence, about the interspecies transfer of the influenza virus strains. Advanced analysis of influenza virus antigenic determinants using monoclonal antibodies, as well as RNA segment analysis of both strains, is needed for the decisive conclusion (Hinshaw *et al.*, 1980; Sriram *et al.*, 1980). These studies are being initiated.

The influenza viruses from coots (non-duck waterfowl), starlings (Lipkind *et al.*, 1979a) and rock partridges were isolated for the first time.

The isolation of influenza viruses from non-waterfowl birds is of particular interest. The point is that the significance of wild ducks as carriers of influenza viruses was investigated thoroughly, including both ecologically and virologically (Slemons *et al.*, 1979, Webster *et al.*, 1976; 1978; Laver and Webster, 1979; Kida and Yanagawa, 1979; Kocan *et al.*, 1980; Hinshaw *et al.*, 1980; Sriram *et al.*, 1980) while corresponding knowledge concerning non-waterfowl birds is rather scant. Starlings, wintering in Israel, offer a remarkable opportunity for the research. They migrate to Israel from the vast area of European part of USSR, congregating in millions in some particular places for the night. (Yomtov *et al.*, 1977; Yomtov, 1980). In the day-time, the birds spread over the countryside searching for food and the invading farm yards. up to now, and H7N7 influenza virus strain (Lipkind *et al.*, 1979a) and a lentogenic strain of NDV (Lipkind *et al.*, in preparation) were isolated from starlings. Thus, the ecological peculiarities of this species in Israel make it of especial interest to investigate non-waterfowl birds as potential reservoirs for circulation of influenza viruses.

TABLE 1
 AVIAN INFLUENZA VIRUSES ISOLATED IN ISRAEL
 WITHIN THE PERIOD OF FEBRUARY 1978 - MARCH 1981

SPECIES		Number of birds Swabbed	Number of influenza virus isolations
FERAL BIRDS	Mallard duck (<i>Anas platyrhynchos</i>)	47	16
	Pintail duck (<i>Anas acuta</i>)	4	1
	Teal (<i>Anas crecca</i>)	31	-
	Coot (<i>Fulica atra</i>)	64	4
	Moorhen (<i>Gallinula chloropus</i>)	12	-
	Rock partridge (<i>Alectoris graeca</i>)	126	2
	Cattle egret (<i>Ardeola ibis</i>)	99	-
	Starling (<i>Sturnus vulgaris</i>)	282	1
	Quail (<i>Coturnix coturnix</i>)	35	-
	Lark (<i>Calandrella cinerea</i>)	20	-
	Pigeon (<i>Columba columba</i>)	31	-
Turtle-dove (<i>Streptopelia turtur</i>)	106	-	
Other species	79	-	
Total		936	24
DOMESTIC BIRDS	Turkey	332	3
	Chicken	56	1
	Duck	85	2
	Total	473	6
Whole total		1403	30

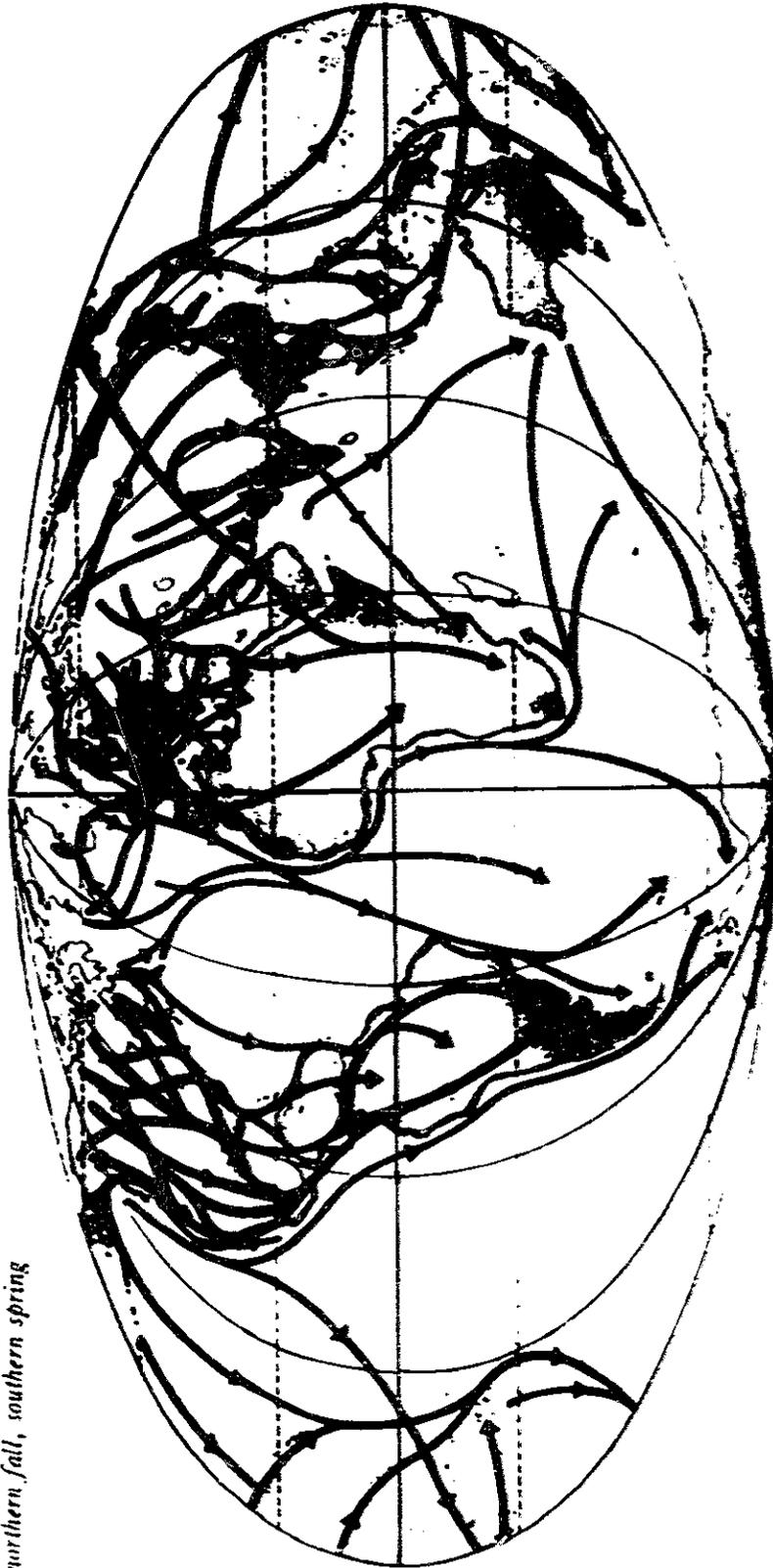
TABLE 2
CHARACTERIZATION OF INFLUENZA OUTBREAKS IN POULTRY FARMS

PLACE	DATE OF OUTBREAK	SPECIES	CLINICO-PATHOLOGICAL DESCRIPTION	MORBIDITY	MORTALITY	SOURCE OF ISOLATION	ISOLATE ANTI-GENIC SUBTYPE
RAMON (moshav)	Mar. 1979	Turkey (breeders)	Rales, sinusitis, nasal discharge, diarrhea, prolapsus of urogenital and intestinal tracts; Sharp drop in egg production, soft egg shells, shells without content.	80%	20%	Cloacal swab	H7N2
DEGANIA (kibbutz)	Jul. 1980	Chicken	Rales, pneumonia, tracheitis, nephritis, poor weight gain.	50%	10%	Brain	H7N2
GAN SHMUEL (kibbutz)	Dec. 1980	Duck	Mild sinusitis, conjunctivitis.	30%	2%	Cloacal swab	H11N2
HATZAV (moshav)	Dec. 1980	Turkey	Rales, sinusitis, nasal discharge,	90%	10%	Liver and lungs combined homo- genate; cloacal & tracheal swabs	H11N2
KPAR VITKIN (kibbutz)	Jun. 1971	Turkey	Rales, sinusitis, torticollis.	90%	5%	Brain	H5N2
RAMON (mosav)	Jun. 1973	Turkey	Rales, pneumonia.	no data	no data	Lungs	H5N2
GAN SHMUEL (kibbutz)	Aug. 1978	Turkey	Conjunctivitis, sinusitis, pneumonia, diarrhea.	90%	30%	Trachea	H5N2

TABLE 3
LIST OF INFLUENZA VIRUS STRAINS ISOLATED IN ISRAEL

Strain	Year of Isolation	Year of Identification	Number of Isolates
1. A/turkey/Kfar Vitkin/H5N2	1971	1980	1
2. A/turkey/Ramon/H5N2	1973	1980	1
3. A/turkey/Gan Shmuel/H5N2	1978	1980	1
4. A/starling/Kinneret/H7N7	1978	1979	1
5. A/mallard/Ramon/H7N2	1979	1979	16
6. A/turkey/Ramon/H7N2	1979	1979	1
7. A/chicken/Degania/H7N2	1980	1980	1
8. A/coot/Shluhot/H10N4	1980	1980	4
9. A/pintail duck/Shluhot/H10N4	1980	1980	1
10. A/rock partridge/Beit Nir/H7N2	1981	1981	2
11. A/duck/Gan Shmuel/H11N2	1980	1981	2
12. A/turkey/Hatzav/H11N2	1980-81	1981	2

Some of the most important flyways of migrants across oceans and continents in September - northern fall, southern spring



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ISOLATION OF INFLUENZA A VIRUSES FROM EXOTIC BIRDS IN GREAT BRITAIN

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INTRODUCTION

Isolations of influenza A viruses from "exotic" caged birds were first made around 1970 when concern for the role of these birds in the devastating epizootics of Newcastle disease virus (NDV) amongst commercial poultry in Europe and the United States of America led to investigations into the freedom of such birds from certain viruses. Since that time many countries have imposed quarantine restrictions on imported captive birds and this close supervision has led to greater knowledge of the viruses infecting these birds.

The number of birds imported into Great Britain each year was estimated as between 350,000 and 600,000 prior to 1975 (Inskipp, 1975; Inskipp and Thomas, 1976) and in 1975 370,679 were imported (Inskipp and Thomas, 1976). Quarantine legislation was introduced on 1st March 1976 and the additional expense involved in quarantine drastically reduced the number of birds imported. Only 60,484 birds were imported during March to December 1976 (Return of Proceedings under Diseases of Animals Act, 1950, 1977). However, since 1976 there has been a gradual recovery of exotic bird trade and figures for imported captive birds in subsequent years are: 1977: 121,507, 1978: 155,782, 1979: 197,120 and 1980: 255,548 (Return of Proceedings under Diseases of Animals Act, 1950, 1978, 1979, 1980, 1981).

ISOLATION OF INFLUENZA VIRUSES FROM EXOTIC BIRDS IN GREAT BRITAIN

Influenza viruses have been isolated from exotic birds from three sources.

1) *Pet and Zoo Birds*

One of the earliest isolations of an influenza virus from exotic birds was made in 1970 by Collings (personal communication) from a parrot, although a virus was also isolated from a cockatoo in that year by Chu and Trow (personal communication). Two further isolates were obtained in 1972 (Alexander *et al.*, 1974) one from three parakeets (*Psittacula Sp.*) also infected with virulent NDV and one from a sulphur-crested cockatoo (*Cacatua sulphurea*). Since 1972 only five other isolations have been reported from zoo or pet birds outside quarantine, three in 1975, two in 1976 and one in 1980. These isolates are listed in Table 1.

2) *Dead Birds Arriving at Heathrow (London) Airport*

Numerous birds pass through Heathrow (London) Airport on world

airlines bound for destinations outside the United Kingdom. Frequently a proportion of these birds die in transit and are removed by the staff of the Royal Society for Prevention of Cruelty to Animals' Hostel for animals at the airport. Dr. Chu of University of Cambridge, realising the potential of exotic birds for carrying influenza viruses, began examining the dead birds for virus. This work was later continued at the Central Veterinary Laboratory, Weybridge after the introduction of quarantine regulations (Alexander *et al.*, 1977). A summary of the viruses isolated since 1975 is given in Table 2.

In Table 2 the influenza isolations have been placed in five groups. Prior to 1976 28% of the consignments yielded influenza viruses which were all of H4N6 subtype (Chua and Chu, personal communication). Of the 188 consignments examined during May 1976 - March 1977 26% were positive, but in this case all the viruses were of H3N8. After a period of 6-7 months during which time no influenza viruses were isolated, the H4N6 subtype reappeared and 14 isolations (19%) were made up to July 1978. Since that time no influenza viruses have been isolated from this source, although over 300 consignments have been examined.

The consignments from which influenza viruses were isolated during 1976-1978 had been dispatched exclusively from India. However, the large number of airports of destination (Table 3) clearly demonstrates the potential for spread of avian influenza viruses as a result of international trade in caged birds.

3) *Birds Dying in Quarantine*

In March 1976 legislation was introduced which enforced the quarantine of captive birds imported into Great Britain (Ashton and Alexander, 1980). Although this legislation was primarily aimed at preventing the introduction of NDV into Great Britain, influenza viruses have been frequently isolated from samples taken from birds dying during the quarantine period (Ashton and Alexander, 1980). Isolations of influenza viruses from this source are summarized in Table 4. A very similar pattern to that seen with the viruses from the airport birds was evident with a sudden change in virus subtype. This was even more marked as the last H3N8 isolate and the first H4N6 isolate were both made in June 1977. Two isolates of H10N7 subtype were made from dead birds from the same quarantine premises in January 1979 and in September 1979 a virus of H7N1 subtype (IVPI = 0.00) was isolated. No viruses were isolated between September 1979 and January 1981. Although there are no figures available for the number of dead birds sampled during this period, the proportion of birds dying was not considered to be dissimilar to previous years, and in 1980 a total of 28 paramyxoviruses were isolated which is an increase on the previous years and probably reflects the greater number of birds imported.

Unlike the airport isolates which came from consignments exclusively imported from Indian airports, the countries of export for the birds in

quarantine from which virus was isolated were more varied: India, Ghana, Taiwan, Holland and Hong Kong. However, the practice of using holding and collecting depots and re-exporting birds means that the country of export is not necessarily the country of origin of the birds. Similarly consignments of birds from different countries may be placed in contact either in transit or in quarantine and infection occur as a result.

Identification of dead caged birds is rarely easy and importers generally use casual non-specific names for the birds. The birds (where some attempt has been made of identification) dying in quarantine, from which influenza viruses were isolated, are listed in Table 5.

ISOLATION OF INFLUENZA VIRUSES FROM EXOTIC BIRDS IMPORTED INTO OTHER COUNTRIES

1) *United States of America*

A series of influenza A viruses were isolated from exotic birds in the U.S.A. during 1971-72 mainly as a result of NDV surveillance. The earliest of these isolations was in June 1971; A/myna/Massachusetts/71 (H4N8), from a bird imported from India (Butterfield *et al*, 1973; Slemons *et al*, 1973b).

Other influenza viruses were isolated during January - August 1972 from a variety of exotic birds, all of which had been imported from Thailand or had been in close contact with birds from Thailand after importation into the U.S.A. (Butterfield *et al*, 1973; Slemons *et al*, 1973a, 1973b). It was concluded that all these viruses were of H4 subtype although the haemagglutinin activity of some of the isolates was also inhibited by sera to other haemagglutinin subtypes.

Since these reports there has been little information published on the isolation of influenza viruses from birds imported into the U.S.A. However, Pearson (cited in Report of the Committee on Transmissible Diseases of Poultry, 1978) reported that in 1977 640 haemagglutinating isolates were obtained from 26,552 samples taken from birds in quarantine. Fifty-three of the isolates were not NDV, one was identified as an influenza virus of H4N8 subtype. Pearson (cited in Report of the Committee on Transmissible Disease of Poultry, 1979) reported the examination of 35,501 samples in 1978 and 20,274 in January - October 1979 from birds in quarantine. These resulted in the isolation 1083 unidentified haemagglutinating isolates in 1978 and 635 in 1979. The agents were mainly isolated from finches, parrots and parakeets and all were apathogenic for chickens and turkeys.

2) *Northern Ireland*

McFerran *et al* (1974) reported the isolation of an avirulent influenza virus from an African grey parrot *Psittacus erithacus* in Northern Ireland: - A/parrot/N. Ireland (Ulster)/VF/-73-67/73 (H7N1). The bird had been a pet which had died. The origins of the bird were impossible to trace.

3) Japan

Nishikawa *et al* (1977) reported the isolation of 11 influenza A viruses from parakeets imported into Japan from India (6) and Thailand (5) during March 1975-April 1976. All the viruses were shown to possess N8 neuraminidase but to fall into three groups of haemagglutinin subtype consisting of one, four and six isolates. The group of four isolates were all identified as of H4 subtype, these came from parakeets imported from Thailand, one in 1975 and three in 1976. One of the other groups was later reported as of H3 subtypes (Matsuoka *et al*, 1979).

Fukumi *et al* (1977) and Nerome *et al* (1978) described the isolation of 22 influenza viruses, during May-August 1976, from 200 birds imported as pets from India or Thailand but found dead or moribund on arrival at Tokyo Airport. The viruses were obtained from nine mynah birds (*Gracula religiosa*) and three parakeets (*Psittacula alexandria facitata*) from India and 10 mynah birds from Thailand. All the viruses isolated from the birds originating in India and two from birds from Thailand were of H3N8 subtype, the other eight isolates were of H4N8 subtype.

During March 1977 - March 1978 six influenza viruses were isolated from budgerigars (*Melopsittacus undulatus*) obtained from pet shops, pet clinics and a pet bird wholesaler in Sapporo, Japan and shown to be of H4N6 subtype. (Matsuoka, 1979; Matsuoka *et al*, 1979).

Ogawa *et al*, (1980) list four influenza viruses isolated from exotic birds in Japan: A/mynah/Tokyo/229/77 (H4N6), A/mynah/Tokyo/252/77 (H4N8), A/mynah/Tokyo/231/77 (H3N8) and A/budgerigar/Aichi/1/77 (H3N8).

4) Austria

Stunzner *et al* (1980) reported the isolation of 17 influenza viruses from 62 pooled samples from 246 exotic birds imported into Austria from Senegal (via Frankfurt, FDR). Sixteen isolates were made from samples taken in May 1978 and one from samples taken in June 1978. The viruses were subtyped as H4N6 (nine), H11N6 (five), H11N1 (one) and H4N8 (two). Isolates were all from passerines, mainly finches and waxbills. Interestingly, attempts to isolate viruses from living birds were unsuccessful. Stunzner *et al* (1980) conclude that these viruses could be endemic in birds in Senegal. While this may be likely it should be noted that in a survey of 616 wild birds trapped during November 1976 - December 1977 for export to Europe 23 paramyxoviruses of PMV-2 subtype were isolated but no influenza viruses (Fleury and Alexander, 1978). The possibility that the birds arriving in Austria had been infected in transit after leaving Senegal should not be overlooked.

DISCUSSION

The isolations of influenza viruses from exotic birds from the various sources mentioned in this paper are summarized in Table 6. The isolates, from all sources, were mainly of H4 haemagglutinin subtype with N6 or N8 neuraminidase up to 1975/1976 when the predominant subtype

became H3N8. However, during 1977, the H4 subtype, again with N6 or N8 neuraminidase, re-emerged as the most frequently isolated subtype. Extreme caution should be exercised in extrapolating these results to the situation in wild birds in the countries of origin. It may be safe to conclude that influenza epizootics have occurred in birds in India and South East Asia since 1970 involving two changes in predominant subtype, but the extreme variations in captive bird trade, the mixing of birds in transit or in quarantine, pooling of birds from two countries of origin while collecting at a third for export to a fourth country and other similar practices may render other inferences completely erroneous.

Many of the birds imported into quarantine in Great Britain have their stated country of origin as European countries. While it is possible that these birds were bred in the country of export it seems more likely that they were merely collected there from other countries of origin before re-exporting. Marked differences can be seen in the sources of birds from one year to the next (Return of Proceedings Under Disease of Animals Act 1950, 1976-1981) and this may have an important bearing on the numbers and types of influenza viruses isolated. For example, from Table 6 it can be seen that birds exported from Thailand were frequently the source of influenza viruses. The number of birds imported from Thailand to Great Britain since 1976 were: 1976: 0, 1977: 0, 1978: 10,409, 1979: 7561, 1980: 10,985. The converse is true of birds imported from India the number of which have fallen drastically: 1976: 6036 (10% of total imports), 1977: 26,700 (22%), 1978: 12,883 (8.0%), 1979: 4,507 (2.3%), 1980: 700 (0.3%). Since most influenza virus isolates in Great Britain have been from birds imported from India this may account for the dramatic decline in the number of isolates in recent years. However, such variations in the number of birds imported may be more representative of changes in travel routes over the years.

Figures for birds held in quarantine indicate that the country exporting the largest number of birds to Great Britain during 1976-1980 was Senegal, 253,394 birds (12,055 psittacines, 241,399 passerines) representing 33% of total number of birds (10% of total psittacines) imported during that period. It is significant, therefore, that none of the influenza viruses isolated in Great Britain during 1976-1980 have been from birds imported from Senegal. However, this finding is in contrast to that of Stunzner *et al* (1980) who were able to isolate influenza viruses from birds imported into Austria from Senegal in 1978.

It would appear that little can be concluded concerning the disease status of birds in their natural environment by the isolation of influenza viruses from exotic captive birds. However, what can be concluded is that a proportion of the extremely large number of captive birds being transported around the world at any given time are infected with influenza viruses. The epizootological significance of this to influenza epidemics in man or other animals remains to be ascertained. Most of the subtypes of influenza that have been isolated from captive exotic birds, H4N8, H4N6,

H3N8, H7N1, H7N7, H11N6 and H10N7, have been isolated from migratory birds (Hinshaw *et al*, 1981). Equally, viruses of these subtypes have been responsible for outbreaks of disease in commercial poultry in Europe and North America (Alexander *et al*, 1981; Alexander and Spackman, 1981; Meulemans *et al*, 1979, Johnson *et al*, 1977, Easterday and Tumova, 1978).

Table 1

Isolations of influenza viruses from
birds kept as pets or in Zoos in
Great Britain

YEAR	BIRD	SUBTYPE
1970	parrot	H4N8 ^a
1970	cockatoo	H4N6
1972	parakeet ^b	H4N8 ^a
1972	cockatoo ^c	H4N8 ^a
1975	parakeet ^b	H3N8
1975	cockatoo ^c	H3N8
1975	parrot	H3N8
1976	parakeet ^b	H3N8
1976	thrush ^d	H3N8
1980	macaw ^e	H7N7 ^f

a: These viruses were originally typed as H4N6 (Alexander et al, 1974) but later shown to be of H4N8 subtype

b: Psittacula Sp

c: Cacatua sulphurea

d: Turdidae Sp

e: Ara sp

f: Intravenous pathogenicity index in six-week-old chickens = 0.00

Table 2

Isolation of influenza viruses from dead
birds arriving at Heathrow (London) Airport

Period	Number of consignments examined	Number of consignments positive	Subtypes
1975 ^a	104	29 (28%)	H4N6
May 1976 - March 1977	188	48 (26%)	H3N8
April 1977 - October 1977	44	0 -	-
November 1977 - July 1978	73	14 (19%)	H4N6
August 1978 - October 1980	300	0 -	-

a: data from Chua and Chu (Chu personal communication)

Table 3

Airports of origin and destination of consignments of exotic birds in transit at Heathrow Airport from which influenza viruses were isolated, 1976-1978

Airport of origin	Number of positive consignments	Airport of destination	Number of positive consignments
Calcutta	29	Amsterdam	12
Delhi	11	Brussels	3
"India"	2	Copenhagen	2
Unknown	20	Dusseldorf	1
		Frankfurt	2
		"Germany"	2
		Lyon	2
		Madrid	2
		Manchester	1
		Milan	2
		Naples	2
		Palma	1
		Rome	3
		Stockholm	1
		Unknown	26

Table 4
Summary of the influenza viruses isolated
from imported captive birds dying in
quarantine after importation into Great Britain

Dates	Number of isolates	Subtypes
March 1976 - June 1977	8	H3N8
June 1977 - December 1978	21	H4N6
January 1979	2	H10N7
July 1979	1	H4N6
August 1979	1	H4N6
September 1979	1	H7N1
September 1979 - January 1981	0	-

Table 5
Identification of birds dying in quarantine
from which influenza viruses were isolated

Influenza subtype	Bird Identification	Number of Isolates
H3N8	Order Passeriformes	
	finches ^a	5
	soft-bills ^b	1
	Order Psittaciformes	
	parakeets ^c	1
H4N6	Order Passeriformes	
	finches ^a	4
	shrike (<u>Lanius excubitor</u>)	1
	red-crested cardinal (<u>Cardinalinae</u> Sp)	1
	silver bill (<u>Enodice malabarica</u>)	1
	Order Psittaciformes	
	parakeets ^c	9
	parrot	1
	cockatoos (<u>genus Cacatuo</u>)	2
	lesser sulphur-crested cockatoos (<u>Cacatua sulphurea</u>)	1
Macaw (<u>genus Ara</u>)	1	
H10N7	Order Passeriformes	
	Wax-bills (<u>genus Estrelida</u>)	2
H7N1	Order Passeriformes	
	African glossy starling (<u>Aplonis</u> <u>parayensis</u>)	1

a: "finch" is the popular name for many small seed eating passerines most or which are of the family Fringillidae

b: "soft-bill" is an agricultural term for predominantly insectivorous, frugivorous and nectar-feeding birds

c: "parakeet" is the term used for any small, long-tailed parrot but more specifically refers to birds of the genus Psittacula.

TABLE 6

Summary of influenza A viruses isolated from pet birds 1970-1980

Date	Country of isolation	Probable country of origin	Virus subtype (No. isolates)	Reference
1970	England	Unknown	H4N8 (1)	Alexander <i>et al</i> (1974)
1970	England	Unknown	H4N6 (1)	Chu, personal communication
1971	USA	India	H4N8 (1)	Butterfield <i>et al</i> (1973)
January-August 1972	USA	Thailand	H4 (18)	Slemons <i>et al</i> (1973a,b)
1972	England	Unknown	H4N8 (2)	Alexander <i>et al</i> (1974)
1973	Northern Ireland	Unknown	H7N1 (1)	McFerran <i>et al</i> (1974)
1975	Japan	Thailand	H4N8 (1)	Nishikawa <i>et al</i> (1977)
1975	England	India unknown	H4N6 (29) H3N8 (5)	Chu, personal communication unpublished
January-April 1976	Japan	Thailand India	H4N8 (3) H3?N8 (6)	Nishikawa <i>et al</i> (1977)
February and June 1976	England	Unknown	H3N8 (2)	Unpublished results
May-August 1976	Japan	India Thailand Thailand	H3N8 (12) H3N8 (2) H4N8 (8)	Nerome <i>et al</i> (1978)
March 1976-June 1977	England	Various (mostly India)	H3N8 (56)	Alexander <i>et al</i> (1977) Ashton and Alexander (1980)
1977	Japan	Unknown	H3N8 (2) H4N8 (1) H4N6 (1)	Ogawa <i>et al</i> (1980)

CONT'D

TABLE 6 (contd)

Date	Country of isolation	Probable country of origin	Virus subtype (No. isolates)	Reference
June 1977-December 1978	England	Various	H4N6 (35)	Ashton and Alexander (1980) and unpublished results
May-June 1978	Austria	Senegal	H4N6 (9) H4N8 (2) H11N6 (5) H11N1 (1)	Stunzner <u>et al</u> (1980)
January 1979	England	Unknown	H10N7 (2)	Unpublished results
July/August 1979	England	Unknown	H4N6 (2)	Unpublished results
September 1979	England	Unknown	H7N1 (1)	Unpublished results
April 1980	England	Unknown	H7N7 (1)	Unpublished results

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INFLUENZA A VIRUS INFECTION OF DOMESTIC DUCKS

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SUMMARY

One hundred and six influenza A viruses belonging to 11 different antigenic types and 25 isolates of Newcastle disease virus were recovered from clinically sick or apparently healthy domestic White Pekin ducks in the eastern United States from 1978-80. Viruses were recovered from 23% of 2-to-5-week-old ducklings sampled, yet no viruses were detected in ducklings under 2 weeks, 6-8 weeks, or breeder ducks. Influenza virus was also isolated from water used for drinking and swimming by the ducks.

Experimental infections of ducklings with 2 different antigenic subtypes of influenza (Hav3Nav1, Hav6Neq2) and a lentogenic strain of Newcastle disease virus produced no disease signs in the birds. Ducklings co-infected with virus (either NDV or influenza) and *Pasteurella anatipestifer*, experienced no greater morbidity or mortality than ducklings infected only with *P. anatipestifer*. These findings indicate that many different influenza A and paramyxoviruses circulate in domestic ducks in the U.S., yet may not produce disease or enhance the severity of the disease produced by a common bacterial agent.

INTRODUCTION

Type A influenza viruses have been frequently isolated from wild waterfowl throughout the world.^{1,2} These viruses have also been detected in domestic ducks in other countries,^{3,6} but, in the United States, only one isolation has been reported from domestic muscovy ducks in Pennsylvania.⁷ Paramyxoviruses, including lentogenic strains of Newcastle disease virus (NDV), have also been isolated from feral waterfowl and from domestic ducks.⁸ Influenza viruses and paramyxoviruses are often recovered from healthy ducks, so it is not clear if these viruses are pathogens for ducks.

This report describes the isolation and antigenic classification of influenza A viruses and paramyxoviruses isolated from domestic White Pekin ducks and provides an evaluation of their ability to produce disease singly or in combination with a bacterial pathogen.

MATERIALS AND METHODS

Collection of samples and virus isolation:

In 1978, influenza A viruses were first isolated from nasal samples from a flock of 18,000 birds showing respiratory signs of disease as swollen sinuses, sneezing, and discharge from nostrils. Morbidity was high but mortality did not exceed 1 percent. Recovery was rapid and uncomplicated except in a few ducks which developed caseous cheesy material in nasal and infraorbital sinuses. Since this was the first indication that influenza viruses circulated in domestic ducks, an effort to monitor ducks for evidence of influenza was begun.

From 1978-80, cloacal and nasal swabs were collected from healthy and sick ducks of different ages. Additionally, water samples (from drinking and swimming water for ducks) were collected. Virus isolation has been described elsewhere.² Briefly, material from cloacal and/or nasal swabs or water samples was injected into 10-to-11-day-old embryonating chicken eggs which were incubated for 2 days at 35°C and then tested for hemagglutinin activity.

Serological tests and virus identification:

Hemagglutinin(HA) titrations and hemagglutination inhibition (HI) tests were performed in microtiter plates with receptor-destroying enzyme (RDE)-treated sera.⁹ Neuraminidase (NA) titrations and neuraminidase inhibition (NI) tests have been fully described.¹⁰ All hemagglutinating agents were identified in HI and NI tests with specific antisera to the isolated surface antigens of reference influenza viruses.²

Experimental infections of ducks:

Two-week-old White Pekin ducklings were used for "in vivo" infectivity studies. No viruses were recovered from these birds prior to infection. Ducklings were kept in Horsfall isolation units throughout the experiments.

The following agents were used for experimental infections of ducks: A/duck/NY/12/78 (Hav3Nav1); A/duck/NY/49/78 (Hav6Neq2); P/duck/NY/22/78 (NDV); and *Pasteurella anatipestifer* (PA). All of these organisms were originally isolated from clinically sick domestic White Pekin ducks.

Ducklings were inoculated intratracheally and orally with 1.0 ml of allantoic fluid containing approximately 10^7 EID₅₀/ml of influenza A. NDV was given intratracheally — 0.5 ml of allantoic fluid per duck. Ducklings were infected with 10^9 organisms of PA by the intrasinus route, 24 hrs. after virus-exposure. Cloacal swabs were taken before and 2, 4, 9 and 14 days after exposure, for virus isolation. Specimens for virus and bacterial isolation were taken from ducklings that died of exposure.

RESULTS

Virus Isolation:

A total of 733 samples from ducks or water were tested. One hundred and six influenza A viruses and 25 paramyxoviruses were isolated from 580 samples collected from 2-to-5-week-old ducklings (Table 1). No viruses were recovered from 140 samples from the other age groups. One influenza virus was isolated from 13 water samples collected on the duck farms.

Antigenic classification of these isolates showed that the influenza A viruses included 11 different combinations of hemagglutinin and neuraminidase antigens (Table 2). On several duck farms, different antigenic subtypes circulated concurrently. All paramyxoviruses were identified as lentogenic strains of NDV except for one isolate which has not yet been classified. The majority of influenza and NDV isolations were made from apparently healthy ducks or ducks which had died due to duck virus hepatitis, *E. coli*, and PA. Infrequently, influenza virus was isolated from ducks showing typical signs and lesions similar to natural influenza outbreaks.

Experimental infection:

Since influenza viruses and NDV were recovered from healthy birds, it was important to determine whether these viruses could produce disease in ducks. PA is a common pathogen for White Pekin ducks, and so the possibility that influenza virus infection may increase the severity of this disease was also examined. Two antigenic subtypes of influenza and a NDV isolate replicated, yet produced no mortality or disease signs in susceptible ducklings (Table 3). Although one duckling died in one of the influenza-exposed groups, no virus or bacteria could be isolated from its organs and no lesions were observed in any of the virus-exposed ducklings. The multiplication of viruses in the gastrointestinal tract, evidenced by virus recovery from the cloaca, was maximum by the 4th day postexposure and absent by day 14.

In the co-infection studies (Table 4), the bacterial infection alone produced 45% mortality in Experiment 1 and 20% in Experiment 2. The birds infected with influenza virus or NDV, or both, and bacteria, showed no greater mortality than those infected with the bacteria alone. These results indicated that there was no enhancement of disease in birds co-infected with virus and bacteria.

DISCUSSION

The recovery of 106 influenza A viruses and 25 paramyxoviruses from domestic ducks in the U.S. from 1978-80 clearly indicates that these viruses circulate in domestic ducks in this country. Their association with disease is not clear since most of the viruses were recovered from apparently healthy rather than sick ducks, and co-infection of ducks with these viruses and a common bacterial pathogen, *P. anatipestifer*, did not enhance the disease produced by the bacteria alone. These results agree

closely with studies by Shortridge et al. on domestic ducks in Hong Kong in which many different antigenic subtypes of influenza A viruses were isolated from healthy Pekin ducks.⁶

It is evident that influenza virus primarily infects ducklings between 2-5 weeks of age. The virus probably re-circulates in successive hatches. It is possible that older ducklings may develop immunity and that breeders carry antibodies which can be passively transferred through egg to progeny to account for the absence of infection in ducklings under 2 weeks.

Shedding of virus in droppings and the presence of virus in drinking water would provide means for transmission of the infection from one hatch to another, as demonstrated for feral ducks.¹¹ Since commercial ducklings are reared outside, on the range, at most duck farms, the source of viruses may be wild free-flying birds, particularly waterfowl which harbor many different influenza A viruses.^{1,2}

Experimental infections of ducklings showed that influenza A and ND viruses produced an inapparent infection. Although these isolates may be avirulent, this does not mean that all of the isolates are incapable of producing disease. These results indicated that there was no interaction between influenza, NDV and PA, a common pathogen of domestic ducks. This was in contrast to the suggestion that introduction of organisms of low virulence after establishment of a virus infection enhances their virulence.³ Additional studies are required to determine the disease potential, if any, of influenza A viruses and paramyxoviruses, as NDV, in domestic ducks.

Table 1. Isolation of influenza A viruses and Newcastle disease viruses from domestic White Pekin ducks from 1978-80.

<u>Source of Specimens</u>	<u>No. of Specimens</u>	<u>Virus Isolations</u>	
		<u>Influenza Virus</u>	<u>NDV</u>
Ducks			
Under 2 wk.	74		
2-5 wk.	580	106	25
6-8 wk.	25		
Breeder ducks	41		
Water			
Ditch-line	13	1	
Total	733	107	25

Table 2. Antigenic types of influenza virus isolated from domestic White Pekin ducks.

<u>Antigenic Types</u>	<u>No. of Isolates</u>
Hav3 Nav1	32
Hav3 N2	6
Hav4 Neq2	2
Hav5 Nav2	2
Hav6 Nav1	11
Hav6 N1	7
Hav6 N2	19
Hav6 Neq2	16
Hav7 N2	7
Hav7 Neq2	4
Hav7 Nav4	1

Table 3. Virus shedding and mortality of ducklings infected with influenza A viruses or NDV.

<u>Viral Type</u>	<u>Virus recovery^a</u>	<u>Mortality^b</u>
A/Dk/NY/12/78 (Hav3Nav1)	18/20	1/20
A/Dk/NY/49/78 (Hav6Neq2)	10/10	0/10
P/Dk/NY/22/78 (NDV)	10/10	0/10

^a No. shedding virus from the cloaca
No. Inoculated

^b No. dead
No. Inoculated

Table 4. Mortality and agent recovery from ducklings experimentally inoculated with influenza A viruses, NDV, and Pasteurella anatipestifer

	Inoculation with:		Mortality ^A	Isolation Result ^B	
	Virus	Bacteria		Virus	PA
Exp. 1					
	IA ^C	--	1/20	---	---
	IA ^C	PA	8/20	4/8	8/8
	--	PA	9/20	---	9/9
	--	--	0/20	---	---
Exp. 2					
	IA ^D	--	0/10	---	---
	IA ^D	PA	0/10	---	---
	--	PA	2/10	---	2/2
	NDV ^E	--	0/10	---	---
	NDV ^E	PA	2/10	1/2	2/2
	IA ^D , NDV ^E	PA	3/10	3/3	3/3
	--	--	0/10	---	---

^ANo. dead/no. exposed.

^BNo. positive/no. dead

^CA/DK/NY/12/78 (Hav3Nav1)

^DA/DK/NY/49/78 (Hav6Neq2)

^EP/DK/NY/22/78 (NDV)

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ECONOMIC IMPACT OF AVIAN INFLUENZA IN DOMESTIC FOWL IN THE UNITED STATES

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Economic loss associated with Avian Influenza virus has been reported by turkey producers in most of the turkey producing states in the United States over the past decade. The incidence and loss varies from area to area and year to year; however, detailed information on the magnitude of the financial losses were not available until 1979.

In 1979, the Extension Department of the University of Minnesota conducted a survey of all commercial turkey flocks in Minnesota following a severe outbreak of Avian Influenza which started in the fall of 1978 and continued into the first two months of 1979. The extent of the problem and the losses that were reported were determined and the total economic loss incurred due to the outbreak was calculated.

The number of poults started in affected flocks totalled 2.2 million or 10% of the 22.2 million poults raised in Minnesota in 1978. The number of breeders affected was 27,680 or 5% of the 593,000 breeder hens in Minnesota flocks in 1978. The highest total mortality reported was over 75% and the highest condemnation was 73% of the birds marketed. Values were assigned to the mortality, condemnation, weight loss and egg loss that was reported and these losses were totalled along with the reported cost of medication, extra clean up and other costs including loss of profit to come up with a total economic loss of 4.2 million dollars. The results of this survey were reported at the United States Animal Health Association Annual Meeting in 1979 and are summarized in Table I.

In addition to losses in turkey flocks during the 1978-79 outbreak, losses also occurred in three (3) flocks of chicken layers. The losses were investigated and reported to be due primarily to a loss in egg production and were calculated to total \$50,000.

Avian Influenza outbreaks also have occurred in Minnesota the past two years and flock owners have been again surveyed each year to determine the economic losses incurred. This three-year history of economic loss indicates the loss per bird in involved flocks is approximately two (2) dollars for market turkeys, eight (8) dollars for breeder hens and 30 cents for chicken layers. The three-year loss history data are summarized in Table 2.

TABLE 2
Economic Loss to Avian Influenza
Minnesota Survey

	Number Flocks	Head Involved 000's	Mortality Percent	Eggs Lost 000's	Total Loss 000's Dollars	Average Per Bird
1978						
Market Turkey	130	2,138	16.7	—	3,948	1.85
Breeder Turkey	11	28	10.2	487	235	8.39
Chicken Layers	3	165	Low	984	50	.30
TOTAL					<u>4,233</u>	
1979						
Market Turkey	30	580	0-31	—	1,185	2.04
Incomplete Cost Data: Extrapolated from 1978.						
1980						
Market Turkey	22	620	—	—	1,300	2.10
Incomplete Survey Data						

Killed vaccines have been made available the past two years and Minnesota growers have been using approximately 4 million doses annually. The vaccine costs the grower 3 cents per dose and with approximately the same cost for additional labor to vaccinate each bird, we need to add another \$240,000 per year to the cost of influenza.

The virus types involved in the influenza outbreaks the past three years in Minnesota listed in Table 3 indicate the problem the grower has in determining the type of vaccine to use each year. A number of flocks were infected with more than one serotype which also complicates the problem. Based on pathogenicity studies at the University of Minnesota, these isolates are mild strains; however, the losses are severe due to other complicating disease agents and environmental stresses that are present in a commercial operation.

TABLE 3
Minnesota Avian Influenza Virus Types

1978	1979	1980
Hav4 Neq2	Hav2 Neq1	Hav1 Nav2
Hav6 N1	Hav4 Nav1	Hav2 Neq1
Hav6 N2	Hav6 N1	Nav4 Nav1
Hav6 Neq2	Hav6 N2	Nav4 N2
Hav9 N2	Hav9 N2	Hav4 Neq2
Hsw1 N1		

In order to stop additional losses from Avian Influenza, growers depopulate their infected farms and clean up and isolate them to eliminate the virus from the premises. This depopulation requirement puts the grower out of business for a while and can more than double the economic loss that is reported in the survey.

When a farm, hatchery, feedmill, or processing plant is not operating because of depopulation and loss of turkey production, their cost of operation does not stop completely. Most of the labor, utility, and supply expenses stop, but they still have their management, security and maintenance labor, basic utilities, interest, taxes, depreciation, insurance, and other fixed expenses which must be paid. These fixed costs are added to the costs of subsequent production, but are actually a loss that is assignable to the disease outbreak that resulted in the depopulation decision.

The magnitude of this depopulation cost can best be described using an example. On a commercial turkey production farm with one brooding building and two sets of finishing buildings, the grower will produce 6 flocks of toms annually by brooding every 8 or 9 weeks for a 6-week period and then finishing alternately in the two sets of finishing buildings by 20 weeks of age. Using a 20,000 bird flock as an example and 1 and 3 square feet of space per bird for brooding and growing respectively, the 6 flocks will provide 120,000 birds and 3 million pounds for marketing annually (22 lbs./sq. ft.). Table 4 and 5 schematically describe the farm and the flock schedule.

An influenza outbreak on this farm would be expected to cause disease losses in two flocks but would then result in the depopulation loss of two more flocks or one million pounds which is one-third of the annual production. Assuming breakeven markets (no profit or loss from average production performance), the cost of depopulation as it relates to the expected disease loss of \$2 per turkey is summarized in Table 6 and totals \$2.50 per turkey which more than doubles the disease loss that is reported in the Minnesota survey.

TABLE I

Economic Loss to Avian Influenza

1978 Minnesota Survey

	<u>Market Turkeys</u>	<u>Turkey Breeders</u>
Flocks Involved	130	11
Birds Started/Infected	2,137,989	27,680
Birds Died	356,441	--
Percent Mortality	16.7%	10.2%
No. of Eggs Lost	--	486,700
No. of Birds Condemned	81,535	--
Percent Birds Condemned	6.7%	--
Value of Eggs Lost	--	\$184,946
Value of Birds Died	\$2,171,355	49,585
Value of Loss in Weight	555,020	--
Loss of Profit	174,488	--
Value of Condemned Birds	759,966	--
Medication Cost	135,480	--
Extra Cleanup Cost	38,743	--
Other Costs	112,859	--
	<u>\$3,947,911</u>	<u>\$234,531</u>

TABLE 4

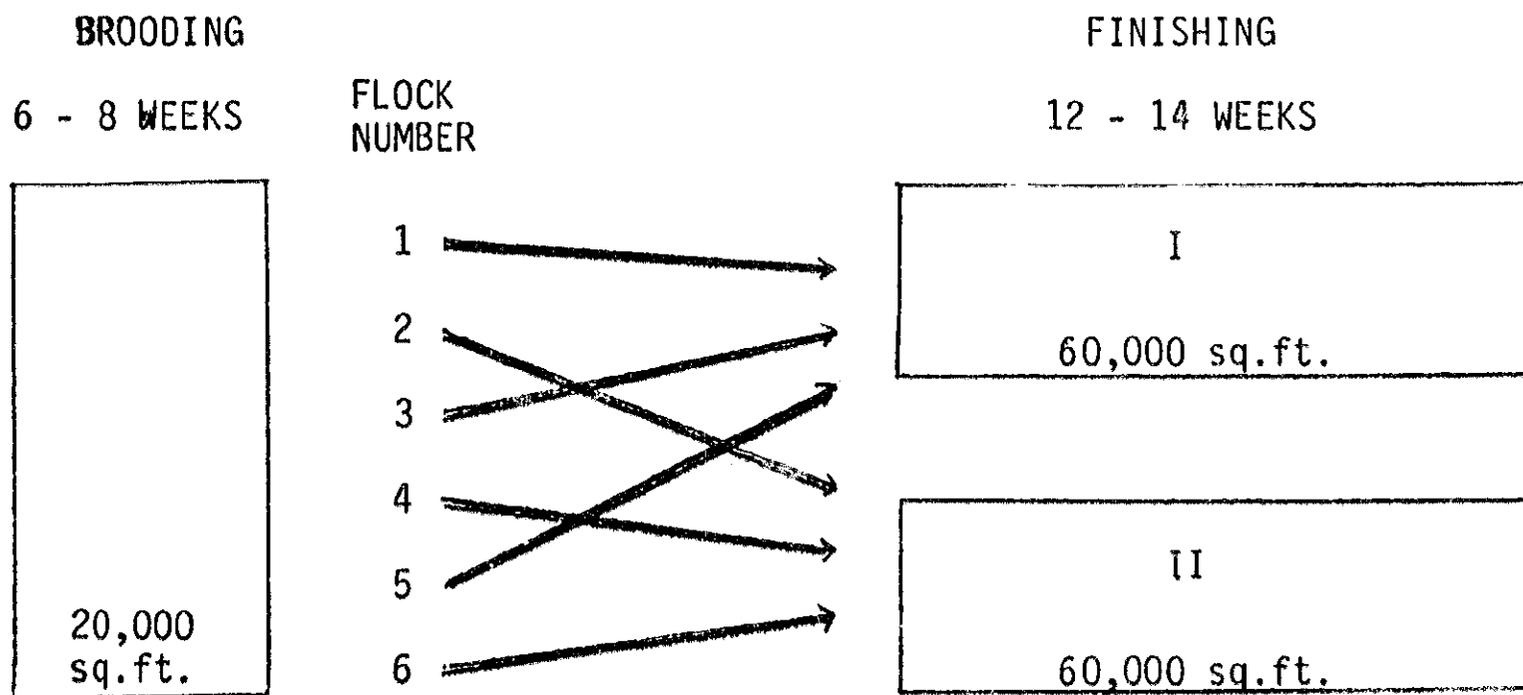
TOM FARM SCHEMATIC
20,000 HEAD per FLOCK

TABLE 5

TOM FLOCK SCHEDULE

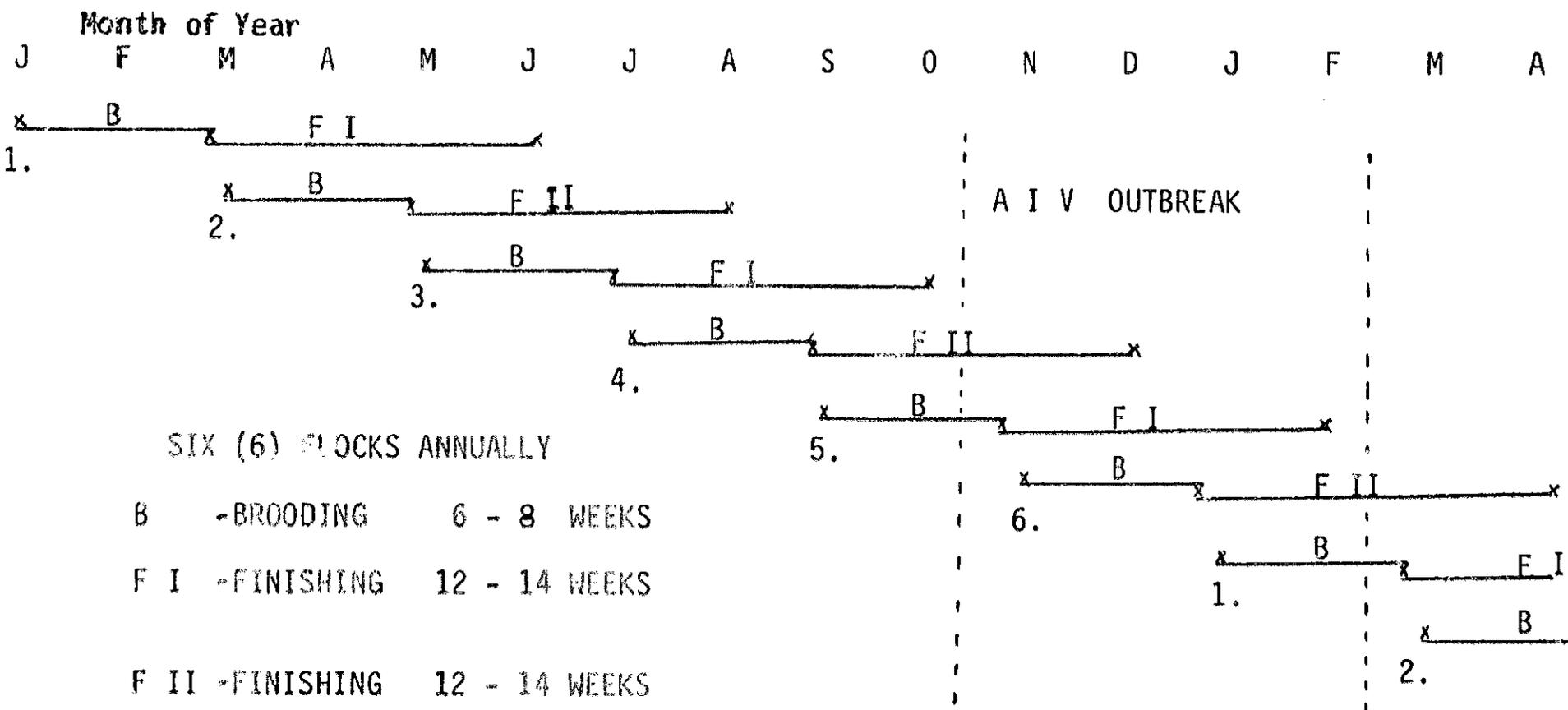


TABLE 6

Estimated AIV Loss With Depopulation

		<u>\$(000)</u>
<u>DISEASE</u>	10,000 Toms @ \$2	80
<u>DEPOPULATE</u>	40,000 Toms/1 mm Pounds	
PRODUCTION FARM	@ 4¢/lb.	33
BREEDER-HATCHERY	@ 20¢/Foult	8
FEED MILL-DELIVER	@ \$4/ton	6
PROCESSING	@ 5¢/lb.	50
<u>OTHER</u>		
AIV VACCINATION	20,000 - 2 Doses	<u>3</u>
SUBTOTAL	\$2.50 per TURKEY	100
TOTAL	\$4.50 per TURKEY	<u><u>\$180</u></u>

DR. EMMETT McCUNE (UNIVERSITY OF MISSOURI): In talking about the distribution of avian influenza viruses, I thought it would be of interest to the group if I briefly discussed the situation as we have encountered it mainly in turkey breeder flocks in Missouri. Year-end and year-out during the winter season from about December to March we have had a number of flocks that show an extremely abrupt drop in egg production. The birds are depressed for about 4 to 5 days and their egg production never does come back. There is very low mortality. Serologically, we have had consistent evidence of avian influenza, Hsw1N1, in these flocks. We have had concurrently with this condition occasional instances of respiratory disease in adjacent market flocks. If the birds are kept under decent circumstances, the effect on the market birds has been practically nil. The same is true of breeders that are not in lay; but in the flocks in production, the estimated cost that we have received from our commercial people is about \$12,000 per flock following an outbreak of avian influenza. This season we have had a similar series of episodes involving flocks in Missouri and flocks in Kansas that supply hatcheries in Missouri. We have two isolates of this agent in the laboratories now. We have been using, on an experimental basis, an Hsw1N1 vaccine manufactured from a Minnesota isolate of this virus. The preliminary information on this looks like the vaccine will work, but the thing that is still not clear is: where this virus came from, and why is it being maintained at a low virulence level persistently year to year in turkey flocks without evidence of antigenic variation? These questions will provide some interesting information if we can get the answers to them.

DR. EASTERDAY: Dr. McCune, I don't think it is so surprising that it stays the same. We have lived with swine influenza virus not changing for at least 50 years, and so it doesn't surprise me that there would be a virus present that doesn't change in any particular way.

_____ question asked to Dr. Easterday (cannot hear)

DR. EASTERDAY: No, what I would hope is that we could adopt this new nomenclature as fast as we can so these hemagglutinins don't have species names on them that interject this bias into where we think they come from.

DR. HINSHAW: One thing too about the change, we do know that, antigenically, these avian strains do undergo drift. That is, they do change slightly; but one has to use specific sera to detect these changes. The hyperimmune sera used for classification are typically not capable of detecting these changes. So you may need to go to more sensitive sera to detect it.

DR. ROSENWALD: My question is aimed at Dr. McCune. What sort of a vaccination history against Newcastle disease do these birds have? It seems to me that along about 1947 we experienced a very similar thing from which endemic Newcastle disease was isolated.

DR. MCCUNE: The history on Newcastle virus, the particular work that I have been following most closely on this is Dr. H. John Barnes from Iowa who has been doing additional studies and we need to do more yet on the response of the turkey to Newcastle disease vaccines. The program that has been followed in Missouri has characteristically given at a very low titer on HI tests in turkeys. We have done a number of these this past year and are finding from organization to organization a very marked difference in the antibody titers in these turkey breeder flocks. In these areas where avian influenza is being detected, we do see clinical Newcastle disease in some of these flocks on the basis of very mild symptomatology and seroconversions. But these may happen with or without this influenza virus. The cases in which there is Newcastle alone, these birds come back on line with egg production in 3 to 4 weeks and stay on the production curve. But, if we have the influenza virus, our experience has been that they do not come back. So that evidently we have a mild enough strain of Newcastle that we are not into serious trouble from this disease. There is one organization that is using a series of 3 avian influenza vaccinations on turkeys and is obtaining HI titers 1:128 and 1:256 on our system which indicates a very high antibody titer. So the turkey is capable of responding if we give them enough of the right kind of antigen at the right time.

DR. LANG: Concerning the epidemiology of avian influenza and sources of infection, you're talking here always about ducks as the source and the animal reservoir of influenza viruses and that the ducks are the danger of our domestic turkeys. From our experience in Canada, we do not see many ducks on our turkey farms. Also our outbreaks occur mostly in the middle of winter. It is very cold and very few wild birds around. The explanation that we have for this is that there are still birds around with virulent levels of virus in blackbirds. We need to concentrate much more in the future on these species too because I don't believe the ducks are really the direct transmitters to the turkeys. They may be the reservoir but other birds are picking up the virus and then transmit it into the barns. And I would remark that the studies that we have seen today list very few birds other than ducks as being carriers of influenza virus. I don't believe it. I think we have to look more into this aspect of it. Then there is another question, Dr. Shortridge talked about paramyxoviruses as a possible element to be considered in our influenza outbreaks. That is true. While we have in Canada very little influenza right now, despite the massive infection detected in the wild ducks, it is the paramyxovirus problem which is most of concern to our industry right now — paramyxovirus 2 and paramyxovirus 3 rather than influenza virus.

DR. HINSHAW: I would comment in response to Dr. Lang. As I mentioned at the beginning of my paper, I think we all clearly realize that there are several different avian species involved in influenza in nature. Because of time, I concentrated on one. Certainly, if you look at the many isolates we have then from shearwaters, terns, gulls, starlings, the

diversity of different species in nature clearly indicates all of them could be involved in the transmission of viruses between different groups. I point to the ducks primarily because they do appear to represent the only group which perpetuates all influenza A virus subtypes that we know. And there are several characteristics of that reservoir which would indicate that they are very important in the maintenance of these and; therefore, they could represent a continual source for other wild birds as well as domestic species. I also alluded to the fact that we have not eliminated the possibility that these are maintained for a long period of time in the domestic birds themselves and certainly an important question to address. I think most of us do appreciate that many different species are involved, but ducks would appear to represent the major continuing reservoir.

DR. EASTERDAY: As long as we are talking about species, let's don't group ducks as one because there is a great variation in ducks, and the number of isolations will vary greatly from one specie of duck to another. And not all of these ducks go the same places and do the same things.

DR. HINSHAW: I would agree 100 percent with Dr. Easterday that there is a great diversity of duck species in the United States as well as in North America. Primarily the mallards and the pintails and teals seem to be most involved.

DR. _____: Although everybody would agree, Dr. Lang, that ducks or that species other than ducks are involved in spreading avian influenza viruses, I would like to make a comment that it is very, very difficult, and people in Europe and also people in the United States have sampled thousands of other birds and tried to isolate influenza, to isolate influenza from these other birds. So I would not overestimate the role of these birds in spreading influenza.

DR. BEARD: Thank you very much. This has been an interesting discussion. I do believe that Dr. Lipkind has actually incriminated starlings in the infection of turkey flocks in Israel, so I think that it is very valid to consider other birds as being involved, besides ducks, perhaps as the link between the large reservoir and the domestic species.

DR. BARNES: I would like to know — it seems like water is the important aspect of this whole cycle. What is known about the stability of the virus to survive in water, and is there anything known about aquatic plants? Is it possible that the virus may live in fish, frogs, turtles, or anything, or plant life? Is there much known about this aspect of the virus cycle?

DR. HINSHAW: We have studied the survival of the influenza A viruses from the ducks in water, and in this case we were using Mississippi water but I think it is all right. The viruses are quite stable in this environment particularly when you are talking about temperatures at 4 degrees. They will survive even for several days when you are talking about 25 degrees. So these isolates will remain viable for a considerable amount of time in water supplies. We have isolated virus from unconcentrated lake water

where these ducks are living indicating that this is a very important mode for them. As far as aquatic life, plants, or things of this nature, or fish, there is no evidence at this point that would indicate that they are involved thus far. The reason people keep concentrating on ducks, of all the surveillance studies, and there have been many, to look at the different species is the characteristics listed. There is only one group that fulfills the many different things listed there, and that is the ducks. When we look at other species, we just don't see that. And these are primarily ducks that live on ponds. Now tomorrow I will be talking about an avian virus which has appeared in aquatic mammals. The mechanism of that may involve water also.

DR. GHAZIKHANIAN: In the relations with water ponds, there are many waterponds, manmade waterponds where we have seen lots of ducks around and we know virus survives as you said. What is the practical control approach? That is the question I have been asked many times.

DR. HINSHAW: That's a tough one (question). You certainly are not going to interfere with the ducks on their ponds in many cases. Now, theoretically, and I know Dr. Poss and I have examined this to a certain extent, is adding a chemical to the water to inactivate the virus, for instance, in a more feasible situation where you are talking about a pond or a small water supply for domestic birds. On these lakes in nature, it certainly would not be a feasible thing. This is a natural occurrence and really we can't interfere. But I think once viruses are introduced into a domestic group, it's not infeasible to consider the possibility because water is very important. If you watch a turkey drool in the water you can see how you can get infectious virus in these areas too. We have recovered it from troughs on turkey farms as well. That possibly inactivation of the water by chemicals or PH treatment something of this (nature will work) because we know something about the stability of the virus in the presence of different chemicals.

DR. KUMAR: Yes, I want to add to Dr. Poss' comments. We had swine influenza, Hsw1N1, isolated from our breeders. We had infection in 42,000 breeders in Colorado. There is no disease problem, but we lost egg production worth \$300,000. And if we didn't have the vaccine available at that time, we would have lost more. The vaccine did help us. The source of this infection probably was from employees attending a pig roast back in early November. I am sure some of them slaughtered and prepared these pigs before they came to work. And that's how I think our birds got exposed. Those people have these pig roasts quite often, and I did not realize that at the time. Now we have a problem.

DR. LANG: I wonder if this was really necessary to enter into such a state of commotion because 3 flocks of chickens showed fowl plague. In our experience we had at least 3 houses in Canada where we had highly pathogenic Hav5 virus, and from our experience it shows that by very

simple confinement procedures, that is, restricted the circulation and sitting out the outbreak, the disease can be confined on all 3 farms. One house was only infected and three other houses were full of turkeys and the infection did not spread. Also since the classical outbreaks at the turn of the century and in 1929 or 26 and I think there was one outbreak in Europe in 1948. In France, for instance, it never generated into a major fowl plague epidemic as it had happened earlier. The consideration is that we are no longer operating under the same circumstances. We do not have as many live chicken markets. Also the transportation of live birds is rather restricted and from my observation it seems that these were instances in the past when the general state and measures of hygiene were not as high as we have now. And I wonder also, for instance, on the British Islands where I think there was one chicken virus isolated, H7N7, with a high neuropathogenic index — why the British at the time did not go into the same state of panic as the Australians did at the time. I think they are sitting it out more and that the outbreak cooled down. I think this is a very wise principle, because nowadays with our large turkey farms where there are hundred thousand of birds congregated, a slaughtering program would cost enormous sums. Therefore should we weigh the cost of loss with the risk involved when the risk is not as high as many people say. Many of these measures are extrapolated from foot-and-mouth disease to the fowl plague situation and that is a wrong extrapolation because the disease is not as contagious. We have to be working with fowl plague viruses in our isolation quarters under very confined circumstances where there were many turkeys and chickens in the same premises, and we never had a single escape of the virus into groups that were not intended for the experimentation.

ECONOMIC IMPACT OF AVIAN INFLUENZA IN DOMESTIC FOWL ON INTERNATIONAL TRADE

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U.S. poultry and poultry products reached another record last year totaling \$600 million. Fresh and frozen U.S. poultry meat is now exported to over 100 countries valued at \$386.5 million in 1980, with another \$14 million of poultry specialty products. Live poultry and egg exports contributed another \$116.8 million.

The major U.S. poultry and egg markets include Japan, the Caribbean countries, Venezuela, Hong Kong, Singapore, and the European Community. In more recent years, the Middle East Countries have become an important market accounting for nearly \$50 million in trade in 1980.

Many barriers to trade in poultry and egg products still exist around the world. Some are in the form of non-tariff barriers, such as licensing or quotas, and some are in the form of hygiene regulations and animal health restrictions.

One of the most significant barriers that U.S. producers have encountered in developing markets around the world is restrictions placed on poultry and egg products originating from countries that have live-virus type vaccine programs to control Newcastle disease.

At present, U.S. poultry exports to Northern Ireland and Ireland, Denmark, Sweden, Norway, and New Zealand are limited to fully cooked poultry products since these countries have declared themselves free of Newcastle disease. Australia is also free and accepts only canned (sterilized) poultry products. It is interesting to note that only Denmark, from the above-listed so-called "Newcastle free" countries, is a major producer and exporter of poultry and egg products.

The U.S. poultry and egg industry has become very dependent on the export markets over the years. Some producers/exporters and some regions of the U.S. are more dependent on exports than others. As a result, producers and industry/government leaders are more and more concerned about the threat of disease outbreaks, such as the large outbreak of exotic Newcastle disease which occurred in California in 1972-74. During the past few years, the USDA has spent \$87 million controlling the disease in poultry and birds.

What would consequences be for U.S. exports of poultry and eggs if a major outbreak of Newcastle disease occurred in U.S. poultry flocks? They would be disastrous, particularly if viewed by other countries as a national outbreak. Exports to our major markets would come to virtually a full stop, with the possible exception of fully cooked poultry products. Confronted with this situation, U.S. officials would most probably push

even harder a concept that is taking on more and more interest — the concept of regionalization.

Veterinary officials of the major meat-producing countries are currently giving serious consideration to the “regionalization concept” for diseases such as African swine fever, hog cholera, and bluetongue in bovine animals. Very little thought, unfortunately, has been given to such a concept as it relates to poultry.

Some U.S. laws, such as the one dealing with controls on foot-and-mouth disease, prohibit consideration of a regionalization concept versus a “free country concept.” However, no such constraints are placed on U.S. veterinary officials in dealing with most other diseases.

In view of the volume of poultry and eggs moving in international trade, a more pragmatic approach to disease control and disease acknowledgement needs to develop. Countries need to take a serious look at the “regionalization concept” to control and certification.

ECONOMIC IMPACT OF AVIAN INFLUENZA IN DOMESTIC FOWL

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I wish to outline some of the costs that were involved in the eradication of a small outbreak of fowl plague that occurred near Melbourne, Australia in 1976.

The economic impact of the outbreak was felt by both government and the poultry industry. Most of the impact arose as a consequence of decisions that were taken not by the Victorian Government but by the governments of the other States of Australia and overseas countries.

To detail the economic impact, I need to give some details of the poultry industry in Australia. The poultry industry of Australia is largely based on internal production and consumption, but nevertheless considerable numbers of eggs and amounts of egg pulp are exported. The poultry industry in the State of Victoria within Australia has considerable internal production and limited distribution to the rest of Australia.

Following the declaration of fowl plague as a disease in Victoria, all the other States of Australia closed off their borders and refused entry to all poultry and poultry products from Victoria. Victorian veterinary authorities declared the infected properties the infected area and an area of some 5 km radius was made the control area. The movement of live poultry and eggs for hatching was prohibited in the control area, except that birds could be transported for slaughter under written permit to an approved processing works.

The infected poultry farms were slaughtered out and the farmers were paid compensation to full market value for the animals, produce, feed buildings and fittings that were destroyed. These farmers were relatively well catered for in compensation.

The poultry farmers in the control area were fortunately few on account of the location of the infected properties. These farmers suffered little loss of income because none were breeder properties or hatcheries. The local egg marketing authority made available separate facilities for the collection and handling of eggs from within the Control Area.

If any of the properties in the control area had been either breeding properties or hatcheries, the economic impact would have been very considerable because all sales would have been stopped. Such a producer would have lost all stock under incubation and would only obtain table egg price for his produce. The social effects of disease control programs on such people can be substantial and the effects continue for as long a period as it takes to eradicate the disease.

I am not aware of legislation in any country whereby losses of the type outlined above are made up to producers suffering hardship under conditions beyond their control. Circumstances do occur whereby it would be better that infection occurred on such properties.

So much for the losses to producers in the infected and control areas. The costs to government were not inconsiderable being some \$A250,000 to destroy 17,000 broilers, 25,000 layers and 16,500 ducks. The costs of mounting the diagnostic and surveillance program over the above normal routine costs was some \$A25,000 and is included within the total cost above.

The principal costs to the poultry industry arose out of loss of interstate and overseas trade. Victoria as a State was isolated by all other States by refusing to accept any of its poultry or poultry products. Overall, the State of Victoria imports most of its genetic stock and exports little except eggs and egg pulp.

The interstate restrictions on trade did seriously financially embarrass those few owners of hatchery and breeding flocks that had a significant proportion of their trade with producers in other States. Whole incubators of eggs had to be destroyed and production could not be started until the outbreak had been declared eradicated i.e. three weeks after the declaration of eradication. Assessments of losses to individual farmers were not made at the time of the outbreak. It can only be assessed that many hundreds of thousands of dollars were lost.

Eggs normally intended for hatching were of course sold to the egg marketing authority at prices considerably less than their value as hatching eggs. The egg marketing authority had to handle additional quantities of eggs and had fewer outlets because no eggs or egg pulp could be exported. Economic pressures built up on the egg marketing authority, and to cover for loss of trade outlets, sales were made at reduced prices to encourage turnover of stocks. To cover the cost of these transactions, charges were increased to producers. The overall direct cost to the marketing authority and to the egg producers was estimated at about \$275,000. This covered such costs related to additional refrigeration and additional pulping requirements.

All these events occurred in a State that was very nearly self sufficient. The outbreak was confined and eradicated within 5 weeks. If the outbreak had occurred in the State of Australia with the major component of the poultry industry of Australia, the economic impact would have been increased many fold and the hardship caused to people would have been multiplied many times.

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THE ROLE OF THE HEMAGGLUTININ IN INFECTIVITY AND PATHOGENICITY OF AVIAN INFLUENZA VIRUSES

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Although both hemagglutinin and neuraminidase change independently during the formation of new influenza viruses, the hemagglutinin is considered the more important surface glycoprotein. It is quantitatively the major surface component, it is responsible for the attachment of the virus particle to neuraminic acid-containing receptors of the host cell, it is involved in the initial stages of virus infection, and it is the antigen against which neutralizing antibodies are directed.

The biosynthesis of the hemagglutinin, like that of other integral membrane proteins, involves translation at membrane-bound ribosomes, insertion into the membrane of the rough endoplasmic reticulum, and transport to the plasma membrane. In the course of transport, the hemagglutinin is processed by glycosylation, covalent attachment of fatty acids to the carboxy terminus of the molecule, and by proteolytic cleavage of the primary gene product HA into the amino terminal fragment HA₁ and the carboxy terminal fragment HA₂. Although these co- and posttranslational modifications must play essential roles for providing adequate structural elements, a definite function can so far be attributed only to the proteolytic cleavage of the hemagglutinin (Rott and Klenk, 1977; Klenk and Rott, 1980).

It could be shown — and this will be the content of my presentation — that proteolytic cleavage of the hemagglutinin is an important prerequisite for the infectivity of influenza viruses and that besides an optimal genome composition, differences in cleavability and host range account for variations in pathogenicity.

PROTEOLYTIC ACTIVITY OF THE HEMAGGLUTININ

Depending on the presence of an appropriate enzyme in a given cell, virus particles with cleaved or with uncleaved hemagglutinin may be formed. Viruses formed with uncleaved hemagglutinin are able to adsorb to the cell surface. They are, however, non-infectious. Such virus particles can be converted into infectious virions by treatment *in vitro* with trypsin or trypsin-like enzymes (Klenk et al., 1975; Lazarowitz and Chopin, 1975). Only a limited number of influenza viruses are produced in a large variety of cell types derived from diverse host species with a cleaved hemagglutinin and thus in infectious form. It should be emphasized that all these viruses, like fowl plague virus, are of avian origin. For most influenza viruses, including all human virus strains, cleavage of hemagglutinin depends on both the host cell system and the virus strain used.

Double infection of a cell type with virus strains containing a cleavable and a non-cleavable hemagglutinin has proved that cleavage of the sensitive strain cannot facilitate simultaneous cleavage of the resistant hemagglutinin of the other strain (Klenk et al., 1977). This means that the individual structural characteristics of the hemagglutinin, rather than activation of cellular enzymes by the infecting virus, determine whether cleavage takes place. Moreover, these as well as other experiments indicate that the active cleavage enzyme is a normal cellular constituent.

SPECIFICITY OF THE CLEAVAGE REACTION

Proteases of different specificities are able to cleave hemagglutinin, but activation is observed only by tryptic enzymes (Lazarowitz and Chopin, 1975; Klenk et al., 1977). These observations suggested that cleavage of a specific peptide bond is required for activation. Therefore, comparative sequence analyses were carried out on the hemagglutinin of virus N (Hav2Neq1) that had been cleaved either *in vitro* using proteases of various specificities, or in the infected host cell (Garten et al., 1981). As shown in Fig. 1 the amino terminus of HA₂ was identical whether the hemagglutinin was cleaved *in vivo* or *in vitro* with trypsin or the trypsin-like enzyme acrosin. It differed, however, by one or three amino acids after treatment with the non-activating enzymes thermolysine or chymotrypsin. The results obtained with thermolysine, demonstrating that elimination of a single amino acid is enough to yield inactive hemagglutinin, show unequivocally that activation of infectivity requires a highly specific amino acid sequence at the amino terminus of HA₂. This observation is compatible with the studies of Richardson et al. (1980) who found by a different approach that activation of infectivity requires a specific sequence at the amino terminus of HA₂.

In contrast to the conserved amino acid sequence at the amino terminus of HA₂, there is more variability of the carboxy terminus of HA₁ (Fig. 2). It therefore appears that activation of hemagglutinin does not require a high degree of structural specificity in this region of the cleavage site. Comparison of the uncleaved precursor and the cleaved hemagglutinin demonstrates that arginine or a series of predominantly basic amino acids is eliminated in proteolytic activation. It is interesting to note that a single arginine is removed from hemagglutinins which are cleavable only in a few host cells, whereas a peptide containing several amino acids is eliminated from those hemagglutinins which are activated in all cell systems tested. These observations demonstrate 1) that, in addition to trypsin-like enzymes, another protease, presumably a carboxypeptidase B, is involved in the activation reaction and 2) that differences in the susceptibility of the hemagglutinin of different influenza virus strains to proteolytic enzymes are determined by the specific structure of the cleavage site (Bosch et al., 1981; Garten et al., 1981).

In conclusion, present observations demonstrate that the structure of the hemagglutinin encoded in the viral genome determines whether a

proteolytic enzyme of a given host cell is capable of reacting with the precursor structure in such a way that cleavage ultimately results in infectivity of the virus particle.

THE ROLE OF THE HEMAGGLUTININ IN VIRUS INFECTION

Since viruses formed with uncleaved hemagglutinin are capable of adsorbing to receptors of the host cell, the cleavage of hemagglutinin must facilitate another decisive function in initiation of infection. There is increasing evidence for an involvement of the hemagglutinin in penetration by triggering fusion of the viral envelope with cellular membranes. This concept is supported by the following observations:

1. Cellular membranes exposed to influenza viruses show fluidity changes similar to those observed after exposure to paramyxoviruses, which are well known fusing agents (Nicolau et al., 1978).
2. Exposure of cells to specifically sensitized cytotoxic T cells early after infection, results in lysis implying that the viral envelope has fused with the cell membrane (Kurrle et al., 1979).
3. The involvement of the influenza virus hemagglutinin in fusion between the viral envelope and the host cell membrane could be demonstrated directly with reconstituted viral membranes (Huang et al., 1980a). Electron microscopic studies showed (Fig. 3) that liposomes containing both influenza virus glycoproteins fused with cell membranes when the hemagglutinin was present in the cleaved form. Liposomes containing the uncleaved hemagglutinin, adsorbed to cells without causing fusion of membranes.

Fusion of viral and cellular membranes could also be imitated by mixing liposomes containing cellular receptors or gangliosides and native virus particles. Virus with cleaved hemagglutinin was able not only to adsorb to these liposomes but electron microscopic observations showed that virus spikes had become incorporated and exposed on the liposomal membrane. Such liposomes in turn fused with the host cell membrane (Huang et al., 1980a, 1981).

All these data support the idea that influenza virus gain entry into cells by fusion of the viral envelope with host cell membranes and that for this process cleavage of the hemagglutinin is necessary. It is therefore not surprising that only virions containing the hemagglutinin in the cleaved form are infectious.

Recent studies have provided evidence that, in addition to active hemagglutinin, neuraminidase is also necessary for membrane fusion (Table 1). Liposomes loaded with activated hemagglutinin but without neuraminidase are strongly adsorbed to cell membranes without causing fusion. To induce fusion under these conditions neuraminidase has to be added to the liposome-cell-mixture (Huang et al., 1980b).

The mechanism underlying the cooperative effect of neuraminidase in membrane fusion and thereby in virus penetration is not known. It might

be that influenza virus infection is a two-step process (Fig. 4): Initial adsorption of the virus to neuraminic acid-containing receptors is mediated by HA₁. During the action of viral neuraminidase a new receptor may become unmasked which reacts with the hydrophobic region of the amino terminus of HA₁ created by the proteolytic cleavage, or alternatively, HA₁ has to be released so that HA₂ can induce the actual membrane fusion.

This hypothesis would explain why exposure of an identical hydrophobic sequence at the amino terminus of HA₂ as a precondition for penetration is a general observation with all influenza virus hemagglutinins. It also would explain why peptides resembling the amino terminus of HA₂ inhibit virus infection (Richardson et al., 1980).

THE STRUCTURE OF THE HEMAGGLUTININ DETERMINES VIRAL PATHOGENICITY

The findings discussed so far made it conceivable that differences in the susceptibility of the various hemagglutinins to proteolytic activation may affect the host range, the ability to undergo multiple cycle replication, and spread of the virus in the host. If an infectious virus particle containing the cleaved hemagglutinin infects a permissive cell that possesses a protease capable of cleaving the hemagglutinin of progeny virus particles, infectious virus is produced and can spread to other permissive cells. If, on the other hand, a cell to be infected does not possess an appropriate activating protease, the progeny virus will have an uncleaved hemagglutinin and will be non-infectious. This would therefore be a dead end for the spread of infection. This could be confirmed convincingly in studies with avian influenza viruses, pathogenic or non-pathogenic for chicken, using the chicken chorioallantoic membrane (CAM) as a model organ system (Rott et al., 1980).

It could be shown that pathogenic avian influenza viruses were produced in infectious form in the endoderm as well as in the ectoderm of the CAM. Formation of infectious, non-pathogenic virus was achieved only in the endodermal cells. Accordingly polyacrylamide gel analysis revealed that the hemagglutinin of pathogenic viruses was cleaved in both germinal layers of the CAM, whereas that of the non-pathogenic viruses was cleaved only after synthesis in the endoderm. Immunohistological studies revealed (Fig. 5) that multiplication of non-pathogenic virus was restricted to the cell layer which was inoculated. Spread of newly synthesized virus was inhibited as soon as the virus reached mesodermal cells. Mesoderm consists mainly of fibroblasts which were found to be nonpermissive for non-pathogenic avian influenza viruses. On the other hand, pathogenic avian influenza viruses spread through the whole membrane and gained entrance into the blood vessels independent of the route of inoculation.

A similar mechanism turned out to function in the chicken host. Pathogenic as well as non-pathogenic avian influenza viruses are produc-

ed in infectious form with cleaved hemagglutinin in the epithelial cells which line the respiratory and intestinal tracts of the bird. Spread of non-pathogenic viruses is inhibited as soon as the virus reaches the *lamina propria mucosa* which is nonpermissive for these viruses. Only the very few pathogenic avian influenza viruses which possess a cleavable hemagglutinin may pass this barrier. The resulting generalized infection leads to the well known disease which is designated by the suffixes "pest" or "plague" (Bosch et al., 1979; Rott, unpublished results).

It is of particular interest that a virus isolated recently from seals by Dr. V. S. Hinshaw, that has the cleavable hemagglutinin of a pathogenic avian virus strain (Hav1). This virus type causes a generalized infection in a mammalian species. On the other hand, infection by virus particles carrying a non-cleavable hemagglutinin of avian origin will remain confined to the primary site of infection. This situation was encountered in the human Hong Kong influenza strain (H3N2) which has the hemagglutinin gene of an Hav7 strain (Scholtissek et al., 1978). These findings underline again the significance of the structure of the hemagglutinin in determining the occurrence of a generalized infection and the manifestation of a specific clinical disease.

All present observations demonstrate that the structure of the hemagglutinin determines whether a proteolytic enzyme of the host cell is capable of reacting with the precursor structure which ultimately results in a cleaved hemagglutinin necessary for infectivity, host range, and spread of the virus particle. Since the capability of rapid multiplication and spread within the host is a precondition for virus to cause acute disease, it is not surprising that the cleavability of the hemagglutinin is essential for pathogenicity. Comparative studies on naturally occurring avian influenza viruses, pathogenic or non-pathogenic for chicken, has shown this correlation without any exception (Bosch et al., 1979). These viruses occur with at least 13 different HA subtypes and in many hemagglutinin-neuraminidase combinations. In addition to the genes coding for hemagglutinin and neuraminidase, there are also considerable differences in base sequence homologies of the other genes. The cleavability of these different avian influenza virus subtypes in MDCK cells, chicken, duck, turkey and quail fibroblasts, their ability to form plaques on these cells as well as their behavior in chicken has been investigated as an indicator for viral infectivity and pathogenicity for chicken. The findings are summarized in Table 2. Only those viruses which are produced in an infectious form in a broad spectrum of host cells are pathogenic. It should be emphasized that differences in cleavability of the hemagglutinin and pathogenicity occur not only between the different subtypes but even between strains within a single subtype. Although all strains in the subtype Hav1 (H7) have a serologically closely related hemagglutinin, they differ in cleavability and pathogenicity (Table 3). Analyses of the genetic relatedness of the hemagglutinin gene of these viruses revealed significant differences in their base sequences

as determined by RNA hybridization. It is remarkable that the hemagglutinins of the non-pathogenic Hav₁ strains appear to have a cleavage site structurally similar to the human influenza viruses, whereas the hemagglutinin of the pathogenic strain have significantly more basic connecting peptides (Bosch et al., 1981).

GENETICS OF VIRAL PATHOGENICITY

The findings pointing to the primary significance of the hemagglutinin for pathogenicity seem to apply only to naturally occurring avian influenza viruses. Genetic analysis of recombinants of influenza A viruses obtained *in vitro*, however, have revealed the polygenic nature of pathogenicity (Burnet, 1959; Kilbourne, 1963; Rott et al., 1978). A large number of such recombinant viruses could be obtained due to the segmented structure of the influenza virus genome which permits a ready exchange of genes during mixed infection. Observations with recombinant influenza viruses obtained *in vitro* lead to the following conclusions with regard to factors determining pathogenicity (for review see Rott, 1980):

1. The pathogenic virus must possess an hemagglutinin that is cleavable in a broad variety of cells.
2. No specific single gene is responsible for pathogenicity. An optimal constellation of all RNA segments is required for the genome of a highly pathogenic virus strain.
3. It is impossible to establish a rule for the combination of different genes indicative of pathogenicity of all influenza viruses.
4. In each reassortment of different virus strains, another genome composition might lead to increase or loss of pathogenicity, depending on the parent virus strains used. Therefore, at present genetic analysis of influenza virus recombinants does not provide us with a specific marker for pathogenicity or attenuation for man and animal.

Thus, in our hands increase or loss of pathogenicity seem to be dependent not only on the hemagglutinin but also on the influenza virus genes involved in viral RNA synthesis (Table 4). It should be stressed, however, that other gene constellations influencing pathogenicity have been described (Rott et al., 1978; Potter and Oxford, 1979). For example, Ogawa and Ueda (1981) using two avian influenza viruses for reassortment, found that cotransfer of the hemagglutinin, M and, to a certain extent, the neuraminidase genes was necessary for expression of pathogenicity. On the other hand, in studies on neurovirulence in mice with the neurovirulent WSN (H1N1) strain, Sugiura et al. (1980) found that neuraminidase, M and NS genes were involved in determining pathogenicity of the recombinants.

We are not able at present to define optimal gene constellation. There is evidence, however, that not all of the 254 ($2^8 - 2$) possible new gene constellations between two virus strains can be isolated from a given host, presumably, because not all resulting recombinants are viable in one par-

ticular host system (Rott et al., 1976). Furthermore, it has been observed that certain groups of genes tend to be transferred together during reassortment. For example, the transfer of the fowl plague virus hemagglutinin was always accompanied by the transfer of the gene coding for the M protein if the other parental virus originated from man or other mammals (Scholtissek et al., 1976). If both parents were avian influenza viruses, such a cotransfer was found to be not essential. A similar situation was found with regard to the polymerase. Cotransfer of all genes coding for polymerase activity was critical for pathogenicity if the parent viruses were unrelated. This is in good agreement with the high base sequence homology among corresponding genes of related viruses and significantly lowered genetic relatedness in the case of non-related strains. Thus, replacement of gene products without loss of pathogenicity is accomplished more easily with related viruses.

THERMOSENSITIVITY AND PATHOGENICITY

Besides the cleavable hemagglutinin, there is also a correlation of pathogenicity and the ability of a recombinant virus obtained *in vitro* to grow at an elevated temperature (Rott, Orlich, Scholtissek, to be published). It turned out that pathogenic recombinants are able to grow equally well at 37° and 41°C, whereas the nonpathogenic recombinants have a significantly lower growth rate at 41°C (Fig. 6). It has not been possible yet to define the precise step during the replication cycle which is blocked by the elevated temperature of nonpathogenic recombinants. According to preliminary results, in a variety of recombinants different steps of the replication cycle can be inhibited by the elevated temperature. It is not yet clear how these *in vitro* observations relate to specific aspects of virus replication associated with clinical manifestations *in vivo*. In any case, growing virus at elevated temperature is a powerful tool for rapid *in vitro* selection of pathogenic recombinants.

These findings underline once more the importance of an optimal gene constellation: It is reasonable to assume that a virus recombinant which has a reduced replication rate at the normal body temperature will not be able to induce clinical signs of disease in the bird before the defense mechanism of the organism comes into action. Rapid multiplication and spread of the virus in the host seem to be the most critical factors in the pathogenicity of avian influenza viruses.

CONCLUSION

Comparing the results obtained with influenza virus recombinants isolated *in vitro* with those obtained with naturally occurring avian influenza viruses, we can assume that nature selects an optimal gene constellation for each individual field strain and that naturally occurring viruses with a suboptimal gene constellation will not survive in nature. The requirements for an optimal genome composition seem to be met by

all avian influenza viruses. If a virus possesses, in addition to the optimally functioning genome, a hemagglutinin which is cleaved and activated in many different kinds of host cells, then it is always pathogenic.

Table 1

Requirement for Neuraminidase for Fusion Activity of Liposomes Containing the Glycoproteins of Orthomyxoviruses

Liposomes containing	Percentage fusion
HA _{1,2} ^a + NA	60
HA ^b + NA	4
HA _{1,2}	0
HA _{1,2} + neuraminidase of <i>V. cholerae</i> ^c	75
HA _{1,2} + soluble NA ^c	50
HA _{1,2} + NA + anti-NA	5
NA	0

^a Cleaved HA

^b Uncleaved HA

^c Neuraminidases containing 0.2 unit of enzyme activity/ml of liposome-cell mixture were used.

(For details see Huang et al., 1980b).

Table 2. Proteolytic Activation, Host Range, and Pathogenicity of Avian Influenza Viruses

Virus strain		Activation occurs in ¹						Plaque formation without trypsin ²	Pathogenic for chicken
		CAM	CEF	DEF	TEF	QEF	MDCK		
A/FPV/Rostock	(Hav1N1)	+	+	+	+	+	+	+	
A/parrot/Ulster/73	(Hav1N1)	+	-	-	-	-	-	-	
A/chick/Germany/49	(Hav2Neq1)	+	-	-	-	-	-	-	
A/duck/England/55	(Hav3Nav1)	+	-	-	-	-	-	-	
A/duck/Czechoslovakia/56	(Hav4Nav1)	+	-	-	-	-	-	-	
A/turkey/Ontario/7732/66	(Hav5Nav6)	+	+	+	+	+	+	+	
A/duck/Scotland/59	(Hav5N1)	+	+	+	+	+	+	+	
A/duck/Germany/1868/68	(Hav6N1)	+	-	-	-	-	-	-	
A/duck/Ukraine/1/63	(Hav7Neq2)	+	-	-	-	-	-	-	
A/turkey/Ontario/6118/68	(Hav8Nav4)	+	-	-	-	-	-	-	
A/turkey/Wisconsin/66	(Hav9Neq1)	+	-	-	-	-	-	-	
A/duck/Alberta/60/76	(Hav10Nav5)	+	-	-	-	-	-	-	

¹ CAM, CEF, DEF, TEF, QEF, MDCK means chorioallantoic membrane cells, chicken embryo, duck embryo, turkey embryo, quail embryo fibroblasts and Madin Darby canine kidney cells, respectively.

² Plaque tests were performed in chicken embryo fibroblasts.
(For details see Bosch et al., 1979).

Table 3. Dependence of Infectivity and Pathogenicity on Cleavability of Hemagglutinin of Hav1 Subtype Avian Influenza Viruses.

Virus strain		HA present in cleaved form ¹	Plaque formation without trypsin ²	Pathogenic for chicken
A/FPV/Rostock	(Hav1N1)	+	+	+
A/FPV/Dutch/27	(Hav1Neq1)	+	+	+
A/fowl/Victoria/75	(Hav1Neq1)	+	+	+
A/turkey/England/63	(Hav1Nav3)	+	+	+
A/carduelis/Germany/72	(Hav1N1)	+	+	+
A/parrot/Ulster/73	(Hav1N1)	-	-	-
A/turkey/England/77	(Hav1Neq1)	-	-	-
A/turkey/Oregon/71	(Hav1Nav2)	-	-	-
A/mallard/Ramon, Israel/79	(Hav1N2)	-	-	-
A/chicken/Degania/Israel/80	(Hav1N2)	-	-	-

¹ in CEF, DEF, TEF, QEF and MDCK cells (see Table 2)

² see Table 2.

Table 4. Correlation between Gene Constellation and Pathogenicity of Recombinants between Fowl Plague Virus (FPV; Hav1N1) and the avian influenza virus A/turkey/England/63 (Hav1Nav3).

Isolate Nr.	pathogenic for chicken	Pol 1 ^{a)}	Derivation of genes either from FPV (●) or turkey (○)							NS
			Ptra	Pol 2	HA	NP	NA	M		
2	+	○	○	○	○	○	○	○	○	○
21	+	○	○	○	○	○	○	○	○	○
8	+	○	○	○	○	○	○	○	●	○
18	+	○	○	○	○	○	○	○	●	○
11	+	○	○	○	●	○	●	○	●	○
12	+	○	○	○	○	●	○	●	●	○
14	+	○	○	○	○	●	○	●	●	○
13	+	●	●	●	●	●	●	●	●	○
9	-	●	○	○	○	○	○	●	○	○
19	-	●	○	○	○	○	○	●	○	○
22	-	●	○	○	○	○	○	●	○	○
1	-	○	○	○	●	●	○	○	○	●
17	-	○	○	○	●	●	○	○	○	○
15	-	●	○	○	○	●	○	○	●	○
3	-	●	○	○	○	●	○	○	●	○
10	-	●	○	○	○	●	○	○	●	○
16	-	●	○	○	○	●	○	○	●	○
23	-	●	○	○	○	●	○	○	●	○
24	-	●	○	○	○	●	○	○	●	○

a) For the nomenclature of the genes see Scholtissek et al. (1976). (For details see Rott et al. (1979)).

Figure 1

Amino terminus of the hemagglutinin fragment HA₂ of virus N (A/chick/Germany/49; Hav2Neq1) after cleavage *in vivo* (chicken egg) or after cleavage *in vitro* by various proteases.

Cleavage	Activation	Amino-Terminus of HA ₂ of Influenza Strain N
In Vivo	+	NH ₂ -Gly-Leu-Phe-Gly-Ala-Ile-x-x-x
By Trypsin	+	NH ₂ -Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-x
Acrosin	+	NH ₂ -Gly-Leu-Phe-x-Ala-Ile-x-x-x
By Thermolysin	○	NH ₂ -Leu-Phe-Gly-Ala-Ile-x-x-x
By Chymotrypsin	○	NH ₂ -Gly-Ala-Ile-Ala-Gly-Phe

Figure 2

Part of amino acid sequences around the proteolytic cleavage site of H2, H3 and Hav1 (H7) hemagglutinins. Arrows indicate the carboxy termini of HA₁ and the amino termini of HA₂ determined on the cleaved forms of H2 (Waterfield et al., 1980), H3 (Ward and Dopheide, 1980), and Hav1 (Klenk et al., 1980).

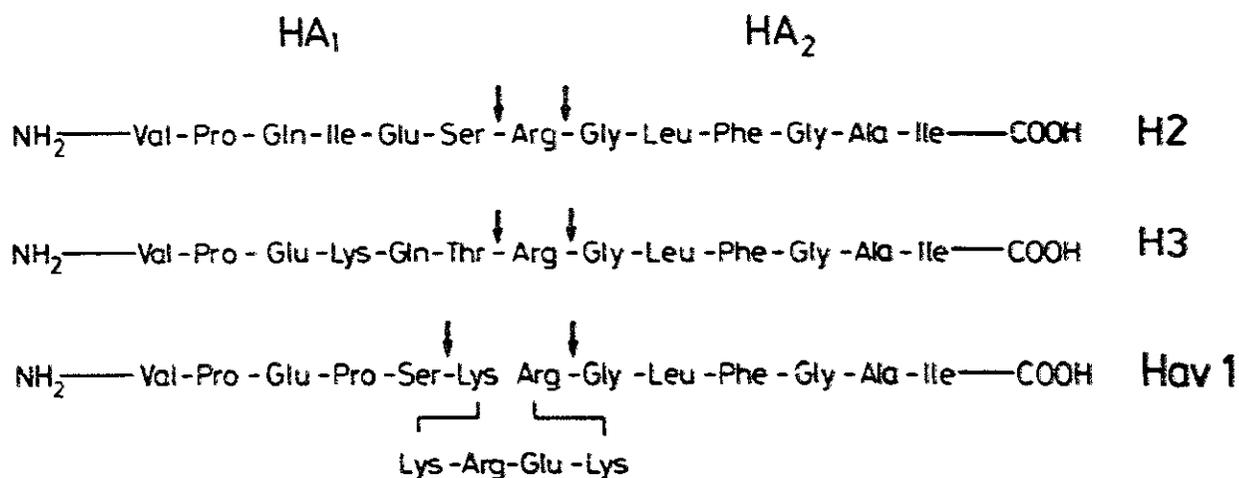


Figure 3
Interaction of chick embryop cells with liposomes containing neuraminidase and uncleaved (a) or cleaved (b) hemagglutinin of virus N (Hav2Neq1); (for details see Huang et al., 1980a).



Figure 4

Proposed mechanism of the cooperative action of hemagglutinin and neuraminidase in influenza virus penetration by membrane fusion.

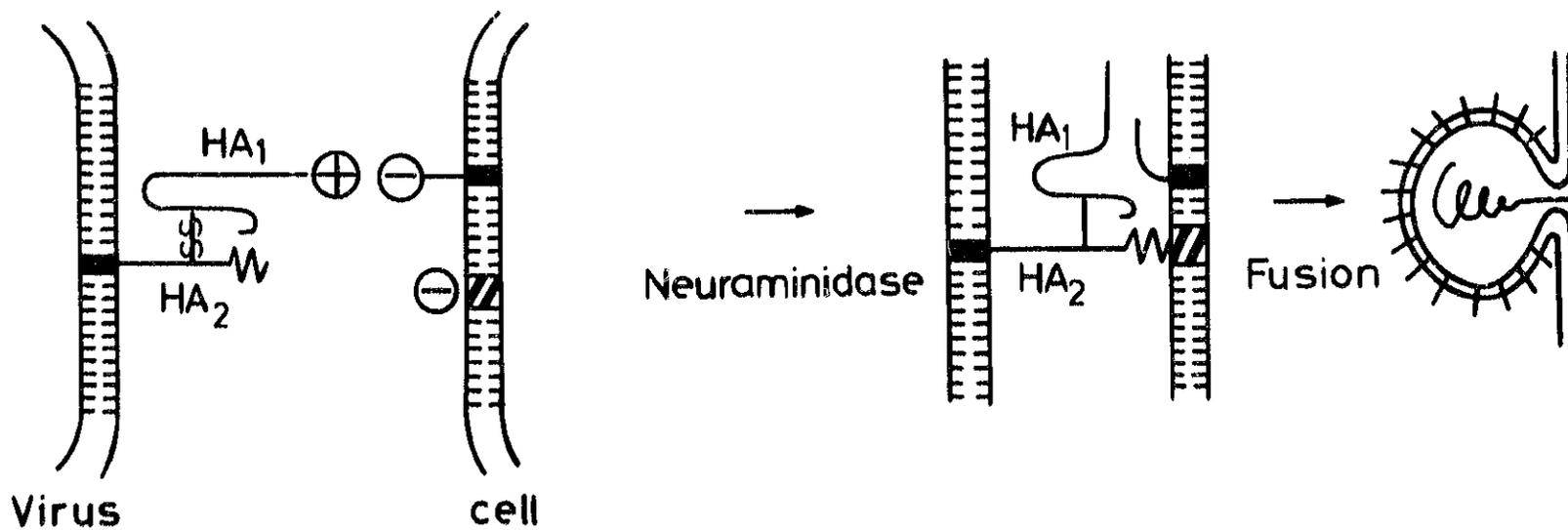


Figure 5

Spread of avian influenza viruses in the chorioallantoic membrane of chick embryo. Chick embryos were inoculated with the non-pathogenic virus N (Hav2Neq1) (a,b) or the pathogenic fowl plague virus (Hav1N1) (c,d) onto the ectodermal layer (a,c) or into the allantoic cavity (b,d). After incubation for 24-48 h after infection, NP antigens were demonstrated in the membranes by the peroxydase-antiperoxydase method (for detail see Rott et al., 1980).

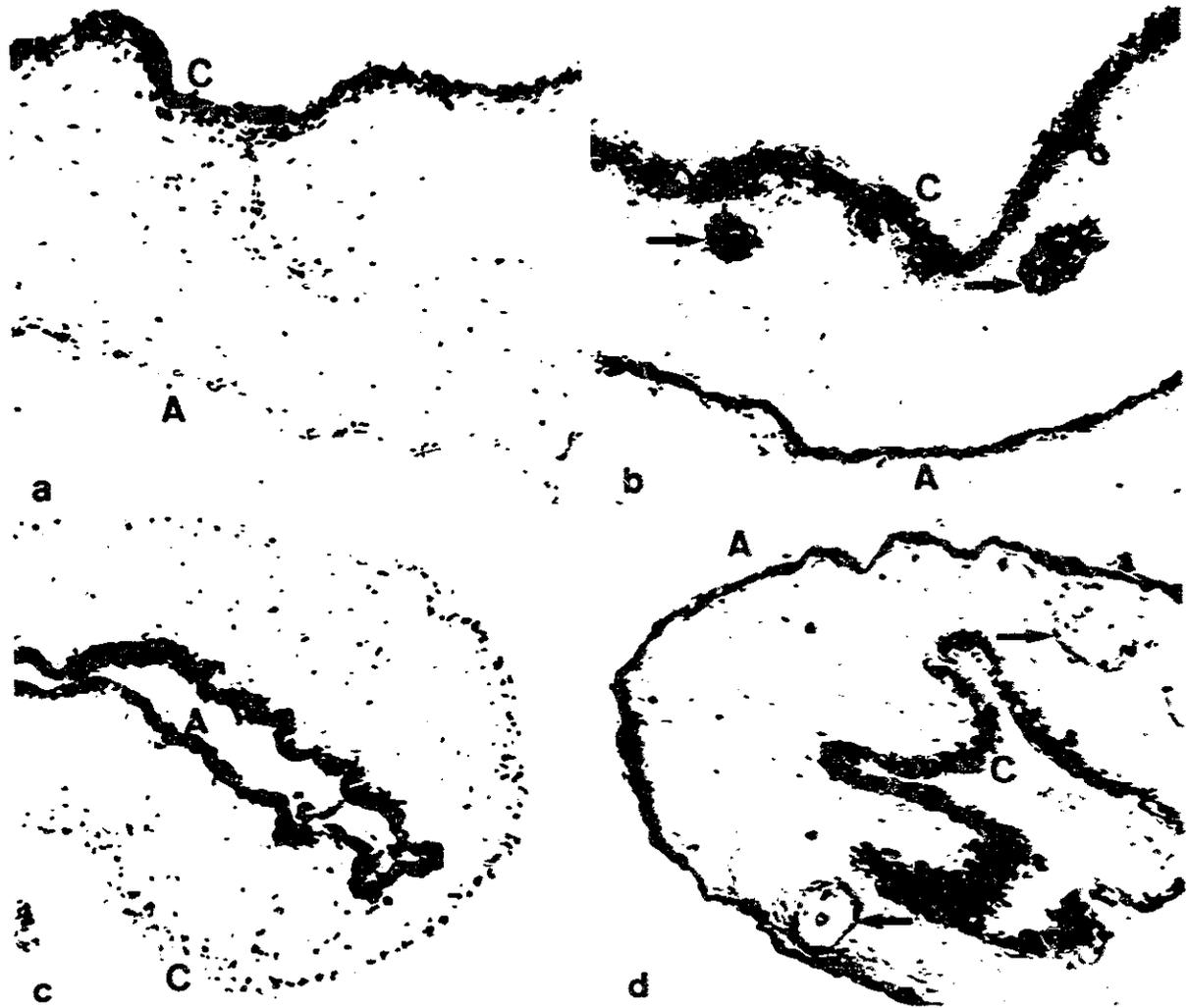
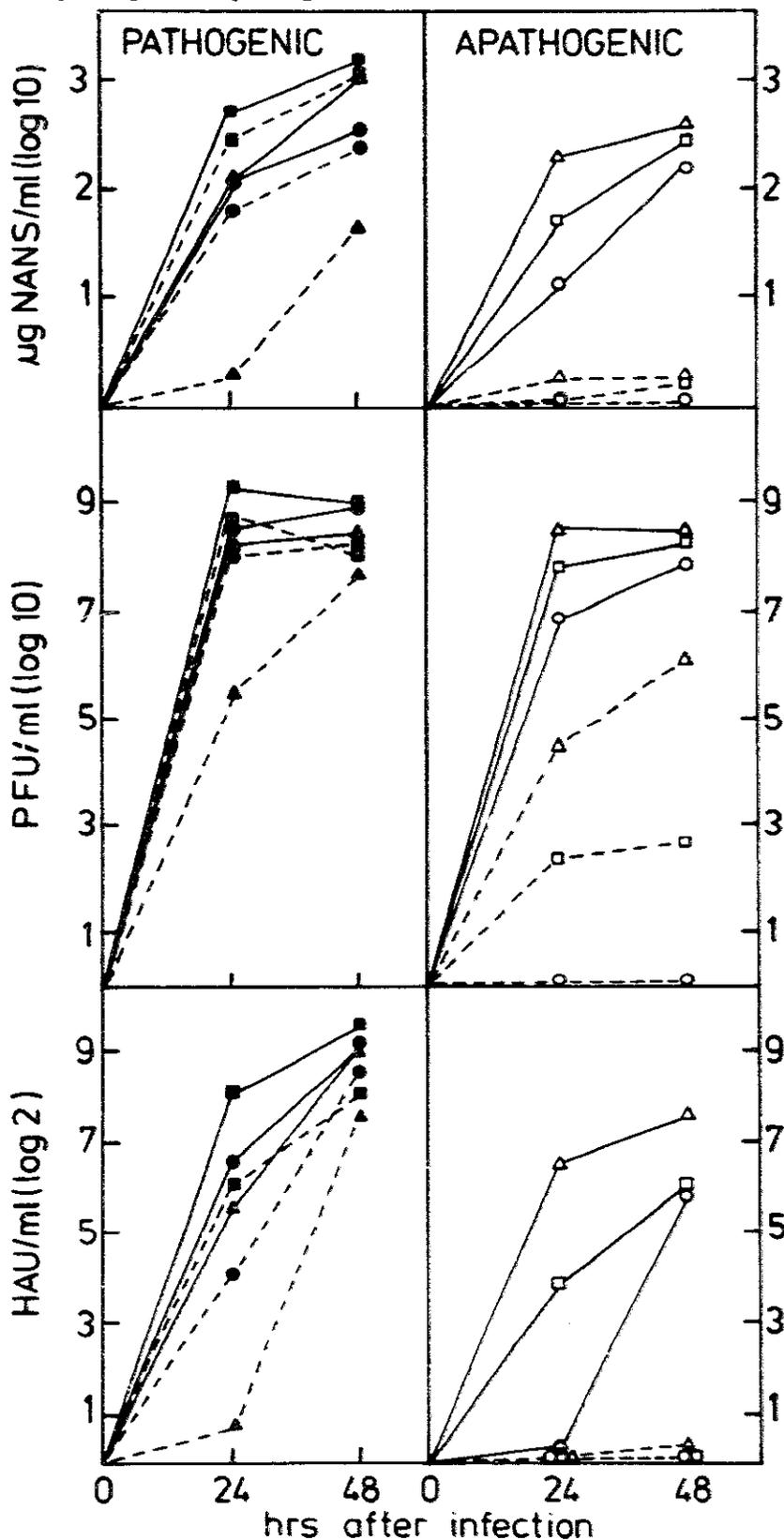


Figure 6

Multiplication of pathogenic and non-pathogenic influenza virus recombinants obtained *in vitro* as a function of temperature.

Infected cultures were kept at 37°C (solid lines) or 41°C (dotted lines). After the time indicated neuraminidase activity (upper row), plaque forming units (middle row), and hemagglutinating units (lower row) were determined. the symbols o, ☆, □ or •, \$, ■ represent different recombinants between fowl plague virus (Hav1N1) and A/turkey/England/63 (Hav1Nav3) non-pathogenic or pathogenic for chicken.



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**APPLICATION OF RECENTLY DEVELOPED TECHNIQUES
TO DETERMINE THE ORIGIN OF INFLUENZA A VIRUSES
APPEARING IN AVIAN AND MAMMALIAN SPECIES
AND TO DEVELOP POTENT AVIAN INFLUENZA VACCINES**

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ABSTRACT

Influenza A viruses in domestic poultry represent a costly disease problem for commercial producers, especially in turkeys and ducks. Unfortunately, we do not know the source of these viruses or the antigenic and genetic composition of the viruses that cause problems. The viruses are either maintained in domestic flocks or are introduced from outside sources, such as feral birds. Current studies, in conjunction with the University of Minnesota, have demonstrated the presence of antigenically indistinguishable viruses in turkeys, feral ducks and sentinel ducks in the same area in 1980. To establish that these viruses originated from the same source, however, it is necessary to examine all eight genes and gene products of these viruses in greater detail. Studies on these viruses are still in the preliminary stages, so the answer is not yet available; however, the successful application of the techniques being used can be demonstrated by results from studies on influenza A viruses from seals. In this case, a virus antigenically related to fowl plague virus was isolated from dead harbor seals (*Phoca vitulina*) in the U.S. Antigenic analyses using both heterogeneous antisera and monoclonal antibodies showed that the surface antigens and the nucleoprotein of the seal virus were most closely related to recent avian isolates. Studies on the RNAs, using competitive hybridization assays, showed that all eight RNA segments were most closely related to the RNAs from avian viruses. These studies indicated that the seal virus originated from avian viruses. Similar approaches are being used for detecting the origin of viruses in the domestic birds — i.e., detailed antigenic and genetic comparisons of isolates from other species, both avian and mammalian, with those appearing in these birds. Information from these studies should enable us to answer the questions as to whether feral birds, such as ducks, are the source of viruses appearing in domestic birds and whether a virus with a particular gene constellation is responsible for the annual outbreaks of disease.

Since avian influenza vaccines are currently being used, available techniques for the development of effective vaccines, such as highgrowing recombinants and quantitation of antigen content, are proposed for preparation of vaccines with high potency.

INTRODUCTION

The history of avian influenza began with the isolation of virus from sick domestic birds almost a century ago (Easterday, 1975). Fowl plague, a rapidly fatal infection for domestic birds, was first described in Italy in 1878 and, in 1955, Schafer showed that the viral agent responsible for fowl plague was an influenza A virus. Shortly thereafter, in 1956, influenza A viruses were isolated from sick ducks in Czechoslovakia and England. Since then, influenza A viruses have been detected during disease outbreaks in many different domestic species, including chickens, turkeys, ducks, quail, pheasant and geese in many areas of the world. Influenza infection in these birds may be asymptomatic, produce mild disease symptoms, or may kill the birds within 48 hours. This wide range of pathogenicity depends both on the virus and the host.

Currently, influenza is a frequent problem in turkeys, particularly in North America. The first isolate from turkeys, A/Ty/Eng/63 (Hav1Nav2), was in 1963 during a severe disease outbreak in England (Wells, 1963). Within the year, viruses were isolated from turkeys in Canada (Lang *et al.*, 1965) and the U.S. (Bankowski *et al.*, 1964). Since then, strains representing various antigenic subtypes have been detected in turkey flocks in many areas; most recently from turkeys in Israel (Lipkind *et al.*, 1979), England (Alexander *et al.*, 1979), and the U.S. (Newman *et al.*, 1981). Within the last three years in the U.S., influenza in turkeys has been a substantial problem and the severity of this problem has stimulated the use of polyvalent vaccines, the efficacy of which is still being evaluated.

These findings indicate that turkeys are currently involved in the circulation of influenza A viruses in nature; however, it is not clear whether the viruses are maintained in the turkeys themselves or introduced from an outside source, such as migratory birds. There is substantial circumstantial evidence to support the possibility that wild birds, as ducks, represent the source of virus appearing in domestic birds (Easterday, 1975; Hinshaw *et al.*, 1980a). These data include the detection of antigenically related viruses in both feral and domestic birds, perpetual circulation of diverse antigenic subtypes in ducks and the occurrence of disease outbreaks in the fall during migration of waterfowl. Current collaborative efforts between our laboratory and the University of Minnesota (Drs. J. Newman, B. Pomeroy, D. Karunakaran, D. Halvorson) are aimed at evaluating this point by detailed antigenic and genetic characterization of influenza A viruses from domestic turkeys, feral ducks and sentinel ducks in the same geographical area within the same year. In this study, antigenically indistinguishable viruses have been isolated from these different groups in 1979-80; however, this does not prove that the viruses share the same origin. It should be recognized that antigenic analyses, even with monoclonal antibodies which are currently being used to characterize avian viruses, involve only two (the hemagglutinin and neuraminidase) of the eight gene products of influenza A viruses. It has been shown that the RNAs of antigenically in-

distinguishable avian viruses may be quite different (Sriram *et al.*, 1980); thus, to determine if viruses originate from the same source, it is necessary to compare not only the surface antigens but the entire genomes of the viruses. Recent advances in genetic analyses provide the opportunity for such comparisons.

In view of the current problem with influenza in turkeys, the following sections will describe techniques which can be used to address two major questions:

1. What is the source of the viruses appearing in domestic birds?
2. How can an effective vaccine be developed?

Although the studies on the viruses in turkeys are in the preliminary stages, the techniques required to answer the above questions will be described and illustrated in the following sections by studies on other viruses.

A. Recent Advances in Antigenic and Genetic Analysis of Influenza A Viruses.

The techniques currently used for detailed antigenic and genetic analyses of influenza A viruses are discussed in the following section. Examples of the application of such techniques, particularly regarding avian strains, are described.

Antigenic Analyses.

Hemagglutinin and Neuraminidase: The current classification of the surface antigens of influenza A viruses includes 12 hemagglutinin and 9 neuraminidase subtypes based on antigenic cross-reactions and genetic homologies (WHO, 1980). All known antigenic subtypes and multiple combinations of these antigens are represented on viruses circulating in avian species, as demonstrated in Table 1. Classification of influenza A isolates can be accomplished by hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) assays with heterologous or preferably with monospecific antisera, e.g., antisera prepared to the isolated hemagglutinin or neuraminidase antigens (Webster *et al.*, 1974). As shown in Table 2, influenza virus within the same antigenic subtype, e.g., Hav4N2, have been isolated from turkeys, sentinel ducks and feral ducks in the fall of 1980 during collaborative studies with Dr. Bruce Turner in Canada and Drs. Newman, Karunakaran, Halvorson and Pomeroy; however, antigenic relatedness does not mean that these viruses are the same.

The above classification, however, was not designed to discriminate between influenza viruses within a subtype. Antigenic variations (drift) occur in all subtypes, including avian strains (Hinshaw *et al.*, 1980a). The availability of monoclonal antibodies (Kohler *et al.*, 1976), which recognize single antigenic determinants, now permits detailed antigenic mapping of viruses within a subtype. Their application in studying antigenic variation in human influenza viruses has recently been established (Webster *et al.*, 1980). Monoclonal antibodies are now proving useful for evaluating

determinants shared by animal and human strains. Studies (Hinshaw *et al.*, 1981b) with monoclonal antibodies to H2 viruses shared antigenic determinants with the human A/Japan/305/57 strain, although none of the avian viruses were identical to the human strain. Although the avian strains were indistinguishable in HI tests with heterologous antisera, these monoclonal antibodies demonstrated variation in the avian strains. For example, two isolates from feral ducks, Dk/Ontario/77 (H2N1) and Dk/Alberta/77 (H2Nav2), shared 4 of 6 determinants with Japan/305/57, whereas, A/Dk/GDR/72 (H2Nav6) did not have any of the same six determinants. The reactivity patterns indicated that the H2 hemagglutinins of avian and human strains were very closely related. Although these detailed antigenic analyses show that none of the H2 avian influenza viruses had a hemagglutinin identical to the human strain in 1957, H2 viruses continue to be isolated from birds and an identical hemagglutinin may well be detected in the future. These findings indicate that avian and human H2 influenza viruses share antigenically identical determinants, suggesting that the genes in human and avian strains had a common origin and that these genes are being maintained in the viruses in the avian population. Monoclonal antibodies can more precisely define the antigenic relatedness between strains and, in combination with genetic studies, suggest whether viruses share the same source.

Internal Proteins.

Influenza A viruses share closely related internal antigens, i.e., the matrix and nucleoprotein (NP); however, recent studies show that the NP does show antigenic variation (Schild *et al.*, 1976; van Wyke *et al.*, 1980). Studies using monoclonal antibodies to the NP of WSN/33 (HON1) indicated that 5 different antigenic determinants (epitopes) could be distinguished and that the NP of various human and animal strains could be divided into 6 groups, based on their reactivity patterns in enzyme-linked immunosorbent assays (ELISA) with these monoclonal antibodies. The NPs of all of the avian strains examined to date share each of the 5 antigenic determinants with A/WSN/33. In contrast, the human strains showed more extensive variation in these determinants. Why the NPs of animal strains are conserved in these epitopes is not clear and further analyses with monoclonal antibodies prepared to avian influenza virus NPs are indicated since RNA analyses of the NP gene of avian strains (Sriram *et al.*, 1980) suggest considerably heterogeneity. As shown from those studies, monoclonal antibodies permit analysis of antigenic variation in the internal, as well as the external, antigens of influenza viruses.

Techniques for Genetic Analyses.

Current techniques for evaluating genetic relatedness between different influenza A viruses include various approaches, depending on the level of sensitivity required.

Comparison of RNA migration patterns of different viruses after separation by polyacrylamide gel electrophoresis indicates whether the

RNAs of various strains are distinguishable, suggesting genetic differences. For example, a series of antigenically indistinguishable isolates from ducks were examined in this way (Sriram *et al.*, 1980). The RNA migration patterns of Hav7Neq2 viruses isolated from Canadian ducks within one week showed that each virus could be distinguished from the other when the RNA migrations were compared, illustrating the genetic heterogeneity of these viruses. These techniques were also used to demonstrate genetic reassortment between avian strains which occur readily in the intestinal tract of mixedly infected ducks (Hinshaw *et al.*, 1980b).

Hybridization analyses are used to evaluate the degree of genetic homology between genes from different viruses. For example, the duck viruses mentioned above were examined by competitive hybridization analyses to determine if the differences in RNA migration patterns truly reflected genetic diversity (Sriram *et al.*, 1980). The hybridization results confirmed the migration differences and showed that heterogeneity occurred in all 8 genes of these avian isolates, demonstrating that these antigenically related strains possessed different internal genes. Earlier hybridization analyses by Scholtissek *et al.* (1978) have clearly demonstrated that the human pandemic strains, A/Hong Kong/1/63 (H3N2), contained seven of the eight genes from the preceding human strain of A/Singapore/1/57 (H2N2); however, gene 4, coding for the hemagglutinin, had little homology with the human H2N2 viruses. This gene was more closely related to the HA gene from Duck/Ukraine/1/63 (Hav7Neq2) and A/Equine/Miami/1/63 (Heq2Neq2). These studies provide additional evidence for relationships between influenza A viruses, indicating that viruses in lower mammals and birds may serve as a source of genes appearing in new human pandemic strains. Hybridization analyses provide the opportunity to determine whether genes in one virus are shared with other strains appearing in different species.

Oligonucleotide mapping can detect a small number of nucleotide changes in the genes of closely related viruses. Desselberger *et al.* (1978) examined isolates from ducks in France for evidence of genetic exchange. In this case, they showed, by oligonucleotide mapping, that two isolates (Hav6N2 and Hav6Nav4) from naturally infected ducks possessed hemagglutinin and matrix genes which were almost identical. They concluded that these genes originated from the same virus and were introduced into these two antigenically different strains by genetic reassortment in a mixedly infected duck, suggesting that oligonucleotide mapping can determine whether viruses share identical genes.

Recent advances in nucleotide sequencing now permit comparisons of the actual nucleotide sequences of different strains. There is an increasing amount of sequencing data available on both avian and mammalian viruses. With regard to avian viruses, A. Porter (1979) has recently described the complete nucleotide sequence of the avian fowl plague virus HA gene from cloned DNA. The availability of sequencing data will

enable very detailed genetic comparisons between viruses, a topic which is too extensive to be covered in detail in this paper.

Application of Recent Techniques to Determine the Origin of An Influenza Isolate.

The following study on an influenza A virus isolated from sick harbor seals (*Phoca vitulina*) demonstrates the application of recent techniques, as described above, to determine the origin of a virus.

In December, 1979, an unusually large number of dead or moribund harbor seals were found on the beaches of Cape Cod, Massachusetts, USA. Postmortem examination of these animals revealed severe lung consolidation typical of primary viral pneumonia (Geraci *et al.*, 1981). Analysis of tissue samples from these animals revealed high titers of influenza virus in the lungs (10^6 to 10^7 EID₅₀/gm) and lower titers in the brain ($10^{1.5}$ to $10^{2.5}$ EID₅₀/gm). All virus isolates were of the serotype, Hav1Neq1 (H7N7) (Lang *et al.*, 1981; Webster *et al.*, 1981). The prototype virus with this antigenic combination is A/Fowl Plague/Dutch/27, a strain that had previously been associated with severe disease outbreaks in domestic fowl. The host range of this virus was tested by experimental infection of several species of birds and mammals (Webster *et al.*, 1981). Replication in birds was sporadic and limited to the respiratory tract. There were no clinical symptoms and no intestinal replication of the virus as is seen with many avian influenza virus strains. In mammals, the virus replicated in the respiratory tract of swine, cats, ferrets, guinea pigs and mice.

The above studies showed that 3 of the 7 structural proteins of the seal virus were antigenically similar to avian influenza viruses. The RNA segments of the seal influenza virus were, therefore, analyzed to determine the extent of genetic homology with influenza viruses from other species. Genetic homologies were measured by competitive RNA:RNA reassociation as described (Bean *et al.*, 1980). The RNA gene segments of A/Seal/Mass/1/80 were isolated by polyacrylamide gel electrophoresis, labelled with ¹²⁵Iodine, and annealed to RNA from seal virus or from other virus strains. Annealing reactions were run at 15° below the homologous melting temperature. This modest level of stringency was used to show overall levels of homology rather than small differences in base sequence which are amplified when the reaction is run at a higher temperature (Sriram *et al.*, 1980).

The influenza virus strains chosen for comparison with the genes of the seal virus are listed at the top of Figure 1. These include representatives of all of the human, equine and swine serotypes and a series of avian influenza isolates from several species representing all of the avian-hemagglutinin and neuraminidase subtypes.

RNA from each of these strains was used in competitive reassociation assays with individual labeled seal virus RNA segments coding for the nonsurface proteins. With all seal RNA segments, the most closely

related corresponding RNAs were found in various avian influenza strains. For example, with RNA segment 3 (Fig. 1), the most closely related strain was A/Gull/Md/5/77, while with RNA segment 5 (Fig. 2), the most closely related strain was A/duck/Alberta/60/76. None of the strains tested contained genes closely related to all of the seal RNAs and only one of the strains with a closely related gene (Ty/Oregon/71, gene 7) also had the appropriate hemagglutinin. This virus, A/Seal/Mass/1/80, provides the first evidence suggesting that an influenza strain deriving all of its genes from one or more avian influenza viruses can be associated with severe disease in a mammalian population.

B. Recent Advances in the Quality and Standardization of Inactivated Influenza Vaccines.

Since inactivated influenza vaccines are currently being used in turkeys in the U.S., it seems appropriate to consider the available techniques which can and should be used to produce effective vaccines. These improvements are primarily concerned with increasing the antigen content (potency) of a vaccine which is critical in producing a vaccine that can induce an adequate immune response to provide protection. A great deal of time and effort has been devoted to the development of good vaccines for human use, thus it is important to consider these techniques in the development of avian vaccines.

There have been several developments since 1968 which have greatly improved human vaccines (Schild *et al.*, 1976):

(1) *The use of high-yielding recombinants for vaccine production:* In this case, high yielding recombinant strains of an influenza isolate can be prepared by recombination of a new antigenic variant with a rapidly growing laboratory-adapted strain, such as A/Pr/8/34 (HON1) as described by Kilbourne (1968).

(2) *The use of rate zonal centrifugation techniques:* The use of these techniques has led to a considerable improvement in vaccine purity and potency.

(3) *The development of the single radial diffusion test* (Schild *et al.*, 1975) to assay the antigenic content of vaccines. In this assay, the antiserum to the HA of the vaccine strain is incorporated into an agarose gel and dilutions of test and reference viruses are placed into wells in the gel. Zones of antigen-antibody reactions develop and can be measured. In this assay, the quantity of HA is directly proportional to the area of the reaction zone and can be used to determine the quantity of HA in a vaccine. This approach has several advantages over standardization by hemagglutinin (chick cell agglutinin) levels and has been used to develop international standards for human influenza vaccines.

Preparation of High-Growing Recombinants for Avian Influenza Vaccines.

In developing vaccines for use in turkeys, it was noted that initial isolates from the turkeys produced low and erratic hemagglutinin yields

in eggs (HA yield of 1:20 to 1:160) which were unsuitable for vaccine use. Thus, our laboratory was asked to prepare high-growing recombinants for use in experimental vaccines. The human strain, A/Pr/8/34 (Mt. Sinai) (HON1), which produces hemagglutinin yields of 1:1280 to 1:2560, was used as the gene donor of the high-growth potential, as previously described (Kilbourn *et al.*, 1968). With two turkey strains (Hav6N1 [H6N1] and Hav1Nav2 [H7N3] isolates), high growing recombinants, which were antigenically indistinguishable from the parental turkey virus, produced HA yields of 1:640-1:1280, greater than 10-fold higher than the original viruses. Repeated efforts to increase the yields of an Hav4Neq2 [H4N2] isolate resulted in only a 2-4 fold increase, i.e., HA yields of 1:160. In cases in which the high-growth potential is not obtained, the use of an antigenically related virus which already has that potential should be considered. As shown with 2 of the above viruses, this technique substantially increased the hemagglutinin yields from eggs. At this time, however, this technique has not been widely applied for other viruses used in avian influenza vaccines. Thus, the antigenic content of these vaccines depends on viruses which may or may not grow to high titers in eggs.

It must be recognized that even with high-growing recombinants, the yields of different harvests during vaccine production may vary due to many different factors. Thus, once a vaccine is produced, it is critical to determine the antigen content by an accurate, standardized method to ensure that minimum antigen content is maintained. The techniques for accurate quantitation of vaccine potency as SRD (described above) have not been applied in avian vaccines. It is clear that additional studies are needed to evaluate the potency of the vaccines being used so that the minimal antigen content required for protection of the turkeys can be ascertained.

CONCLUSIONS

The above studies indicate that recent advances in the antigenic and genetic analyses of influenza A viruses can be applied to address questions of importance to commercial poultry producers, i.e., determining the source of the viruses and developing potent vaccines.

If the viruses appearing in turkeys during disease outbreaks are introduced by feral birds, as ducks, an aspect currently being evaluated, the producer may consider containment as the most effective means for preventing introduction of the viruses. If this is not feasible or if the viruses are maintained in the turkeys themselves, the producers may have to rely on preventing disease problems by vaccination.

There are many questions on avian influenza A viruses which remain to be answered, yet the recent advances in antigenic and genetic analyses should prove extremely helpful in answering these questions. Such studies should enable us to better understand the biology of influenza A viruses circulating in birds and, when necessary, to prevent such infec-

tion. The techniques to develop effective avian vaccines are available; yet, their application in avian vaccines has received little attention thus far. Since avian vaccines are relatively new, it is important to apply the available techniques now. By these methods, effective, potent avian vaccines can be produced.

The advances in influenza research can well be applied to the solution of practical problems with avian influenza which confront the poultry industry today.

TABLE 1

HEMAGGLUTININ AND NEURAMINIDASE SUBTYPES OF INFLUENZA A VIRUSES
ISOLATED FROM BIRDS^a

Hemagglutinin Subtype		Isolates from birds ^c
H0, H1, Hsw1 ^a	(H1) ^b	Dk/A1b/35/76
H2	(H2)	Dk/GDR/72
H3, Hav7, Heq2	(H3)	Dk/Ukr/1/63
Heq1, Hav1	(H7)	Ck/Brescia/02
Hav2	(H10)	Ck/Ger/N/49
Hav3	(H11)	Dk/Eng/56
Hav4	(H4)	Dk/Cz/56
Hav5	(H5)	Tern/S.A./61
Hav6	(H6)	Ty/Mass/65
Hav8	(H8)	Ty/Ont/6118/68
Hav9	(H9)	Ty/Wis/66
Hav10	(H12)	Dk/A1b/60/76
Neuraminidase Subtype		
N1	(N1)	Ck/Scot/59
N2	(N2)	Ty/Mass/65
Neq1	(N7)	Ck/Dutch/27
Neq2	(N8)	Dk/Ukr/1/63
Nav1	(N6)	Dk/Cz/56
Nav2-3	(N3)	Tern/S.A./61
Nav4	(N4)	Ty/Ont/6118/68
Nav5	(N5)	Shearwater/Aust/1/72
Nav6	(N9)	Dk/Mem/546/74

^aCurrent subtype designation. (WHO, 1971).

^bProposed subtype designation. (WHO, 1980).

^cThe earliest recorded viruses with the designated subtypes isolated from birds are presented. (Hinshaw *et al.*, 1981a).

TABLE 2

ANTIGENICALLY RELATED INFLUENZA VIRUSES ISOLATED
FROM FERAL DUCKS, SENTINEL DUCKS
AND TURKEYS FROM SEPTEMBER - DECEMBER, 1980^a

Antigenic Subtype	Feral Ducks in		Sentinel Ducks in	Turkeys in
	Canada ^b	Minnesota ^c	Minnesota ^d	Minnesota ^e
Hav2Neq1			+	+
Hav3Nav6	+		+	
Hav4N2	+		+	+
Hav4Neq2		+	+	+
Hav4Nav1	+		+	
Hav5N2	+		+	
Hav6Nav1			+	+
Hav7N2	+	+	+	
Hav7Neq2	+	+	+	

^a Viruses were isolated in embryonated chicken eggs and classified in HI and NI tests with monospecific antisera, as previously described (Palmer et al., 1975; Aymard-Henry et al., 1973).

^b Isolates from live ducks during banding studies in Alberta, Canada, by Dr. Bruce Turner, as previously described (Hinshaw et al., 1980a).

^c Isolates from hunter-killed ducks in Minnesota.

^d Isolates obtained from sentinel ducks placed on lakes in August, 1980, adjacent to turkey farms in Minnesota by Drs. Newman, Halvorson, Karunakaran, Pomeroy at the University of Minnesota.

^e Isolates obtained from turkeys in Minnesota by the above investigators at the University of Minnesota.

Figure 1.

Competitive hybridization analysis of RNA gene segments of A/Seal/Mass/1/80 with influenza virus strains of avian and mammalian origin. Labeled RNA segments of A/Seal/Mass/1/80 were prepared as described (Bean *et al.*, 1980) and annealed with homologous complementary RNA in the presence of increasing concentrations of RNA from the virus strains listed at the top of the figure. RNAs 1, 2 and 3 code for the 3 polymerase proteins.

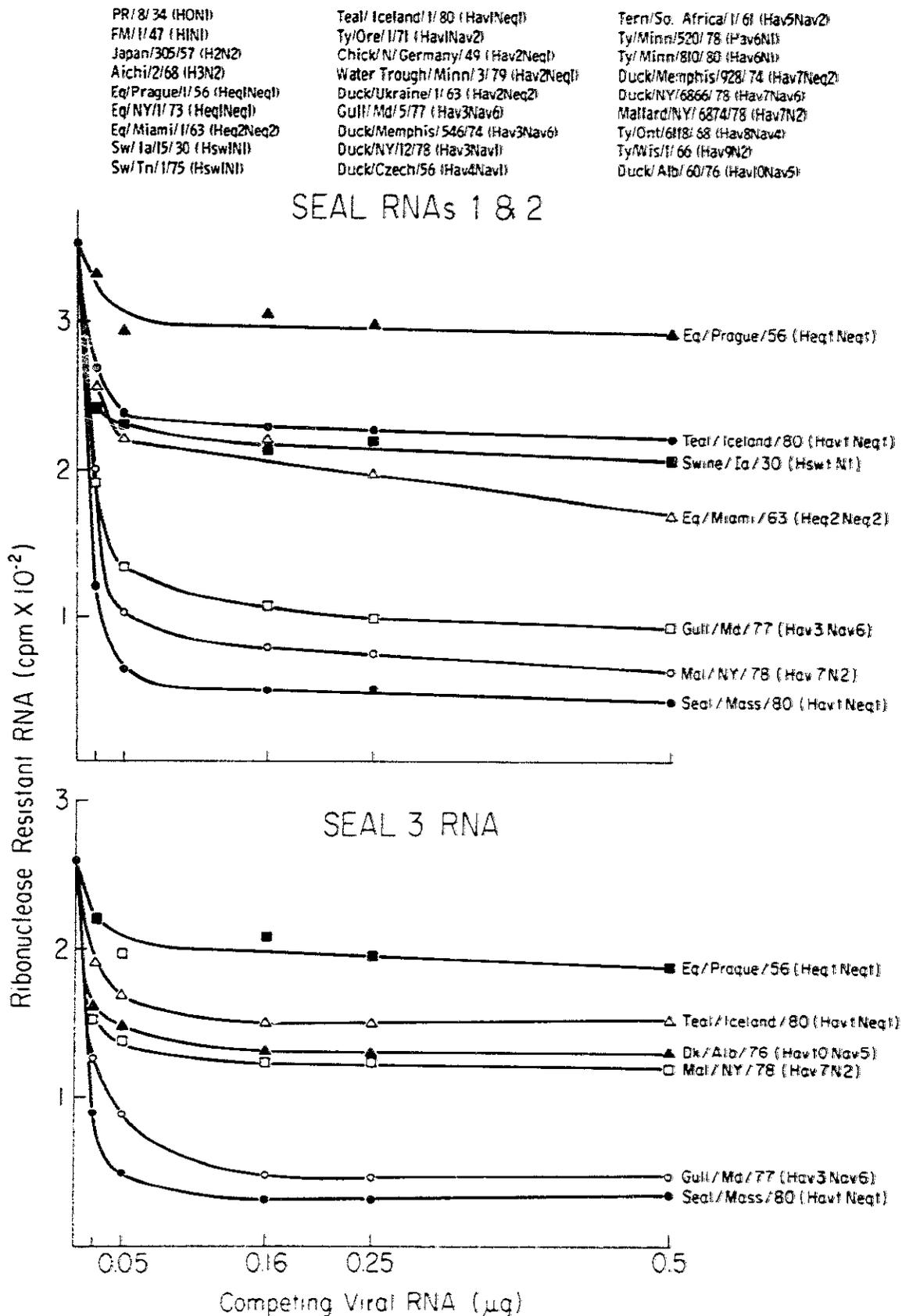
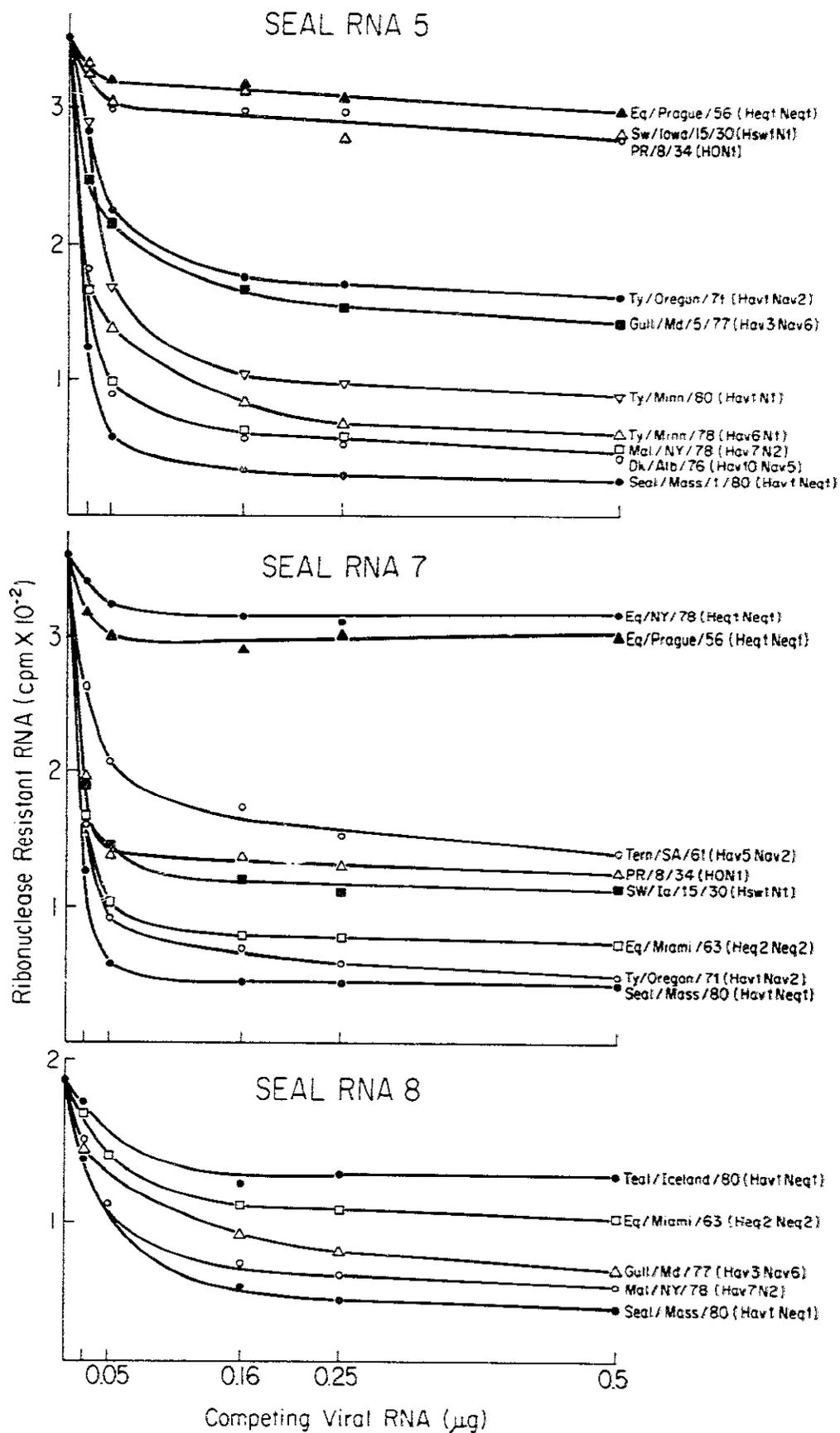


Figure 2.

Competitive hybridization of RNAs 5, 7 and 8 of the seal virus. These RNAs code for the nucleoprotein, matrix and nonstructural protein, respectively. Experimental details are given in the legend to Figure 1.



DR. LANG: In matters of vaccination, I would like to give you an opinion as a veterinarian. Another parameter has to be added in the evaluation of the vaccine protection and this is the duration of immunity induced by a vaccine which is very rarely given in immunization studies in influenza in all species. And the second parameter which has to be specified is which species was the virus derived. It is not adequate to develop a vaccine just in chickens and then use this in both chickens and turkeys. The turkeys are not as well immunizable as the chicken is. The immunity is very short. Also I would like to comment on this whole area of information about vaccination against fowl plague. During the war years, the U.S. Government did extensive studies and there were many studies in the countries of Europe on immunization for fowl plague; and so far we have not been able to accept the vaccination program for the control of fowl plague.

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PLAQUE-FORMING ABILITY IN MDCK CELLS AND STRUCTURE OF THE HAEMAGGLUTININ OF INFLUENZA A VIRUSES WHICH DIFFER IN VIRULENCE FOR CHICKENS

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INTRODUCTION

In influenza A viruses the haemagglutinin spike consists of a trimer of three identical glycopolypeptides with an apparent molecular weight of 75,000 - 80,000 (HA polypeptide) or as a disulphide-bound complex of smaller glycopolypeptides of about 50,000 (HA₁) and 30,000 (HA₂) produced as a result of proteolytic cleavage of the monomer (Klenk *et al.*, 1977). Such cleavage is extremely important, as virus particles assembled with uncleaved HA are non-infectious, although infectivity may be restored by protease treatment *in vitro* (Klenk *et al.*, 1975). All influenza viruses have cleaved HA polypeptide when grown in chick embryos or isolated chorioallantoic membrane cells, but in other cell systems, such as Madin Darby Canine Kidney (MDCK) cells, not all viruses are produced with cleaved HA (Rott, 1979).

Plaque formation in cell cultures is dependent on the release of infectious particles from the originally infected cell and in influenza virus infections it is related to the nature of the HA polypeptide. So that, in cell systems such as MDCK cells, viruses that are produced with a cleaved HA will produce plaques under normal conditions whereas those viruses produced without a cleaved HA will only form plaques in the presence of a protease such as trypsin (Klenk *et al.*, 1975, 1977). It has also been demonstrated that the pathogenicity of influenza A viruses for chickens shows a strict correlation to cleavability of the HA and plaque formation in a wide range of cell types (Bosch *et al.*, 1979). It is considered that only viruses produced with cleaved HA in a broad range of cell types are able to spread to and infect vital tissues and organs, while other viruses are restricted to replication in less important sites (Bosch *et al.*, 1979; Rott, 1980). This has been confirmed, to some extent, by the demonstration that after infection of the chorionic layer of cells in chick embryos, pathogenic viruses were able to spread to and replicate in all layers of the chorioallantoic membrane, while nonpathogenic strains did not spread beyond the site of inoculation (Rott *et al.*, 1980).

Studies aimed at assessing the virulence of influenza viruses for chickens have produced results that are in agreement with the concept that pathogenicity is chiefly governed by the cleavability of the HA polypeptide in that viruses have tended to show one extreme or the other in terms of virulence for chickens (Allan *et al.*, 1977, Ogawa *et al.*, 1980).

However, there have been some reports of viruses showing intermediate levels of virulence for chickens in laboratory studies (Allan *et al.*, 1977; Alexander *et al.*, 1978; Alexander & Spackman, 1981; Wetsbury *et al.*, 1979). In the present study influenza isolates, selected to show a full spectrum of virulence, have been examined in MDCK cells for infectivity, plaque-forming ability and the structure of the HA polypeptide.

MATERIALS AND METHODS

Viruses

The viruses used in the present study are listed in Table 1. Each virus had been, at sometime, cloned by two passages to limit dilution in 9-10-day-old embryonated fowls' eggs. Chick embryo grown virus was produced by inoculating about 10^3 EID₅₀ into the allantoic cavity of 9-10-day-old embryonated fowls' eggs.

Cell culture

MDCK cells in 5cm diameter plastic Petri dishes were used for plaque assays. Cell cultures were washed well with phosphate buffered saline pH 7.4 to remove serum before the addition of virus. Overlay medium contained no serum but, when applicable, 10ug/ml trypsin was added. Plaques were measured and counted 72 hours after infection. Viruses for glycopolypeptide analysis were grown in MDCK cell cultures in 175 cm² flasks which were infected with about one EID₅₀/cell.

Glycopolypeptide analysis

Viruses were purified by sucrose density gradient centrifugation and subjected to polyacrylamide gel electrophoresis (PAGE) on 13% acrylamide gels in the presence of 1% sodium dodecylsulphate (SDS) and 2% dithiothreitol as described (Alexander & Collins, 1981). Glycopolypeptides were visualized by staining with Schiff's reagent.

Pathogenicity

Where necessary, infectious allantoic fluid was diluted with PBS so that six-week-old chickens were each infected with about 10^6 EID₅₀ of virus in 0.1ml and the intravenous pathogenicity indices (IVPI) calculated as described (Allan *et al.*, 1977).

RESULTS

Pathogenicity

The pathogenicity of the six viruses was estimated by calculating IVPIs in chickens after administration of approximately the same infectious dose for each virus. The values obtained indicated a broad spectrum of virulence for the six viruses (Table 1), ranging from all birds dead within 24 hours for A/chicken/Germany/34 (ck/Germ/34) to a complete absence of clinical signs with A/parrot/Northern Ireland/73 (pt/N.I./73). All birds infected with A/chicken/Australia/75 (ck/Aust/75) died within the 10-day observation period, while 9/10 birds infected with A/turkey/England/384/79 (ty/Eng/384/79), 6/10 birds infected with A/turkey/England/69

(ty/Eng/69) and 3/10 birds infected with A/parakeet/England/138/75 (pkt/Eng/138/75) died.

Plaque formation

All six viruses showed some evidence of plaque formation in the absence of trypsin in the overlay. Plaque morphology varied considerably, from large well-defined plaques produced by ck/Germ/34 to tiny, poorly-defined plaques produced by pt/N.I./73. The six viruses fell into three groups on the basis of plaque size in the absence of trypsin: 1) ck/Germ/34 with plaques about 2mm in diameter 2) ck/Aust/75, ty/Eng/384/79 and pkt/Eng/138/75 with plaques about 1 mm in diameter 3) ty/Eng/69 and pt/N.I./73 with plaques about 0.5 mm in diameter (Table 2).

The presence of trypsin in the overlay medium caused little or no enhancement of plaque size for ck/Germ/34 and ck/Aust/75. However, a three-fold enhancement was seen for ty/Eng/69, ty/Eng/384/79 and pkt/Eng/138/75 while plaques formed by pt/N.I./75 showed a six-fold increase in size (Table 2).

Titration of egg-grown virus in MDCK cells in the presence and absence of trypsin in the overlay medium enabled similar groupings of the six viruses to be made. Little or no increase in titres were obtained for ck/Germ/34 or ck/Aust/75 by the incorporation of trypsin into the overlay medium. In contrast, pt/N.I./73 showed a 1500-fold increase in titre; while the other three viruses showed an intermediate 25-35-fold enhancement of titres (Table 3).

Glycopolypeptides

The six viruses were grown in chick embryos and MDCK cells in the absence of trypsin, purified by sucrose density gradient centrifugation and subjected to SDS-PAGE. The glycopolypeptides were detected by staining with Schiff's reagent and the apparent molecular weights estimated (Table 4). Four glycopolypeptides may be detected in gels of influenza viruses:— HA, its cleavage products HA₁ and HA₂ and the neuraminidase polypeptide (N). All four were seen in gels of ck/Germ/34 and the estimated molecular weights of 73/76,000 (HA), 57/58,000 (HA₁), 48,000 (N) and 30,000 (HA₂) are comparable to those reported by Bosch *et al.* (1979) for this virus.

In gels of ty/Eng/384/79 and pt/N.I./73 the N polypeptide appeared to migrate to a position close to the HA polypeptide as close examination of the gels revealed a double band at the position of the highest molecular weight polypeptide. The differences in the glycopolypeptide profiles of pkt/Eng/138/75 and ty/Eng/69 compared to the other viruses could be explained if the HA₁ and N polypeptides of these two viruses migrated to the same position, but there was no evidence of this.

The overall results indicated that all six viruses grown in embryonated fowls' eggs had a cleaved HA polypeptide but that only ck/Germ/34 and

ck/Aust/75 had a cleaved HA polypeptide when the viruses were grown in MDCK cells in the absence of trypsin.

DISCUSSION

Bosch *et al.*, (1979) established that the single most important factor governing the pathogenicity of influenza A viruses for chickens is the property of the HA polypeptide to be cleaved in a wide range of cell types. The results of the present study are in agreement with this finding as the two most virulent viruses for chickens, ck/Germ/34 and ck/Aust/75, were the only two to show cleavage of the HA polypeptide when grown in MDCK cells. Similarly, these two viruses failed to show enhancement of titre or plaque size as a result of addition of trypsin to the cell culture medium. However, ck/Germ/34 was measureably more virulent for six-week-old chickens than ck/Aust/75. This may be related to the difference seen in the size of the plaques formed by these two viruses in MDCK cells. Plaque formation over a specified time can be regarded as a measurement of the speed of replication of the virus. The smaller plaques seen with ck/Aust/75 virus may, therefore, indicate a longer replicative cycle and this, in turn, may mean that the virus would take longer to cause disease and death *in vivo*.

There is no immediately obvious explanation for the differences seen between pt/N.I./73 and the other viruses which were produced in MDCK cells with an uncleaved HA polypeptide. Low levels of infectious progeny produced in MDCK cells by viruses which normally have uncleaved HA polypeptide have been recorded (Rott, 1979) and this may account for the plaques formed by similar viruses in the present study. It is possible that such infectious particles are produced with a greater frequency for ty/Eng/384/79, ty/Eng/69 and pkt/Eng/138/75 than with pt/N.I./73 and this may offer some explanation for the greater infectivity in MDCK cells in the absence of trypsin and the higher virulence. However, differences in infectivity and plaque size were seen amongst these three viruses that do not relate to the differences in virulence and are probably indicative of the complexities of the properties that may be important in determining minor variations in virus virulence.

In conclusion, the results obtained in the present study are in agreement with those of Bosch *et al.* (1979), who concluded that the single most important factor in determining the pathogenicity of an influenza virus for chickens is the structure of the HA polypeptide, but also suggest that the rate of replication and the proportion of infectious particles produced may play a role in determining the level of pathogenicity of a virus.

Table 1

Intravenous pathogenicity indices
of influenza A viruses used
in present study

Virus	IVPI ^a
A/chicken/Germany/34 (H7N1)	3.00
A/chicken/Australia/75 (H7N8)	1.74
A/turkey/England/384/79 (H10N4)	1.34
A/turkey/England/69 (H3N2)	0.89
A/parakeet/England/138/75 (H3N8)	0.53
A/parrot/N.Ireland/73 (H7N1)	0.00

a : Each virus was diluted to give approximately 10^6 EID₅₀ per 0.1ml which was injected intravenously into each of ten six-week-old chickens.

Table 2

Effect of trypsin (10 μ g/ml) in overlay on size of plaques formed in MDCK cells by influenza A viruses

Virus	Mean diameter of plaques (mm)		Ratio with/without
	without trypsin	with trypsin	
A/ck/Germ/34	2.0	2.2	1.1
A/ck/Aust/75	1.0	1.3	1.3
A/ty/Eng/384/79	0.9	2.9	3.2
A/ty/Eng/69	0.4	1.2	3.0
A/pkt/Eng/138/75	0.7	2.2	3.1
A/pt/N.I./75	0.5	2.9	5.8

Table 3

Effect of trypsin (10µg/ml) in overlay on plaque formation in MDCK cells

Virus	PFU ^a /ml without trypsin	PFU/ml with trypsin	Ratio with/without trypsin
A/ck/Germ/34	5.9×10^5	8.5×10^5	1.4
A/ck/Aust/75	3.3×10^6	3.8×10^6	1.2
A/ty/Eng/384/79	1.3×10^5	3.2×10^6	24.6
A/ty/Eng/69	1.1×10^6	4.0×10^7	36.4
A/pkt/Eng/138/75	5.6×10^5	2.0×10^7	35.7
A/pt/N.I./73	1.2×10^4	1.8×10^7	1500.0

a : PFU = plaque forming units

Table 4

Glycopolypeptides of influenza viruses grown in chick embryos and MDCK cells

Virus	Molecular weights of glycopolypeptides x 10 ⁻³							
	chick embryo grown				MDCK cell grown			
ck/Germ/34	73	57	48	29	76	58	48	30
ck/Aust/75	74	54	50	30	75	58	51	31
ty/Eng/384/79	73 ^a		51	31	76 ^a			
ty/Eng/69	75	58		27	81	62		
pkt/Eng/138/75	75	55		22	77	60		
pt/N.I./73	68 ^a		47	29	72 ^a			

a: These polypeptides appeared to run as double bands.

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AVIAN INFLUENZA DIAGNOSTIC PROCEDURES

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SUMMARY

Two different approaches to influenza diagnosis have been used at the National Veterinary Services Laboratories (NVSL). Specimens submitted from domestic birds were inoculated into embryonating chicken eggs by the allantoic route. At 4 days post-inoculation, the amnionic-allantoic fluid (AAF) was tested for hemagglutinating (HA) viruses. Any HA virus isolated was first screened on the immunodiffusion test for type A ribonucleoprotein antigen. The envelope antigens of the virus were then identified using first the neuraminidase-inhibition test to identify the neuraminidase and then the hemagglutination-inhibition (HI) test to identify the hemagglutinin using an antiserum produced against a virus with a different neuraminidase.

The second approach was used on submissions from import birds. Specimens were inoculated into embryonating chicken eggs which were then incubated for 5 days. The AAF from the eggs with dead embryos was tested for HA viruses. If none were detected, a second passage was made. The HA viruses were tested against Newcastle disease antiserum with the Newcastle disease HI test.

All the HA isolates from import and domestic birds were inoculated into 4- to 6-week old susceptible chickens and turkeys by the caudal thoracic air sac route. In addition, the influenza isolates made at NVSL from domestic birds were inoculated by the intranasal route into chickens and turkeys and the intravenous and intracerebral pathogenicity indexes of the isolates were also determined. Influenza A viral isolates from 2 of the 1,348 lots of import birds tested were pathogenic.

The following hemagglutinin-neuraminidase subtypes have been isolated at NVSL from domestic poultry: Hav2 Neq1, Hav6 N1, Hav4 Neq2, Hsw1 N1, Hav 6 N2 and Hav4 N1. Since the USDA import bird program started in 1973, approximately 6,000 HA viral isolates have been made. Of 413 selected isolates from 1979 and 1980 that were characterized, only 8 were influenza viruses.

INTRODUCTION

The laboratory diagnosis of influenza is based on the isolation and identification of the virus and/or detection of specific antibody against the virus. The basic isolation method for influenza viruses is the inoculation of embryonating chicken eggs. The first step in influenza virus characterization is to identify its ribonucleoprotein (RNP) using the immunodiffusion (ID) test. All influenza viruses that have been isolated from avian species have had type A RNP; therefore, the ID test is used as

a group specific test to identify avian influenza isolates. The envelope antigen subtyping of the virus is determined using the HI and neuraminidase inhibition (NI) tests.

Pathogenic and non-pathogenic influenza A viruses with serologically identical surface antigens have been isolated. This led to the suggestion by Beard and Easterday⁵ and later by Allan et al.² that the virulence of the isolate must also be determined. The method used is the inoculation of susceptible poultry, usually chickens and/or turkeys.

The details of the avian influenza diagnostic techniques used at the NVSL are described in this paper. All influenza A virus strains referred to in this paper are classified according to the 1971 nomenclature.¹² The nomenclature was recently changed.¹³ The new subtype designation for the influenza A viruses is provided (Table 1).

MATERIALS AND METHODS

Virus Isolation: Depending upon the source of the specimen, two different isolation procedures were used. The specimens from birds submitted for importation into the United States were tested for pathogenic embryo-lethal viruses. The procedure for collection and testing of samples has been described.⁸ Two changes were made since this publication. The sample collection was reduced to the first 15 days of the 30-day quarantine. Tissues were collected into brain-heart-infusion broth from 30 of the dead birds and cloacal swabs were collected from the remaining dead birds up to 150 per day. Swabs from five birds were pooled into one tube. The specimen fluid was mixed with antibiotics and 0.3 ml was inoculated into each of four 8- to 11-day embryonating chicken eggs by the allantoic route. Embryos that were alive after five days' incubation were considered negative and discarded. If an embryo died, the amniotic-allantoic fluid (AAF) was checked for HA viruses. If no hemagglutination was observed, a second passage was made.

The procedure for the isolation of influenza virus from domestic birds was to inoculate 0.3 ml of a 10% tissue suspension or medium from the swabs into four embryonating chicken eggs by the allantoic route. The AAF from all embryos that were alive at 4 days and all embryos that died was tested for hemagglutinating activity.

Antiserums: For virus identification, antiserums were produced against 43 different strains of virus. Antiserums were produced against 9 recombinant viruses which had 9 of the 10 described neuraminidase subtypes. A recombinant for Nav4 was not available. The recombinant viruses had Heq1 or H0 hemagglutinin. Antiserums were produced against at least 2 viruses with the same hemagglutinin, but with different neuraminidase antigens. One of the viruses for each hemagglutinin subtype was a recombinant with N1 or N2 neuraminidase. All the recombinant viruses and some of the prototype strains were supplied courtesy of V. S. Hinshaw, Department of Virology, St. Jude Children's Hospital, Memphis, Tennessee. The other prototype strains were supplied

courtesy of B. C. Easterday, University of Wisconsin, Madison, Wisconsin. The chickens were inoculated intravenously (IV) with 3 ml of AAF. The birds were exanguinated at 10 days post-inoculation if they had an HI antibody titer of 1:64 or an N1 titer of 1:10. If the titer was low, a second IV dose was administered and the birds exanguinated 10 days later.

Virus Characterization: All hemagglutinating viruses were tested by the Newcastle disease HI test using the previously described procedure.⁶ The isolates other than NDV from imported birds were tested for pathogenicity for chickens and turkeys. At least one isolate from each species in each submission was saved for later characterization.

The isolates from domestic birds and the selected isolates from import birds were tested for influenza type A RNP using the ID test. The positive control antigen was prepared from the chorio-allantoic membrane (CAM) of embryonating chicken eggs inoculated with a type A influenza virus using a method similar to that described by Beard.⁵ After freezing and thawing 3 times, the CAM was homogenized, frozen and thawed 3 more times and then homogenized again. The CAM suspension was then centrifuged and the supernate saved. Additional antigen was extracted from the sediment by treating overnight at 4 C with glycine-Sarkosyl^a buffer⁹ and then sonicated. The sediment was diluted, shaken vigorously, centrifuged and the supernate pooled with the original CAM supernate. The AAF from the embryonating eggs inoculated with influenza virus was acid precipitated following the previously described procedure.⁹ This acid precipitated AAF antigen was added to the CAM antigen. The antigen was inactivated with 0.1% beta propiolactone. The agar used for the ID test was 0.7% Oxoid L28^b in phosphate buffered saline (PBS) with 8% NaCl. The test was performed in 100 mm dishes containing 15 ml agar. The pattern was cut with a 7 well template with 6 wells evenly positioned around a center well. The wells were 5.2 mm in diameter and 2.4 mm apart. A positive antiserum was placed in the center well with positive control antigen and unknown antigen added to alternate wells around the center well. Three unknown antigens can be tested on each pattern. The unknown isolate was identified as influenza if it produced a line of identity with the positive control antigen. The unknown antigens were untreated AAF from inoculated eggs. If the titer of the unknown antigen was low, it was acid precipitated.⁹

The neuraminidase of the type A influenza virus was identified using a procedure modified by Van Deusen¹⁰ from a procedure described by Aymard-Henry, et al.³ The test was performed in white polystyrene microplates. An unknown antigen was identified by testing 0.025 ml of 1:12 dilution of the antigen against 0.025 ml of each of the standardized

^aGeigy Industrial Chemicals, Division of Geigy Chemical Corporation, Ardsiey, New York, 10502.

^bOxoid Limited, London SE19HF, England.

antiserums. The incubation time after adding fetuin was decreased from 18 hours to 3 hours and a 56 C water bath was used instead of a 100 C water bath. The plates were observed for an inhibition of the pink color reaction indicating the subtype of neuraminidase antigen of the unknown isolate. Extracting the reactants with butanol was not required.

After the neuraminidase subtype of the isolate was established, the hemagglutinin subtype was determined using the HI test as described in the Public Health Service Procedure⁹ or the Newcastle disease HI procedure.⁶ To avoid steric inhibition, the HI antiserums selected did not contain the same neuraminidase as the unknown isolate.

Pathogenicity Testing: Influenza isolates from domestic birds and the HA isolates from birds submitted for importation into the United States were inoculated into four 4- to 6-week old chickens and turkeys by the caudal thoracic air sac (CTAS) route. Up to 40 HA isolates from import birds were pooled into one group of birds. However, most groups received a single isolate or pools containing 2 to 10 isolates. The birds were inoculated with 0.2 ml of a 1:10 dilution of AAF using a 25 gauge 5/8 inch needle. The site for injection into the CTAS was ventral to the junction of sternal and vertebral portion of the last rib. The tip of the needle was pointed dorsally under the last rib. The inoculated turkeys and chickens were held in isolation cages for 10 days. The birds were observed for evidence of clinical disease. Necropsies were performed on all birds inoculated with HA isolates from pet birds between 1973 and 1975

Six different serotypes of influenza virus from domestic poultry were also pathotyped by inoculating chickens and turkeys intranasally. Also, the intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) were determined as described by Allan, et al.¹ The inoculum used was 0.5 ml of a 1:50 dilution of AAF for the IVPI and 0.05 ml of a 1:10 dilution for the ICPI. Further pathogenicity testing was performed on a Hav4 Neq 2 virus isolated from a flock of Alabama chickens.

Serologic Tests: Serums submitted for avian influenza tests were first screened by the ID test using the same procedure described for virus characterization except that the positive control antigen was added to the center well with the unknowns and positive control serum added to alternate peripheral wells. The neuraminidase (N) subtype of the positive serums is determined by the NI test using a procedure similar to that described for virus characterization. The serum was diluted 1:2 and heat inactivated to 56 C for 30 minutes. A 0.025 ml sample was tested against 0.025 ml of each of the standardized N antigens. The hemagglutinin was determined using either the Public Health Service⁹ or Newcastle HI⁶ procedure. The samples were not heat inactivated or treated with receptor destroying enzyme. Turkey serums were absorbed with chicken red blood cells prior to performing the HI test. This absorption was performed in microplate wells by placing 0.05 ml serum in 0.1 ml of PBS and

adding 0.05 ml of a 1:10 suspension of rooster red blood cells. The plate was shaken and allowed to stand for 30 minutes at room temperature. The resulting 1:4 dilution of the serum was used for the HI test. The serum samples were tested against each of the hemagglutinin subtypes using antigens that did not contain the N subtype of the serum.

RESULTS

Import Birds: Since the present USDA import bird program was started in October 1973, 1,348 lots of birds have been submitted for importation through the privately owned, USDA approved, quarantine facilities. The size of the lots ranged from 25-15,000 birds. Hemagglutinating viral isolates other than NDV were isolated from 338 of these lots. Between 28,000 and 40,000 specimens were submitted each year from 1977 through 1980. Hemagglutinating viruses were isolated from almost 50% of the lots in 1973 through 1974, but only 18 to 26% of the lots from 1977 to 1981. The number of velogenic viscerotropic NDV positive lots was also higher from 1973 to 1975. Of the 413 1979 and 1980 isolates saved for characterization, one subtype of influenza virus, Hav7 N1, was identified from 8 specimen from 3 lots of 149 tested. The isolates were from 5 parrots, 1 finch, 1 robin, and 1 mynah.

Hemagglutinating viruses pathogenic for chickens and turkeys that were not NDV were isolated from 2 lots of imported birds. These pathogenic isolates were from finches and mynah birds. The isolates caused death in chickens and turkeys inoculated and the virus was reisolated from the inoculated birds. Both of the lots were refused entry into the United States. The viruses were identified as Hav7 Neq2 and Hav4 Neq2. The isolates were not pathogenic for parakeets and conures. None of the other HA viral isolates were pathogenic for chickens or turkeys. Significant lesions were not observed in the clinically normal chickens and turkeys that were necropsied between 1973 and 1975; therefore, necropsy of healthy chickens and turkeys was discontinued in 1975.

Domestic Birds: Six subtypes of influenza A virus have been isolated and identified at NVSL (Table 2). Several of these subtypes have been isolated from a number of different flocks. During the 1978 outbreak of influenza A virus in Minnesota, Hav6 N1 was isolated from 72 turkey flocks and from one chicken flock which was in close proximity to infected turkeys. Subtypes Hav4 Neq2, Hav6 N2 and Hsw1 N1 were each isolated from 1 turkey flock during the same outbreak. The Hav4 Neq2 subtype was also isolated from chickens in Alabama in 1975. The Hsw1 N1 influenza virus was also isolated from turkeys in Minnesota in 1979, Minnesota, Kansas and Iowa in 1980, and Colorado in 1981. The Hav4 N1 subtype was isolated from confinement-reared wild ducks at the same time duck virus enteritis virus was isolated.

In addition to the isolations made at NVSL, 11 different subtypes were identified from isolates sent to NVSL for characterization between

November 1978 and February 1981. The different subtypes were from turkeys 9, ducks 5 and geese 1. The following hemagglutinin and neuraminidase antigens have been identified either in isolates made at NVSL or isolates characterized at NVSL: Hsw1, Hav1 through 4, Hav6, Hav7, Hav9, N1, N2, Neq1, Neq2, Nav1 and Nav2.

Of the influenza A viruses isolated at NVSL, none were pathogenic for chickens or turkeys when inoculated by the intranasal or CTAS route. The IVPI and ICPI were all less than 1.0. Serotypes sent to NVSL for typing were not pathogenic when inoculated by the CTAS route.

The Alabama chicken isolate was not pathogenic to chickens, turkeys, ducks, chuckers and pheasants. A total of 61 chickens were inoculated. The age of the chickens was 6- to 12-weeks and 52 weeks. The CTAS, intranasal and intraocular routes of inoculation were used. The inoculums were AAF or original specimens with antibiotics or the original specimens without antibiotics. The virus was reisolated and a seroconversion was detected in each group of inoculated birds. There were no significant lesions found at necropsy.

Serology: Negative serology has confirmed that influenza A virus was not the problem in some flocks where no isolation could be made. However, positive serology has allowed verification of infection and usually identification of the subtype responsible for antibody stimulation. Serology has allowed confirmation of infection when samples were collected too late to isolate virus.

DISCUSSION

Almost every year influenza viruses have been isolated from domestic turkeys with clinical disease. However, using the common pathogenicity testing methods, the disease could not be reproduced by inoculation of susceptible birds. The CTAS inoculation method that was used at the NVSL has proved to be satisfactory in that it insured that the inoculum was introduced into the respiratory system and almost no aerosol was produced. Examination of lesion patterns at necropsy has confirmed that the inoculum is introduced into the air sac.

The Alabama chicken isolate was of particular interest because severe clinical disease occurred in the source flock.⁷ In spite of various inoculation methods, including inoculation of the tissues from the infected chickens without antibiotics, no significant clinical disease or post mortem lesions were produced in experimentally inoculated birds.

In the last 2 years, 8 of the 12 hemagglutinin subtypes described in the 1980 nomenclature have been isolated from domestic avian species. Only H2, H5, H8 and H12 have not been isolated. The number of influenza isolates from import birds in the last 2 years has been surprisingly low, only 3%. A larger rate of infection was found between 1973 and 1975. However, a large number of isolates from only a few lots were typed while isolates from many other lots were not.

The diagnostic methods described have identified pathogenic viruses in import birds. In domestic birds, both influenza virus and its antibody have been identified. The laboratory results from domestic birds have provided epidemiological information and the influenza A isolates from disease outbreaks have been used for vaccine production. However, more work is needed to develop laboratory methods to effectively characterize the pathogenicity of strains isolated.

Table 1. The revised nomenclature for hemagglutinin and neuraminidase subtype of influenza A viruses.

Hemagglutinin		Neuraminidase	
1971 Subtype Identification	1980 Subtype Identification	1971 Subtype Identification	1980 Subtype Identification
H0, H1, Hsw1	H1	N1	N1
H2	H2	N2	N2
H3, Heq2, Hav7	H3	Na2, Nav3	N3
Hav4	H4	Nav4	N4
Hav5	H5	Nav5	N5
Hav6	H6	Nav1	N6
Heq1, Hav1	H7	Neq1	N7
Hav8	H8	Neq2	N8
Hav9	H9	Nav6	N9
Hav2	H10		
Hav3	H11		
Hav 10	H12		

Table 2. Pathogenicity testing of influenza viral isolates made at NVSL from domestic birds. The A/Chicken/Scotland strain was included as a positive control.

Subtype	Prototype Strain	Intravenous Pathogenicity Index		Intracerebral Pathogenicity Index	
		Chickens	Turkeys	Chickens	Turkeys
Hav2 Neq1	A/Ty/MN/24834/79	0.68	NT	0.35	0.63
Hav6 N1	A/Ty/MN/2928/79	0.00	0.05	0.00	0.04
Hav4 Neq2	A/Ck/AL/7394/75	0.00	0.00	0.47	0.40
Hsw1 N1	A/Ty/KS/4880/80	NT	0.00	0.06	0.00
Hav6 N2	A/Ty/MN/3575/79	NT	0.00	0.00	0.18
Hav4 N1	A/Dk/TX/4734/80	NT	0.00	0.10	0.13
Hav5 N1	A/Ck/Scotland/59	0.72	0.19	1.55	1.37

NT - Not Tested

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DIAGNOSTIC PROCEDURES — RESPONSE

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Once avian influenza is suspected in a poultry flock, diagnosis is usually a straightforward procedure provided that specimens are received during the viraemic phase of the disease which in some cases may be of limited duration.

Even in such cases a serological diagnosis can be made provided that the diagnostic laboratory has a set of antigens. In other cases serodiagnosis can be based on the Agar Gel Precipitin test of Beard and serum samples may be sent to a central laboratory for full haemagglutination inhibition (HI) testing and Neuraminidase inhibition testing (NI). Serodiagnosis however has the disadvantage that the presence of antibody to avian influenza may not on its own, indicate that a clinical episode was attributable to the virus. For this reason virus isolation attempts should be made wherever possible.

In the case of clinically evident disease, especially in turkeys in which there are respiratory symptoms, a drop in egg production, air sacculitis and in some cases sudden death, avian influenza should be considered. Differential diagnosis includes pasteurellosis and Newcastle disease and this disease may be missed if the diagnostician isolates a pasteurella and fails to go on to carry out virus isolation.

Although the disease is rare in chickens, its symptoms are sufficiently close to that of Newcastle disease that virus isolation for the latter can be relied on to yield influenza virus.

In ducks and some other species virus may be present in a completely asymptomatic form and isolation from such cases is usually the result of routine monitoring rather than a specific attempt to diagnose disease. This may also apply to wild birds and birds in transit or quarantine in the captive bird trade.

In our laboratory, if a clinician reports that he suspects avian influenza we request the immediate submission of three or more fresh carcasses (where any possibility of notifiable disease exists). In other cases the clinician is asked to send specimens of trachea, spleen, intestine and/or cloacal swabs. We have found that it is important to have specimens early in the disease process and this necessitates early consultation with the Veterinarian and suspicion of a virus disease at the outset.

Pooled organs from three or more carcasses are sent in sterile containers without transport medium in such a way as to arrive within 24 hours. Specimens are chilled and transported at +4°C where possible and may be frozen in dry ice if transport is likely to be prolonged

although we are not aware of any cases where failure to isolate virus could be attributed to delays in transit.

For monitoring purposes sets of cotton wool swabs in groups of ten or twenty without transport medium are required.

While there is usually sufficient documentation with the material for the virologist to make some judgement on the suitability of the specimens, in a few cases the clinician is telephoned and further material may be requested; for example tissues taken from a house where colisepticaemia now predominates will yield virus less often than an adjoining house where the viral phase of the infection is just beginning. To form an epidemiological picture of the case it is often useful to have blood samples from all houses on the site so that acute and convalescent titres may be compared.

In a busy virus laboratory, cross contamination is always a risk and to minimize this the serological and virus isolation work is carried out in different laboratories.

In the virus laboratory, the operator takes the package to a clean room in which no myxovirus work has been carried out since the benches were cleaned off and two sets of possible myxovirus material are never handled by the same person in the same laboratory on any one occasion. Where any doubt exists, the laboratory day book is consulted to trace the movement of operators and specimens. For this to be done effectively, a number of rooms have to be available and ample sets of sterile glassware must be on hand.

In the case of material from psittacines all work is done in a Class III safety cabinet to avoid the risk of chlamydial infection.

Approximately 5 grammes of pooled tissues of each organ are ground with sterile sand in a pestle and mortar with two to three volumes of sterile antibiotic saline. The routine diluent contains 200 units of penicillin and 200 microgrammes of streptomycin per ml., but tetracycline or mycostatin may be added especially in the case of cloacal swabs.

Tissues/saline suspensions are placed in universal bottles and held at bench temperature for one hour. Cotton buds from the swabs are nipped off with bone forceps and pools of five are placed in universal bottles with antibiotic saline and also held for one hour.

Centrifugation is avoided and the supernatant is injected into the allantoic cavity of either 5 or 7 eggs of 9 to 11 days embryonation for each pool. In exceptional cases more eggs may be used. The volume injected is 0.1 ml but equally good results have been obtained with 0.2 ml inocula.

Eggs are incubated at 37°C and candied twice daily. While it is the normal practice to discard eggs that die within 24 hours, in the case of acute disease these are also examined. Dead embryos are opened and a rapid

plate Haemagglutination (HA) test is carried out on the allantoic fluids and the condition of the embryo is noted

Incubation is continued for seven days at the end of which period all embryos are chilled at + 4°C for one hour and the allantoic fluids tested for haemagglutinin. Where no haemagglutinin is seen, the fluids are pooled and a second set of eggs is inoculated with undiluted fluids and with fluids diluted to 10^{-4} in antibiotic saline to prevent possible development of the von Magnus state. The fluids from any egg which dies during the 7 day period or which on chilling is found to show traces of HA activity are handled separately.

With experience we have found that virus of high virulence can yield HA in 24 hours, milder virus will yield HA in 48 hours and the avirulent strains may take slightly longer.

Where a diagnosis is urgently required, two eggs from each set are chilled for an hour and the fluids are examined. Eggs are opened in a negative pressure HEPA cabinet to prevent cross contamination and harvested. Separate egg inoculations are not carried out in the same room.

The initial plate test on the fluids consists of mixing platinum loopfuls of allantoic fluid with equal volumes of a 10% suspension of washed red blood cells. This is followed by a preliminary Haemagglutination inhibition (HI) test using micro plates and a 1% suspension of red blood cells. Traditionally all fluids with haemagglutinating activity were tested against SPF normal serum, Newcastle disease serum and Fowl Plague serum; due to the number of HA serotypes of influenza this practice has been discontinued and the preliminary test is used to differentiate ortho and paramyxoviruses where possible.

All fluids showing HA activity are tested for bacterial sterility and where contamination is found, the fluids are filtered and sub-inoculated.

A guide to myxovirus differentiation has been found by examining the nature of the HA activity on the rapid plate test. Generally influenza viruses cause a rapid haemagglutination with the formation of large clumps while Newcastle disease virus haemagglutination is slower and results in finer aggregates. Bacterially sterile Haemagglutinin which is not inhibited by Newcastle disease serum or other paramyxovirus sera is presumed to be influenza and the full range of HI tests and later the NI tests are carried out.

In most cases the HA titre is 2^7 or more, but with some recent isolates a titre of 2^4 or 2^5 has been all that has been available for serological identification. If this titre cannot be raised, serotyping may be difficult.

Serotyping is based mainly on monospecific antibody produced from infected chickens kept in isolators using the type strain for each group wherever possible. The sera has a known titre to its homologous virus and an HI titre to within 1 well of the homologous virus titre is con-

sidered diagnostic. The beta procedure based on serum dilution is mainly used, but in some instances the virus dilution (alpha) test may also be employed.

The HA procedures used are detailed in Poultry Biologics and the HI titres are obtained by carrying out two-fold dilutions using the same system.

Neuraminidase Inhibition tests are carried out using fetuin as a substrate according to the methods defined in "Advanced laboratory techniques for influenza diagnosis 1975." To economize a spot test using 0.1 ml of infected allantoic fluid and 0.1 ml of each neuraminidase serotype are reacted and mixed with 0.05 ml of fetuin in 0.25 ml and reacted overnight. The full test is then carried out using a range of virus and serum dilutions. The N acetyl neuraminic acid is assayed by the thiobarbituric/butanol method at 549 nms.

Simultaneously with the serotyping, pathotype characterization is carried out. We have not found the Intracerebral pathologic Index (ICPI) of Poultry Biologics to be of value in this work and all pathogenicity testing of influenza virus is done by the Intravenous Pathogenicity Index (IVPI) by the injection of 0.1 ml of fresh infected allantoic fluid to each of ten 6-week old SPF chickens. These are observed daily for ten days and the results are calculated according to the method of Poultry Biologics. This test gives a maximum score of 3.0 (when all birds die within 24 hours) and avirulent viruses yield a zero score.

The Classical Fowl Plague serotypes give scores of 3.0 or greater than 2.5 as do the two most virulent Hav5 serotypes and other isolates have yielded a lower spectrum of activity.

At the lower end of the scale, it is often noted that only one or two birds die with the remaining birds appearing healthy throughout and hence the reproducibility of lower values may not be high. In the case of an emergency it has been possible to inject two adult fowls and obtain a good guide to the level of lethality.

Serology

Because of the number of HA types it is not practicable to test all sera by the full HI test except in special cases. For this reason the Agar Gel Precipitin test of Beard (AGP) is used for screening purposes (unless a known serotype is being traced). Where precipitin lines are seen which match with the positive control the sera are tested against all HA types by the micromethod. Usually a selection of the samples are screened and the whole batch is tested once the HA type has been identified. This procedure seems to work well with turkey sera but the AGP test has failed to be of value with duck sera.

Infected turkey flocks may show mean HI titres of more than 2^7 or in the case of milder disease the mean values may be as low as 2^5 . In our ex-

perience these titres have remained for several months although no further virus isolations have been made.

Electron microscopy

Most haemagglutinating viruses are examined in the electron microscope by PTA staining, where size morphology and the dimensions of the helical RNP allow rapid differentiation between para- and orthomyxoviruses. In a rare case this method of examination has revealed the presence of both types of virus in one sample.

SUMMARY

Our diagnosis of avian influenza can be divided into two parts, the investigation of suspect disease (generally in turkeys) and the finding of influenza virus or antibody to influenza virus in the course of routine monitoring of sera sent in for other purposes or from swabs delivered as a routine.

Virus isolation from cloacal swabs taken from captive birds that have died and have been stored for up to 3 weeks at -15°C regularly yielded avian influenza virus from a significant proportion of batches examined. Similarly cloacal swabs taken from ducks and other aquatic birds have yielded virus where no disease has been suspected.

In the diagnosis of influenza in turkeys, the first clue has sometimes been the detection of an AGP reaction in sera sent in for other purposes after which it has been possible to make a virus isolation.

Where clinical disease has been promptly investigated, virus isolation has seldom proved to be a problem. In a few cases where disease has been investigated at a late stage in the process serological evidence of infection has been positive but virus isolation attempts have failed.

IMMUNIZATION APPROACHES TO AVIAN INFLUENZA

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The poultry industry uses many approaches to reduce or prevent losses from disease.

Where practical, they eradicate the disease as with pullorum. When they must co-exist with the disease, they use drugs when economically feasible, to prevent losses as with coccidiosis. Rigid flock isolation practices are used by some to reduce losses by preventing exposure of flocks to the causative agents of disease.

The industry has, however, relied most heavily upon vaccines to prevent or reduce losses from disease. The vaccines have been viruses isolated from other species and used in the hatchery as with Marek's disease in chickens. Immunization has been the timely administration of virulent virus to prevent vertically transmitted virus and resulting disease in chicks as with avian encephalomyelitis. Vaccines have also been naturally occurring viruses of low virulence that share common antigens with their virulent cousins as with the B1 and LaSota strains and Newcastle disease.

Rather than rely on the other means of disease control and prevention, vaccine use by poultry producers is continuing to expand to include other diseases. Viral arthritis and infectious bursal disease vaccine in breeders used to pass on parental antibodies to broilers and new strains of infectious bronchitis in broilers have joined the long list of effective poultry vaccines. A new vaccine to control the egg drop syndrome has gained wide acceptance in Europe. The poultry industry has grown to rely upon vaccines to solve many of their disease problems. It is because of this very positive experience with vaccines that the poultry industry will again look for, and expect, help from vaccines if avian influenza continues to mature into another major disease that causes them serious economic loss.

The poultry industry differs from some of the other livestock industries which make vaccination an attractive approach to reducing disease losses. For instance, the poultry industry is generally confined to certain areas of high population density. In the U. S. we have the heavily populated broiler areas of Delmarva, Arkansas, Georgia, Alabama — the turkey areas of Minnesota, California, North Carolina, Arkansas — the layers of California, Georgia, Florida, Pennsylvania. The close proximity that results from such concentrations of population can make effective flock isolation practices more difficult and vaccine an attractive alternative.

Intensive husbandry practices with large numbers of individuals on a

single premise and in a single building make vaccines a reasonable approach to economically protecting many thousands and even millions of birds on a single farm. Should flock security measures fail and a disease agent gain entry to a large flock without the benefit of vaccine-induced immunity, they could be completely susceptible with severe disease and financial losses.

The integrated nature of the industry with large, well organized companies in charge of all steps of production from feed mixing to slaughter make it a challenge to provide reliable flock-to-flock disease separation because of heavy supervisory personnel traffic and shared equipment.

Flock isolation with turkeys can present unique challenges because of open ranges and the requirement of artificial insemination of breeder hens.

In summary, I believe we can expect the industry to consider the use of vaccines to help solve any existing or potential problems with avian influenza (AI) as they have for other diseases.

Although there has been a limited effort toward accumulating information on vaccines for avian influenza, results have been both encouraging and discouraging, as the magnitude of the problem becomes more evident.

Experimental avian influenza vaccines have been both viable and inactivated. All of these findings are published so I won't review them in any detail today. Turkey/Ore/71 was an avirulent isolate that had the same hemagglutinin (HA) as the classical fowl plague. Prior infection with the avirulent virus was demonstrated to protect, experimentally, chickens and turkeys against disease and death from fowl plague virus.

The inactivated influenza vaccines have been prepared as water in oil emulsions with one, and up to 4, HA antigens in a single product. They were successfully used in both chickens and turkeys, resulting in measurable serologic responses and disease resistance when challenged with virulent viruses possessing the same or similar HA. Inactivated products have been used in the field in several states as an experimental vaccine with good reports.

There are some problems associated with relying on AI vaccines however, be they viable or inactivated:

1. With as many as ten or twelve known possibilities, it will be difficult if not impossible, to successfully predict the particular HA antigen needed to protect flocks against influenza losses. There have been situations where the same, or a similar HA occurs in an area for several consecutive years. There have been other situations, however, such as in the serious Minnesota turkey outbreak in 1978-79, when 4 or 5 viruses of different HA makeup were isolated in a single winter. The prediction of needed antigen type in a vaccine will be difficult; as a direct result of

having to deal with so many antigen possibilities — a concept that may be difficult for the poultry industry to readily accept for it is a drastic departure from the usual and more familiar antigenic sameness, but pathogenic dissimilarity among the Newcastle disease viruses.

2. Widespread vaccination can seriously hamper needed epidemiologic studies that rely upon serology to define the extent and antigenic nature of AI in poultry. Serologic studies will be more difficult to interpret unless careful attention is given to vaccination histories.

3. Immunity resulting from AI vaccines can be very difficult to measure. There are some highly virulent AI strains possessing a particular hemagglutinin that are suitable to serve as reliable challenge viruses. In these instances, vaccine evaluation can be accomplished in a conventional manner not unlike that for Newcastle disease. There are other cases where these highly virulent representatives of a vaccine hemagglutinin are not available — therefore, vaccine evaluation becomes more difficult and perhaps more subjective. Without a good challenge virus that can be readily expected to produce quantifiable disease, or death in non-vaccinates, how do you measure immunity resulting from vaccination? Should we use serologic response in vaccinates, or the amount of antigen contained in a vaccine, or a combination of the two? Regardless of the type of vaccine prepared (viable or inactivated), there may be serious problems in demonstrating potency or efficacy characteristics acceptable to those who are responsible for regulating the products.

The final problem associated with both viable and inactivated vaccines concerns the localized and sporadic nature of the disease. This may continue to result in a relatively low demand for a vaccine, reducing the likelihood that commercial vaccine producers will invest in adequate research and development, and in the costs of meeting licensing requirements.

There are some problems that are unique to viable vaccines and of no concern with inactivated vaccines:

1. Until we have a thorough understanding of virulence with AI viruses, it may be considered too risky to use viruses as field vaccines because they have been demonstrated to be avirulent in the laboratory.

As you know, a classical problem has been experienced when AI isolates from flocks with serious death losses in the field have been taken into the laboratory. More often than not, a disease as occurred in the field is not produced in the controlled laboratory challenge trials. Many of the viruses from lethal outbreaks such as in Alabama and Minnesota chickens appear relatively innocuous in the laboratory. The use of viable vaccines must be approached with great care until we know why there are such differences between field and laboratory experiences with the viruses. Also, the possible role of viable vaccines in the development of harmful field recontaminants cannot be ignored.

2. Viable vaccines may serve to seed areas, complicating results from diagnostic laboratory virus isolations in subsequent years. Serology could be positive not because of an AI virus moving into the area, but because of infections with recirculating viable vaccine.

3. There may be difficulty in finding an avirulent member of each of the 10 or 12 hemagglutinins that can serve as a satisfactory vaccine strain.

Although there are many potential problems, there are, however, some great advantages that could be offered by the use of viable vaccines which would make them acceptable to the industry.

1. The mass administration of vaccines in drinking water or by aerosols makes the viable vaccines particularly attractive to poultry husbandry situations where 10,000 to 90,000 birds may be in a single house. Labor costs and flock disruption losses associated with individually administered vaccines make mass administration techniques much more acceptable to the poultry industry.

2. Viable vaccines are more economical to make in that the actual immunizing antigen is produced by each host and not by the vaccine manufacturer. Because of the relative low cost of production, perhaps lyophilized products representing the different HA antigens could be stockpiled for up to several years awaiting possible use. If certain lots were not needed, there would be no great economic loss as outdated lots of vaccine are discarded and new lots prepared to replace them.

3. Viable vaccines are generally believed to provide more rapid protection than inactivated vaccines. If AI vaccines could be mass administered by spray, a flock might be expected to have some resistance to infection with a field strain of the same HA in only a few days. By contrast, with inactivated vaccines it would take 2 or more weeks for flocks to develop significant resistance.

There are other disadvantages to inactivated vaccine —

1. By their very nature of having to contain relatively large amounts of antigen, they are more expensive to produce.

2. They must be individually administered and may require 2 doses to result in adequate immunity, especially in turkeys.

3. The requirement for individual administration of vaccine will probably result in the necessity for vaccination crews that travel farm to farm. The disease spreading potential associated with such movements of personnel and equipment are well known to all of us.

But inactivated AI vaccines have some distinct and very important advantages.

First — They are safe — with proper inactivation during manufacture, they will not result in a disease problem. Their use will not be hampered by lack of understanding of the virulence mechanisms of AI as with viable

vaccines once a means for product potency testing has been devised and found acceptable to the regulatory agencies.

Second — large quantities of inactivated egg fluids could be frozen back until such time that AI outbreaks indicate that a virus with a specific HA is causing a problem. The frozen product, previously safety tested, could be converted into a finished oil emulsion product overnight and vaccination implemented. Such frozen antigens might be stable for many years. Such a possibility would depend primarily on the availability of vaccine industry expertise, and the economic incentive to pursue such a program. Regulatory acceptance of such an approach should not present unsurmountable problems.

In summary — there are advantages and disadvantages to the possible use of both viable and inactivated vaccines.

Yet, it is highly probable that as the problem of AI becomes more widespread and more costly to the turkey industry, they will seek ways of reducing or preventing these disease losses.

They will do it by improving their flock security and thereby reducing the chances of introduction of the disease through the artificial insemination crews or from growing turkeys on open ranges.

As a second line of defense against possible severe losses, they will probably want vaccination, particularly for their breeders since egg production is a marginal situation for turkeys, at best. The reduction of egg production due to even a comparatively mild strain of AI which causes no mortality can be an expensive disease. Depending on losses from death, decreased feed efficiency and increased condemnations at slaughter, they may consider influenza vaccination for their meat birds as well.

It is doubtful that any vaccines will be capable of preventing a significant rate of infection after field exposure to AI. We don't accomplish that with the other respiratory viruses, so we probably should not expect it with AI. Hopefully vaccines will prove to be effective in reducing losses from the disease even though infection does occur.

As you will hear from Dr. Price, there is increasing interest in vaccines from the turkey industry. Hopefully, those of us in the commercial, university, and government arenas who dedicate our professional lives to reducing losses in poultry will be able to meet the challenges associated with their needs.

If by chance AI becomes a problem in chickens as rapidly as it has for turkeys during the past 18 years, inadequate information on vaccination for the control of losses from AI may prove to be even more costly than imaginable.

With the ubiquitous nature of AI viruses in free-flying avian species, it may be that vaccination, although plagued with the shortcomings and imperfections I have reviewed here today, may be the most feasible tool

that we will be able to offer the poultry industry to soften the sting of influenza. Classically used methods of disease control at the borders of territories and countries fail to stop the free-flying species and therefore will fail to prevent the entry of unknown numbers and subtypes of AI viruses.

We may need to re-think our plans and adjust them to a unique problem of a virus being shared by both domesticated and free-flying avian species. Perhaps conferences of this nature, drawing on the knowledge and experience of scientists the world-over will help us resolve what is fast developing into a worldwide problem. The possible extent of the losses in poultry in future years is beyond the realm of any defensible prediction. There would, however, be little disagreement among the attendees at this Symposium on the capability of AI to someday impose severe losses to the world's poultry.

COMMERCIAL AVIAN INFLUENZA VACCINES

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An investigation following a 1908 outbreak of foot-and-mouth disease attributed to imported contaminated seed virus for smallpox vaccine revealed that many veterinary biological products on the market at that time were worthless, contaminated, dangerous, or harmful.

This led to enactment of the Virus-Serum-Toxin Act of 1913 which empowered the Secretary of Agriculture to regulate the importation and interstate distribution of veterinary biological products.

Regulations designed to insure that all such products are pure, safe, potent, and efficacious are published in the Code of Federal Regulations, Title 9, Animals and Animal Products, Parts 101 through 117.

Regulatory authority has been delegated to the Deputy Administrator, Veterinary Services, Animal and Plant Health Inspection Service. Day-to-day operation of the biologics program is carried out by Regional Biologics Specialists located in Scotia, New York, and Englewood, Colorado, by National Veterinary Services Laboratories, in Ames, Iowa, and by Veterinary Biologics Staff in Hyattsville, Maryland.

Routinely, manufacturers apply for licensure providing proof that they have facilities, personnel, and expertise needed to consistently prepare products which will perform as claimed on the labeling and in advertising when administered as recommended.

Low economic returns have made manufacturers reluctant to make substantial expenditures sometimes needed to develop certain products. This has been true for products for use in less numerous or minor species and especially for complex products, such as Avian Influenza Vaccine. This has also been of great concern to those livestock owners who suffer losses due to diseases known to be controllable if pharmaceutical or biological products were made available to them.

In order to assist in reducing losses due to an outbreak of influenza in Minnesota turkeys in October 1978, a plan was devised which involved two sections of the regulations. The first section involved was 9 CFR Part 103.3 generally used for field safety trials with new products but which may also be used to permit and encourage important research projects by authorizing interstate shipment of unlicensed experimental biological products. The second section involved was 9 CFR Part 103.3(d) and here an exemption was granted by the Deputy Administrator to allow the product to be sold rather than the usual requirement that experimental products be distributed at no cost to the recipient.

This plan made it feasible for Maine Biological Laboratories under the direction of Dr. Kenneth Eskelund to prepare and ship vaccine that would not have been available on short notice if normal licensing procedures had been followed. Dr. Virginia Hinshaw of St. Jude's Children's Hospital, Memphis, Tennessee, contributed to this early effort by preparing recombinant viruses which resulted in improving the quality of the vaccines made with HAV4 and HAV6 strains isolated from Minnesota.

In order to reach the goal of having pure, safe, potent, and effective licensed vaccine available in the future, each request for vaccine was authorized with the provision that an industry organization or governmental agency designated by the State Veterinarian would be responsible for receipt, inventory, and distribution of the product. These organizations were also obliged to obtain information on results of the use of the vaccine, especially regarding safety and effectiveness. Outbreaks in Texas, Wisconsin, Iowa, Missouri, Kansas, Ohio, and Colorado as well as additional subtypes isolated in Minnesota were handled in the same manner. Reports received thus far have been disappointing because they have been incomplete. We have refined the plan for gathering information and have attempted to emphasize the need for improved data. Hopefully, this will result in more useful information in the future.

Most problems encountered in developing standards for licensure of Avian Influenza Vaccine are the result of numerous immunologically distinct subtypes occurring in the host populations. Some of the isolates prove to be good candidates for vaccine production, others may be improved by recombination, while others may resist all efforts to achieve satisfactory immunizing characteristics. We have indicated our willingness to accept serological response at levels which would be likely to demonstrate protection as a serial potency test. It is very unlikely that all subtypes will be capable of producing a statistically acceptable response using current methods. We are hopeful that work underway by Dr. John Newman may give us a better measure of vaccine response.

National Veterinary Services Laboratories has assumed the responsibility for maintaining a supply of virus strains which will be available for use in vaccine production by qualified manufacturers. Seventeen isolates are in storage at the present time. We expect that current procedures will continue to be followed for the immediate future and for handling outbreaks attributed to new subtypes.

Licensed manufacturers will undoubtedly find it difficult and expensive to maintain an inventory of all the completely tested vaccines which might be needed. We will attempt to develop systems which will allow reduction in the time needed to produce, test, and release vaccine to the absolute minimum consistent with acceptable quality.

AVIAN INFLUENZA: APPROACHES IN THE CONTROL OF DISEASE WITH INACTIVATED VACCINES IN OIL EMULSION

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Brescia (Italy)

As years go by Avian Influenza (AI) is more and more recognized as a disease of considerable economic importance particularly in countries with intensive poultry industries, even if the values of losses are not easily definable. In any case, if AI is considered a big problem for avian pathologists, the ecological significance of the increasing spread of infection in the birds is not to be undervaluated.

The infection and its negative effects have been occurring for some time in Italy, primarily in turkeys, where, however, it appears mostly associated with other involvements, particularly *E.coli* and *Pasteurella* infection; consequently a more or less marked decrease of the breeding performances can be observed. Many isolations of AIV have been done in our country from turkeys; some of the isolates have also been characterized antigenically as Hav2N2, Hav5N2, Hav6N2 serotypes, with prevalence of this last serotype (Franciosi et al., 1981 in press).

Attempts to immunize birds against AI have been done by some workers, using monovalent or polyvalent inactivated vaccines, sometimes with apparently good results (Allan et al., 1971; Bankowski & McCapes, 1974; Graves, 1975; Gough et al., 1975; Bahl et al., 1977; Brugh et al., 1979). At the present time, however, no vaccines are commercially available in the world, probably because of the wide antigenic variability of the viruses.

The great importance of turkey's breeding in our country, the high concentration of them in some restricted areas of the northeast (over 25 million per year) and the frequent isolations of AIV as well as Yucaipa virus (Franciosi, 1979; Zanella, 1979 pers. obs.) from cases of respiratory syndrome in turkeys, lead us to undertake research in the control of disease by vaccination, above all on the grounds of our long and wide experience and excellent results obtained in the field with inactivated vaccines in oil emulsion against Newcastle disease, Egg drop syndrome (EDS'76), Infectious bronchitis and other viral and bacterial diseases (Zanella et al., 1966 a,b, 1969a,b, 1978, 1980, 1981).

The purpose of this study is to report some results of vaccination of chickens and turkeys with monovalent and polyvalent killed vaccines prepared with the three prevalent serotypes of AIV in our country, in combination with NDV. These laboratory tests induced us to use such a vaccine in field, limited for the moment to turkeys, with excellent results, even if not easily evaluated.

MATERIALS AND METHODS

Virus

a) AIV strains, serotypes Hav2N2, Hav5N2, and Hav6N2, isolated in these last five years, in northeastern Italy, (Franciosi et al., 1981), kindly supplied by Dr. Franciosi; b) NDV highly immunogenic strains (LCBS 15, 65 and 429) maintained at the "Eurobio" Laboratories, Brescia (Italy).

The viruses have been propagated in the allantoic cavity of 11-day-old embryonated eggs, that were incubated at 37°C for 48 hours and then chilled at 4°C. The viruses (amnio-allantoic fluids) were inactivated with 0.1% propiolactone for 2 hours at 37°C; the absence of residual infectivity was confirmed by two subsequent passages in embryonated eggs. Viral concentration of AIV and NDV strains, estimated by standard methods, has been constantly $10^{9.5}$ EID₅₀/ml of fluid, with haemoagglutinating (HA) titres 1: 640-1280.

Vaccine preparation

30% of AIV + NDV were mixed with 70% of Freund's incomplete adjuvant and treated to obtain an emulsion with optimal viscosity and stability.

Vaccination

Groups of 3-week-old chickens or turkeys were inoculated intramuscularly or subcutaneously with 0.5 ml of different batches of vaccine and revaccinated after 3-4 weeks. The chickens were of SPF origin; the turkeys were commercial stock obtained from local hatcheries.

Challenge

Vaccinated and control birds have been challenged at different times after the second vaccination, by eyedrop instillation of $10^{7.2}$ EID₅₀ of AIV Hav6N2 and observed for 14 days.

Control of immunogenic condition

The response was determined by the hemagglutination-inhibiting (HI) test on individual serum samples, collected after the first and second vaccination, against the 3 serotypes of AIV, by the clinical conditions and by reisolation of the virus from the trachea after challenge. HI tests were done by standard methods, using 4 hemagglutinating units (HAU) of homologous and heterologous AIV antigen or 10 HAU of NDV antigen and using 0.5% of chicken red blood cells as the indicator system. The titres of sera have been recorded on log₂ scale.

RESULTS AND CONSIDERATIONS

The results of this study are reported in tables 1, 2, 3 and in fig. 1. Even if the degree of protection induced by a single dose of inactivated vaccine in oil emulsion has not been tested by challenge, we believe that the HI antibody levels are quite low, particularly in turkeys; also in comparison with the levels of antibody against NDV, in spite the same viral concen-

trations were used (fig. 1). After a second vaccination the serological response, in terms of H₁ antibody, appears to reach rather good levels (table 1) and the degree of protection against the same serotype to be rather significant (table 2). The potency tests (in terms of HI antibody) of various batches of vaccine, done only in chickens, confirmed the apparent good immunity response (table 3). The experimental trials here reported have shown that a very low or no cross-protection was induced, at least in terms of reisolation of the virus after challenge, even with two doses of monovalent vaccines. These results confirm the more importance of hemagglutinin than of the neuraminidase antigen in the immunization. In attempts to maintain control over the disease it seems to be very important: a) the availability of polyvalent vaccines; b) the verification of H and N antigen of the strains involved at times, especially in cases of breaks of immunity and c) the monitoring tests of the antibody spectrum in big integrated farms or in areas with a high concentration of birds. In fact, just last year we introduced in the polyvalent vaccine the serotype Hav5N2, never isolated in our country up to that time. Even if the viral concentration is a major determinant of immunogenicity of AIV vaccines (Brough et al., 1979) as well as of other vaccines (Zanella et al., 1966b, 1969a), the screening of several AIV isolates with common H or N antigens would appear rather important in order to select the most immunogenic strains for the preparation of vaccine. In our research the Hav5N2 induced levels of HI antibody higher than the other two strains used in the vaccine.

SUMMARY

Results of vaccination of chickens and turkeys by mono- or polyvalent inactivated vaccines prepared with Hav2N2, Hav5N2 and Hav6N2 serotypes of Avian Influenza virus in combination with Newcastle Disease virus are reported.

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INTERNATIONAL RESPONSIBILITY FOR CONTROL OF AVIAN INFLUENZA

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INTRODUCTION

The control of avian influenza depends on a clear definition of the disease, the identification of the causal virus and procedures for international reporting.

The virulent nature of classical fowl plague is associated with rapid death and high mortality. As a result, many countries now designate fowl plague as a notifiable disease and subject to national disease control measures.

In 1955, Schafer demonstrated that fowl plague virus had the characteristics of type A influenza virus. However, despite the identification of the causal virus, the disease continues to be known as fowl plague.

There are at least two international agencies which receive and report information on outbreaks of fowl plague. The Animal Health Yearbook is published annually by the combined international agencies of FAO-WHO-OIE. The World Reporting Service on the Evolution of Epizootics is published regularly by the Office International des Epizooties, Paris. However, for both these publications, it is the responsibility of individual countries to establish the criteria used to define fowl plague. Another journal which commenced publication in mid 1980, is entitled Animal Disease Occurrence, and is published by the Commonwealth Agriculture Bureaux.

NATIONAL AND INTERNATIONAL RESPONSIBILITIES

National responsibilities for the control of avian influenza have varied greatly. Thus, in recent years, both Australia and Great Britain considered it a responsibility to eradicate outbreaks of fowl plague occurring in their countries.

In other countries, stamping out measures have not been adopted by the national government. Thus, in the Soviet Union, an inactivated vaccine has been used to control a number of serious outbreaks (Butterfield, 1976a). In the epidemic of influenza in turkeys during 1978-79 in Minnesota, USA, a Declaration of Emergency was not made, although the loss to the turkey industry in Minnesota during that time was approximately \$5. million (Bahl *et al*, 1979).

Similarly, in Canada, the influenza outbreak in turkeys reported by Lang *et al* (1968) was not considered fowl plague. As a result, no eradication measure was undertaken by the Canadian National Veterinary Services.

These are examples of widely differing views on the significance of out-

breaks of avian influenza and the actions taken. It is also clear that many countries do not classify all avian influenza A outbreaks as fowl plague under their national veterinary legislation. As a result, the question "when is fowl plague, fowl plague" has been asked by national animal disease control agencies. This question is of special interest to the 40 or more countries in which fowl plague is a named and reportable disease.

DEFINITION

It is well known that there is a wide variation in the host response (Narayan *et al*, 1969). Only a small number of strains or isolates of the virus result in classical fowl plague in poultry (Ogawa *et al*, 1980). The majority of isolates cause very diverse clinical disease (Lang *et al*, 1972). Thus, strains of influenza virus, antigenically related to classical fowl plague (Hav1), have been isolated from clinically normal free flying wild waterfowl (Hinshaw *et al*, 1978; Slemmons *et al*, 1974); a parrot (McFerran, 1974) and turkeys (Beard and Easterday, 1973). It must also be noted that there exist avian influenza A subtypes antigenically related to subtypes of human, swine and equine origin (Schild *et al*, 1980).

A clear definition of classical fowl plague presents difficulties (Narayan *et al*, 1969), and therefore, it has been suggested that fowl plague should not be given special regulatory significance (McFerran, 1974). The presence of fowl plague antigen may or may not be associated with virulence (McFerran, 1974). Conversely, viruses other than classical fowl plague viruses produce high morbidity and mortality (Butterfield, 1976b). Thus Beard and Easterday (1973) considered that virulence may be the only reasonable criterion of the seriousness of an avian influenza isolate. Butterfield (1976b), also regarded pathogenicity as an important criterion. However, Alexander *et al*, 1978 concluded that the assessment of the seriousness of an influenza isolate on the basis of pathogenicity indices may require very careful interpretation. Perhaps organ tropism may also play a role in the pathogenicity of influenza viruses (Scholtissek *et al*, 1977).

Nevertheless, it is proposed that the following scheme be examined and modified if necessary. This scheme is based on the publications of Allan *et al*, 1977; Alexander *et al*, 1979; Ogawa *et al*, 1980, and personal communications from G. A. Cullen, Great Britain.

Table I

Virulence indices of avian influenza viruses in chickens¹

VIRUS	SUB-TYPE	IVPI ²	ICPI ³
A/Chicken/Germany/34	Hav1N1	3.00	2.00
A/FPV/Dutch/27	Hav1Neq1	2.87	1.65
A/Turkey/England/53	Hav1Nav3	2.97	1.84
A/Chicken/Victoria/75	Hav1Neq1	1.74	1.90
A/Parrot/Ulster/73	Hav1N1	0.00-0.11	0.00
A/Turkey/England/647/77	Hav1Neq1	0.00	
A/Chicken/Germany "N"/49	Hav2Neq1		0.00
A/Duck/England/56	Hav3Nav1		0.00
A/Duck/Minnesota/RWR25/73	Hav4Nav1	0.02	0.12
A/Chicken/Alabama/75	Hav4	0.21	
A/Duck/Czeck/56	Hav4Nav1		0.00
A/Chicken/Scotland/59	Hav5N1	2.87	1.91
A/Tern/S.Africa/61	Hav5Nav2	2.53	1.75
A/Turkey/England/N28/73	Hav5N2	0.00	
A/Turkey/Minnesota/BF/72	Hav6N2	0.00	0.00
A/Turkey/England/110/77	Hav6N2	0.00	
A/Shearwater/E.Australia/1/72	Hav6Nav5		0.00
A/Turkey/England/69	Hav7N2	0.81	0.17
A/Finch/England/76	Hav7Neq2	2.10	
A/Duck/Ukraine/1/63	Hav7Neq2		0.00
A/Turkey/Ontario/6118/68	Hav8Nav4		0.00
A/Turkey/Wisconsin/66	Hav9N2		0.00

¹Data selected from Allan *et al.*, 1977; Alexander *et al.*, 1979 and Ogawa *et al.*, 1980.

²Intravenous pathogenicity index.

³Intracerebral pathogenicity index.

Two suggestions are made, namely:—

First

The classification of all serotypes of avian influenza A virus isolated from poultry and birds, into three major groups, namely velogenic, mesogenic and lentogenic types. This classification would be based on field mortality and laboratory pathogenicity in chickens. The indices would be calculated as in Newcastle disease virus studies (Hanson, 1975; Allan *et al.*, 1978).

In making this suggestion, it is recognized that some years ago, the terms "malignant" and "benign" were suggested. Recently, Ogawa *et al.* (1980) have suggested the terms "high virulent"; "low virulent" and "avirulent" strains. I suggest that these terms "high" and "low" may present some difficulty in translation and interpretation when used in inter-

national classification. The classification now being proposed is based on both the intracerebral pathogenicity index and the intravenous pathogenicity index (Allan *et al*, 1977).

Second

It is suggested that the term fowl plague be replaced by the term "Velogenic Avian Influenza." To use the term "Avian Influenza" (Narayan *et al*, 1969) is not considered sufficiently definitive. As stated the term "Velogenic Avian Influenza" would be based on both the intracerebral and intravenous pathogenicity indices using chickens as the test animal.

It is recognized that further studies on the means of defining fowl plague are needed. There is also a need for National Veterinary Services to examine this proposed classification of avian influenza viruses and the proposed definition of fowl plague. Eventually, an international consensus of opinion may be reached on suitable and acceptable definitions.

DIAGNOSIS

Having suggested definitions for the classification of avian influenza and fowl plague, it is necessary to consider diagnostic procedures and facilities. (personal communications from G. Meulemans and J. Moulthrop). Influenza virus reference laboratories have been established to characterize isolates of the virus, usually on the basis of serological tests. In respect of these tests, three situations arise:—

The first is the risk of transmission of other viral agents in the avian material sent to a reference centre.

The second is the risk of escape of virulent fowl plague virus from a laboratory. An accident of this kind has caused an extensive epidemic among domestic poultry (Dardiri, 1975).

The third situation associated with the characterization of avian influenza virus, is the proposal that pathogenicity tests be conducted on live chickens.

It follows, that if international control of avian influenza is to be developed, then countries have a responsibility to examine and recommend minimum laboratory requirements. These minimum requirements might relate only to the isolation of avian influenza virus. A further development could include laboratory procedures to identify the virus as influenza A. Other laboratories may have or develop facilities to subtype the isolate using the 10 or more different haemagglutinin antigens and a similar number of neuraminidase antigens. Avian influenza A virus diagnostic kits have been produced by the Food and Agriculture Organization (Butterfield, 1976b). A final stage in the development of avian influenza reference centres would be the establishment of strict isolation facilities and a supply of susceptible chicks, in order to conduct pathogenicity tests and determine pathogenic indices.

INTERNATIONAL REPORTING

International agreements are already in place for recording outbreaks of fowl plague in the publications of the Food and Agriculture Organization and the International Office of Epizootics (OIE). In this present paper it is suggested that consideration be given to recording avian influenza virus in four categories, namely, (1) undifferentiated virus; (2) velogenic (classical fowl plague); (3) mesogenic and (4) lentogenic. This system is comparable to the recording of Newcastle disease virus in the current Animal Health Yearbook.

CONTROL, AS AN INTERNATIONAL RESPONSIBILITY

It is proposed that international control be directed against the velogenic avian influenza A viruses. This concept does not prevent countries from developing control measures against the mesogenic and lentogenic forms of the virus. However, a number of factors would have to be considered before control measures could be extended beyond the velogenic types of virus. Thus the epidemiology of the different avian influenza viruses is not well known, (Winkler *et al*, 1972; Slemons *et al*, 1974). In very many areas of the world, this lack of epidemiological information presents difficulties in establishing control measures. Therefore, there exists a clear responsibility for countries to initiate studies on the aspects of epidemiology. It is clear that the origin of many outbreaks of avian influenza virus infection in domestic poultry have not been determined (Alexander *et al*, 1979; Gee, 1976; Johnson *et al*, 1977; Lang *et al*, 1972). Nevertheless, national control measures cannot always wait for precise epidemiological data, especially that relating to spread of the virus between species (Slemons and Easterday, 1978).

In the control of animal diseases, economical considerations are important. Thus, it is necessary to estimate the economic consequences of *not* taking measures to control velogenic avian influenza. These economic consequences vary from country to country and from region to region within a country. In addition, there is the ever increasing world demand for poultry and poultry products. National and international responsibility is clear and direct, namely, the maintenance and development of a world food supply.

A basis for the international control of velogenic avian influenza viruses is available in the *International Zoo Sanitation Code*, prepared by the Office International des Epizooties. The relevant sections of the Code provide some flexibility in the measures available to national veterinary services. Within this concept of choice, the following are suggested.

First: A definition of freedom from velogenic avian influenza viruses.

(a) A country, or a zone of a country, may be considered free from velogenic avian influenza when it can be established to the satisfaction of the importing country that the disease has *not* been present for at least the previous three years.

(b) For countries in which a stamping out policy is practised, the country or zone of a country, may be considered free if at least six months have passed since the last outbreak of the disease.

In making this latter suggestion on freedom from velogenic avian influenza virus, it is recognized that a country has been declared free from avian influenza 21 days following completion of a stamping out policy (Gee, 1976).

Second: The application of control measures

The control measures will apply to domestic and wild caged birds of any age, eggs for hatching, poultry meat, feathers and other products as defined by an importing country.

Third: Limitation of movement

The National Veterinary Services of importing countries may prohibit introduction into or transit through their territory, of products from countries considered infected with velogenic avian influenza.

Fourth: Veterinary Certification and Quarantine Requirements

These are considered separately for:—

- (a) domestic birds, and
- (b) wild caged birds, including birds bred in captivity.

The veterinary requirements will include:—

- (a) Freedom from any clinical disease in birds being exported.
- (b) Attestation that the birds were *not* vaccinated against velogenic avian influenza.
- (c) Details relating to the origin of the birds or hatching eggs.
- (d) Required isolation measures and tests to be conducted while in quarantine.
- (e) The treatment of avian meat products originating in countries infected with velogenic avian influenza.
- (f) The required certification of consignments of fresh meat from any avian species.
- (g) The required treatment to destroy the avian influenza virus in meat meals, feathers and other poultry products.
- (h) The destruction of all containers, feed and water.

DISCUSSION

International control has to be adequate to prevent or at least reduce, the spread of velogenic avian influenza viruses through international trade in live poultry, poultry products and live captive birds (Pearson *et al.*, 1975). The development of rapid means of transportation and the increasing numbers of animals and animal products being moved internationally, increases the risk that poultry diseases of economic importance

will eventually extend to all countries where there is a sizeable and increasing poultry population (Cockrill, 1971). Thus, the number of exotic birds imported into the USA during 1975 was approximately 125,000 (Pierson, 1975). As a result, it has been suggested that avian shipments from which any haemagglutinating virus has been isolated during quarantine should be refused entry (Butterfield, 1976b).

The part played in the spread of avian diseases by the very large numbers of live cage birds moving in international trade requires continued study. It has been estimated that in 1975, more than 5,000,000 cage birds moved in international trade in one year (Inskipp, 1975). Published reports have emphasized the danger of spread of influenza A viruses from wild to domestic avian species (McFerran *et al*, 1974; Alexander *et al*, 1974). In addition, surveys conducted on imported captive birds have yielded influenza A viruses having a wide range of virulence for 6-week-old chickens (Ashton and Alexander, 1980).

Thus, a discussion on the international aspects of the control of avian influenza infections must include, in addition to domestic poultry (Meulemans *et al*, 1979) the influence of free flying birds (Rosenberger *et al*, 1974) and the trans-shipment of captive cage birds (Nerome *et al*, 1978). Control over the global spread of the virus requires the establishment of clearly defined and generally acceptable import-export veterinary health certificates. The establishment and the acceptance of veterinary safeguards is an international responsibility. Research into the character of avian diseases and into the means of control must be supported both by governments and by private interests to a much greater extent than at present (Cockrill, 1971). Thus within the influenza group of viruses, the property of mutation under natural conditions (Bankowski, 1973) represents an important research activity.

In addition to the need for further study on the regulatory control of avian influenza; vaccination also requires additional investigation. A live avirulent influenza virus (Hav1 Nav2) has resulted in 100 per cent protection of chickens against virulent strains of the virus (Butterfield and Campbell, 1978). However, these authors and Smolenskii *et al*, 1978, considered that the immune chicken may become a virus carrier. Thus, at the present time eradication rather than vaccination, should be the procedure to adopt in the control of velogenic avian influenza in domestic poultry. However, in certain regions of the world where losses due to avian influenza have been severe, vaccination may provide some reduction in mortality (Butterfield, 1976a). Control with inactivated vaccines has been effective (Brugh *et al*, 1979).

SUMMARY

Disease is a serious restriction to the expansion of national and international trade in poultry and poultry products (Cockrill, 1971). Thus the development of national and international effort, and the acceptance of

international responsibility in the control of velogenic avian influenza is a wiser long term policy than the erection of trade barriers.

However, national policies for the short term are less easily defined. The resources available must be examined. Outbreaks of classical fowl plague, with rapid death and serious mortality present economic problems in many countries. In response to this situation, countries where the classical disease is *not* recognized have established strict import regulations.

In reviewing international responsibility, it is proposed that the term fowl plague be replaced by the term velogenic avian influenza. In addition, a classification is outlined whereby isolates may be grouped according to the field mortality and laboratory pathogenicity tests. A classification of this kind, although having limitations, overcomes the difficulty of basing field control measures solely on the antigenic structure of the isolate. A classification based only on antigenic structure does not permit the development of meaningful regulations for international trade. A clear definition of the term fowl plague is needed if countries are to compare disease legislation, research findings and the results of field investigations.

It is suggested that international responsibility for the control of avian influenza is urgent and is not a situation to be neglected until the global problem becomes more serious.

DR. LANG: I think there are several points we should take into consideration. I think there is no country in the world that can state at the present time that it is free of fowl plague on the basis of the dozens of viruses in the wild bird population. Not Great Britain, nor Canada, nor the United States, nor all of Europe can make such a statement. And therefore, all our measures are based on the principle of considering fowl plague an exotic disease. Fowl plague is no longer an exotic disease. And therefore, by instituting the measures as are proposed here I think they are trying to overkill; we are creating more harm by overburdening the system with regulations than really the situation (requires). The next point is the definition of fowl plague is based on the pathogenicity in chickens. All of you have heard during the last two days that 98 percent of the outbreaks were in turkeys. Then it is necessary the pathogenicity in chickens to the pathogenicity in turkeys. An example here, this morning, Chicken/Scotland was given to have an intravenous pathogenicity index in chickens of 1.98 or something and has .12 in turkeys. We have a virus in Canada which does just the reverse — it is pathogenic to turkeys but not to chickens. So would you kindly tell me which specie is the specie that should be considered as the type specie for defining fowl plague. That's one thing. The second thing is that fowl plague is dangerous because it has a certain diffusibility. Is the intravenous (index) test giving us any information as to the degree of contagion of the virus? Wouldn't it be much better to put the virus in the drinking water and find out how

many chickens or turkeys catch the infection by this more natural route of infection.

DR. HALVORSON: Dr. Lancaster, there are a couple of things that I took exception to in your presentation. One of these is to include the field mortality as part of the method of classifying this virus. I think all of us agree that disease is related to not only the pathogenicity of the virus but also the dose of virus and the resistance of the host. And as Dr. Newman showed on the slide yesterday, we had the same virus that produced 72 percent mortality in young poults, 20 or 25 percent mortality in other birds, and essentially no mortality in another flock of birds. And I don't think that taking field data is a very scientific approach to classifying a virus. The second thing I would like to mention is that all during this Symposium up until now when we talked about the epidemiology of the avian influenza we have talked about wild birds, we have talked about waterfowl, we have talked about all these things, and nowhere have I heard anybody mention today or yesterday or ever that hatching eggs or eggs or the transportation of poultry meat is involved in the mechanical transmission of avian influenza from one country to another. And I think until we have research data to show that this is even possibly a problem, it is premature to even suggest regulations for the same.

DR. POMEROY: Dr. Halvorson and I come from the same State of Minnesota and philosophically we may be 25,000 miles apart. So I just wanted to say that I don't agree with Dave Halvorson and some of his interpretations.

DR. PETERSON: Well I guess I am little more in line with Dave Halvorson, personally. And Dr. Lancaster, would you define (what you mean when you say) "high mortality."

DR. LANCASTER: Of course, I make no pretends to be a scientist, virologist, I am perhaps an armchair pathologist. But the point, Mr. Chairman, ladies and gentlemen, is that we must never overlook in a symposium of this type and a topic of the type given to me is that other countries do not have the technical expertise nor the laboratory facilities to conduct many of the sophisticated test which we in North America regard as routine. And this is something that we should bear in mind. Now to try to answer some of the questions made. Really I can't. Dr. Peterson asked me to define high mortality. I have no special definition, Pete, of high mortality. I would consider anything above 80 percent in a flock as high mortality. This is the area of mortality which I have in mind. Dr. Halvorson has found a very obvious weakness in the classification system proposed and that is to try to integrate field mortality as a matter of classification. Now, certainly in the case of Newcastle disease this would not apply. But how do we deal with an isolate of avian influenza that has been recovered from a flock with high mortality, that is over 80 percent, and yet that same isolate in the laboratory gives low pathogenicity indices? We have an obvious conflict between the field

observations of uncomplicated, uncomplicated avian influenza of high mortality and an isolate in the laboratory which gives low pathogenicity indices—a conflict. And so from the general concept of regulatory veterinary medicine we would put the field mortality, the field data, and the field picture in over and above laboratory data. We have to use it in field veterinary work. We have to use primarily the disease situation we see in the field and whether or not that is supported by subsequent laboratory typing and serological identification and pathogenicity test. It is well known, Mr. Chairman, ladies, and gentlemen, that dealing with problems like velogenic Newcastle disease for example, hog cholera for example, in many cases the flock or herd is killed and questions are asked afterwards. The virus is typed, searched for afterwards. Dr. Halvorson, Mr. Chairman, reminded me, of course, that there is no evidence as yet of avian influenza A virus in hatching eggs or in poultry meat. My response to that is to refer to a very important point that Dr. Price made this morning when he talked about the development of control for biological products. And he indicated clearly that the legislation period required in the United States was 5 years, if I remembered Dr. Price took, to get the serum toxin legislation in place—approximately 5 years. So really what I am looking at, Dr. Halvorson, is something that perhaps there is no supporting data today. But in international thinking, as the example from Dr. Price's experience, we have to think 5 years ahead. And maybe 5 years from now there will be evidence of influenza virus in hatching eggs and poultry meat. Dr. Lang spoke about the diffusibility, the value of this is attached to virulence. To me, I must say that to study diffusibility as a means of criteria is too dangerous a procedure. Usually we have to act far, far quicker in order to control any serious disease of any kind. He also questions the use of pathogenicity test in chickens why not in turkeys. My answer to this, Mr. Chairman, is again looking at this internationally, there are many, many countries in the world that have a limited supply of specific pathogen free chicks. They have limited facilities for chicks. I don't think that they have comparable experimental animals in turkeys. And, therefore, it is for this reason that I took chickens as my experimental animal. Thank you very much.

DR. POMEROY: Now I think the key word that was said here to me is uncomplicated, Dr. Halvorson, uncomplicated avian influenza. I think if we keep that in mind when we are talking about influenza in Minnesota we are talking about complicated influenza, pasteurellas and all the other things with it. So that we have got to say when we are talking about influenza we are talking about the uncomplicated or the influenza virus itself and what it will do.

DR. BEARD: I don't want to disagree with anything that you said Dr. Lancaster. I admire you very much for standing up here and making proposals on such a controversial subject. It takes a person that's weathered many storms to do such a good job, and I appreciate your effort. I would like a clarification rather than to challenge what you said. When you talk

about freedom from influenza, are you talking about freedom from the velogenic, as you described it only, or are you talking about freedom in domestic fowl, or are you including freeflying birds? And the last question I have is how do you correct for those countries that don't look for avian influenza or don't have the expertise or the facilities to do so? And you noticed I didn't challenge a single proposal you made.

DR. LANCASTER: Dr. Beard, you paid me the compliment of being an old ham. Yes, I am getting old all right. But so too are you Dr. Beard, you are a well experienced man, and so you don't contradict me you just make it difficult. Mr. Chairman and Dr. Beard, freedom—yes I quite agree, I didn't explain clearly what I meant by freedom. What I really was trying to say was freedom from velogenic avian influenza. This being my suggested terminology for the old term fowl plague. I think it will be quite impractical to consider a country free from all of the other kinds of avian influenza A viruses. So I thank you very much, Charlie, for bringing this point to my attention. I referred to freedom. Looking at it from country to country then we must look at freedom from velogenic avian influenza in the same way as we have in our interpretation of freedom from viscerotropic velogenic Newcastle. Then, Charlie, you had another question.

DR. BEARD: Which specie, domestic or freeflying?

DR. LANCASTER: I am not at all sure. I don't see how we can deal with freeflying birds. I have listened, Mr. Chairman, carefully to the comments from Dr. Hinshaw and from Dr. Lipkind of Israel of the evidence for and the evidence still waiting to show a clear connection between freeflying birds and domestic poultry. So I think to answer your question, Dr. Beard, I would have to say that I am speaking only of domestic poultry. I can't see that we can deal with control measures for freeflying birds. We seem to have to regard free-flying birds as a serious hazard in this particular disease.

DR. HINSHAW: I would like to make one comment about the velogenic influenzas. Historically, the H_{av1} and the H_{av5} have been associated with the severe disease outbreaks which Dr. Lancaster would characterize as velogenic. But there are enormous numbers of influenza A viruses in nature. Many of which possess those surface antigens and they are totally avirulent. So you cannot use possession of the H and N as the factor which dictates virulence. There are a lot of avirulent ones out there.

DR. ALLAN: I would like to congratulate Dr. Lancaster on plunging into the deep end. I think really what he has done is he has formulated the skeleton from which the reasoning will involve, and I think the vigorous discussion that is taking place now shows in fact that now we are going to build up from this. I'd like simply to make one point and that is that in regard to the highly virulent strains we have and the isolates from them, I believe the total amount of money that has been lost directly due to

disease has been very, very small in comparison to the more enzootic disease of lower virulence and because there has been very little tendency for these viruses to spread as far as we know. It appears to me that fowl plague virus may well have been an emotive term. And if we are really looking to the interest of controlling animal disease, we may want to differentiate between a sporadic outbreak of disease which is contained and enzootic disease. If it is enzootic disease, it may then be the economic importance within any one area. And Dr. Lancaster has identified a way we can look at this. I think we are going to have to work very hard to take it on from there.

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IS AN INTERNATIONAL REGULATION OF AVIAN INFLUENZA FEASIBLE?

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Avian influenza is known world-wide as a disease which is caused by different antigenic types of virus that may be virulent, of low virulence, or avirulent, and are capable of infecting many species of domestic and wild birds.

The disease, misnamed "Fowl Plague", is a potential cause of embargoes in the poultry trade because there are no uniform international regulations.

Research advancements achieved in birds over the past few years mainly emphasized studies of the ecology of influenza viruses and the possibility of re-assortment of genetic information between human and animal influenza A viruses, and are concerned with the antigenic structure of the virus. Less attention was given to the evaluation of the pathogenic effects of the virus in birds, including the economic impact on the poultry industry.

Nevertheless, some information regarding virulence of Avian Influenza Virus (AIV) in domestic birds is available and may constitute basic markers for drafting international regulations for avian influenza for the poultry trade, including hatching eggs, live birds (especially one-day old chicks), and poultry consumer products.

1—MEASUREMENT CRITERIA FOR AIV VIRULENCE AND DEFINITION OF THE DISEASE

The 1971 WHO classification of AIV mentioned that virulent strains had to be considered as belonging to the HAV1 subtype but at the same time a completely avirulent strain (A/turkey/Oregon/71) having the hemagglutinin of classical fowl plague HAV1 was isolated by Beard and Helfer. Later MacFerran isolated a HAV1N1 serotype of AIV (A/parrot/Ulster/73) which was avirulent in chickens.

More recently, many other isolates showed evidence that no correlation could be established between virulence and the antigenic type of AIV. Consequently, virulence remains the only criteria to classify the AIV isolates.

Different methods, similar to those used for Newcastle disease virus, were studied to test the degree of virulence of AIV.

Mean embryo death time and stability of hemagglutinins

These tests used for Newcastle disease virus are not well correlated

with virulence. In the future new technics, involving other genetic markers should be developed.

Intravenous and intracerebral pathogenicity tests

Intravenous and intracerebral pathogenicity tests used for Newcastle disease virus may be proposed for measurement of virulence. The technics, intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI)* are described in "Methods for the Examination of Poultry Biologics" (National Academy of Sciences—National Research Council—Washington DC, 1971).

Allan in 1977 tested 13 AIV strains and showed a good correlation between the virulence and the value of the IVPI and to a lesser degree the ICPI. From that time, the opportunity was given to isolate avian influenza viruses in several countries and study their virulence.

Meulemans in 1978 and 1979 isolated three strains of AIV (two isolates were HAV3NAV1 and one isolate was HAV6N2) from poultry flocks. All were of low virulence and the correlation with IVPI values was confirmed.

In 1979 Alexander reported the isolation of several serotypes in the U. K., especially in Norfolk. Some of them (HAV1Neq1) isolated from turkeys with severe clinical disease, have high values of IVPI (2.49 to 3.00). The others were of low virulence and had low values of IVPI (0.00 to 0.16) except one (HAV2NAV4); of medium virulence, it had an intermediate values of IVPI (1.34).

In 1980, Duee isolated an AIV strain from broiler-breeder flocks in the northern part of France with clinical symptoms (enteritis) and mortality (10-20%). It was a HAV6N2 subtype showing some inhibition with HAV9 antiserum. The values of the IVPI and ICPI were zero. In all the experiments undertaken in our laboratory to reproduce the disease in 4 week old SPF chickens inoculated with different doses by different routes were unsuccessful except in the group infected by the ocular route. In these birds conjunctivitis and sinusitis, mild weakness and enteritis but no mortality were observed after two to three days.

Nevertheless, if IVPI and ICPI are good criteria of virulence for the virologist, other useful parameters may be of interest as the mortality rate observed in the field and the spread of the disease in a geographic area. So, strains of AIV classified as mesogenic according to the IVPI and ICPI values and eventually responsible for high mortality affecting numerous poultry flocks must be considered as velogenic strains.

Furthermore, some strains are more pathogenic for one species than for another or for younger birds than for older and the criteria to be

* IVPI: The intravenous pathogenicity index is estimated from the time taken for six-week-old birds to die or show disease signs after intravenous inoculation of virus. The results are based on a scoring system in which the maximum index possible is 3.00 (100% dead in one day) and the minimum 0.00 (no recorded signs during the 10-day observation period).

mainly considered is the most pathogenic effect for chickens and/or turkeys. From this point of view it would be interesting to experimentally determine the value of the IVPI test comparatively carried out in turkeys and chickens.

According to the ICPI and IVPI values and field criteria, AIV strains may be classified into three types as for Newcastle disease virus: velogenic strains (pathogenic isolates), lentogenic strains (apathogenic isolates), and mesogenic strains (intermediate isolates).

Consequently avian influenza may be defined as a disease caused only by velogenic or mesogenic strains of AIV. This excludes infections by lentogenic strains of AIV which may be confused with a clinical disease caused by infections with lentogenic AIV strains concurrently with other avian pathogens. Consequently the term "Fowl Plague" should be discarded because AIV other than a "Fowl Plague" strain of virus may cause high mortalities and non-pathogenic strains of AIV are incorrectly designated as "Fowl Plague."

2—NATIONAL RULES TO PREVENT AND ERADICATE THE DISEASE

2.1. *Rules to prevent the disease*

Classical sanitary rules must be applied to prevent the occurrence and spread of AIV viruses. Nevertheless, some particular features of the disease must be taken into account in order to prescribe specific rules.

2.1.1. *AIV viruses are capable of infecting many species of domestic birds: chickens, turkeys, ducks, guinea fowl, etc.*

So, the fact that breeders of different species must not be mixed on the same farm to prevent cross-contamination between species has to be emphasized.

2.1.2. *AIV viruses are able to infect many species of wild birds and consequently the domestic birds*

According to Alexander, it seems likely that the origin of the outbreaks of avian influenza that occurred in turkey flocks in Norfolk in 1979 was in relation with the presence of many starlings in the area during this period. It was in winter and winds and snowfalls had caused damage in turkey houses allowing access to wild birds.

Moulthrop and Langston in a report on avian influenza outbreaks in Minnesota turkeys said that wild bird exposure was less important in housed birds than for range birds.

Consequently, the control of AIV infection must include the keeping of breeders in closed houses with wire-netting on the air inlet and outlet to prevent introduction of wild birds in the poultry houses. In warm countries where the wall of the house is replaced by a wire-netting, a nylon-netting at one or two meters from the wire-netting can be used in order to avoid contact between wild and domestic birds.

2.1.3 *Infected AIV breeder flocks may be a source of infection as the possibility of egg transmission cannot be ruled out*

Narayan had observed AIV in eggs laid by infected turkeys and as a matter of fact the embryo cannot be killed by some strains of AIV. Furthermore, during the hatching process, vertical like transmission cannot be excluded if no sanitary measures are taken to prevent the contamination of the hatchery especially by personnel, eggs and egg trays coming from breeder farms.

As breeder flocks are examined for other infections, *Mycoplasma gallisepticum* and *synoviae*, in 1% of birds at 3 month intervals, the same blood samples may be used to check the flock for AIV infection.

Immuno-double diffusion test (agar gel precipitation) is a good serological method to check the AIV infection (Beard, Bankowski). An antigen prepared with a virulent strain (A/turkey/England/63—Langham HAV1NAV3) share the same components with the other AIV and some influenza viruses of other species according to the new classification of AIV proposed by Schild, Newman, Webster, Diane Major and Virginia Hinshaw (National Institute for Biological Standard (London) and the St. Jude Research Hospital (Memphis)).

Consequently, blood testing by the agar gel precipitation test may be proposed on 1% of the breeder flocks to control AIV infection when birds are more than 4 months of age and retested at 90 day intervals.

2.2 *To eradicate the disease*

When poultry flocks are suspected to be infected by a pathogenic strain of AIV the entire area around the infected premises must be considered as "infected" until the national authorities prove that the virus involved is not velogenic (or a mesogenic strain of AIV or have eradicated the infected birds by slaughter and destruction).

If eradication of the birds is not decided, or if the decision is made too late and it is proven that a velogenic (or mesogenic) strain of AIV is involved, then the area is still to be considered as "infected."

The size of the area depends on the virulence and ability of the strain of virus to disseminate. This should be correlated to the number of flocks of susceptible birds in the infected area (between ten square kilometers and 100 square kilometers). The area is once again considered to be clean of infection if no new outbreaks occur during the two weeks following the last case.

If during the quarantine period other farms are infected outside of the area, a portion of the country should be considered as "infected". Possibly the entire country should be considered as "infected" if the virus is disseminated in several areas of the country.

In the event of mild forms of the disease, caused by mesogenic strains, the opportunity for applying the rules above would depend on the ability

of the virus strain to disseminate rather than on the relative pathogenicity of the strain.

3—REGULATION CONCERNING INTERNATIONAL POULTRY TRADE

3.1. *Hatching eggs and one-day old chicks*

These are the main concern of the international trade of live material and consequently the risk of spread of the disease, including avian influenza is less than with older birds. In fact the trade of the latter birds is limited to neighboring countries or countries belonging to the same economic organizations like E.E.C. For hatching eggs and one-day old birds the origin of infection may be the breeders and/or the hatchery.

It's the reason why the biological material to be exported must originate from breeder flocks serologically tested as mentioned above and free of infection of AIV.

Furthermore, the breeder farms and hatcheries must be located in areas free of infection by a velogenic or mesogenic strain of AIV during the last two months.

The knowledge of the world epidemiological situation requires that each country report the influenza outbreaks to an international organization like the "International Office of Epizootics". The problem remains to define the "influenza disease" as the disease caused by velogenic or mesogenic strains and consequently to discard the term of "Fowl Plague". Actually, in most countries, two avian infectious diseases must be reported, Newcastle disease and fowl plague, because they are considered by the national authorities as severe diseases. If the term of "Avian Influenza" is adopted with a general concept of mild disease more than severe disease, it may be possible that national authorities won't agree to report outbreaks due to AIV, especially when mesogenic strains are involved. In fact, the problem is the same for Newcastle disease considering the fact that mesogenic strains are observed sometimes to be circulating in poultry flocks in some countries or areas with low mortality and no severe clinical symptoms, without national authorities reporting the occurrence of Newcastle disease outbreaks.

3.2 *Poultry meat*

The risk of spread of AIV in international trade of poultry meat is mainly, as for Newcastle disease, related to the consumption of some parts of the carcasses, like viscera and by backyard birds, but these risks do not concern ready-to-cook chickens.

Resistance of AIV to physical and chemical agents must be studied in order to get more information on the real risk of spread of AIV from poultry carcasses.

But until results are available, it may be prescribed, for international trade of poultry meat, that flocks and processing plants for export must be located in an area without any case of acute influenza disease caused by a velogenic or mesogenic strain during the last month.

4—CONCLUSION

International regulation of avian influenza is feasible but requires further research on the evaluation of pathogenicity and resistance of the AIV strains.

Nevertheless, some rules can now be proposed after discussion of this paper and may constitute a draft regulation that could be submitted to the "International Office of Epizootics" by its representative, Dr. J. Lancaster, to include the conclusions in the O.I.E. International Zoo. Sanitary Codex.

Nevertheless, if IVPI and ICPI are good criteria of virulence for the virologist, other useful parameters may be of interest as the mortality rate observed in the field and the spread of the disease in a geographic area. So, strains of AIV classified as mesogenic according to the IVPI and ICPI values and eventually responsible for high mortality affecting numerous poultry flocks must be considered as velogenic strains.

Furthermore, some strains are more pathogenic for one species than for another or for younger birds than for older and the criteria to be mainly considered is the most pathogenic effect for chickens and/or turkeys. From this point of view it would be interesting to experimentally determine the value of the IVPI test comparatively carried out in turkeys and chickens.

According to the ICPI and IVPI values and field criteria, AIV strains may be classified into three types as for Newcastle disease virus: velogenic strains (pathogenic isolates), lentogenic strains (apathogenic isolates), and mesogenic strains (intermediate isolates).

Designation of AIV isolates	ICPI	IVPI	Field criteria
1 - Velogenic	<2.00	<3.00	High mortality rate Rapid spread of the disease
2 - Mesogenic	<1.00	<1.50	Medium or low mortality rate -- Slow spread of the disease
3 - Lentogenic	<0.5	<0.5	No mortality

DR. KUMAR: My question is that if we are vaccinating breeding flocks, how do you test that breeding flock for AI? Because it will be positive on AGP unless you test it with every strain of virus possible.

DR. POMEROY: I guess we have a problem here of using vaccination. Vaccinating the breeding flock, we are going to have titers, AGP positive, serologically positive, and the question is then how do we differentiate on your scheme there of being negative? Would you like to make any comments about that.

DR. BENNEJEAN: It is difficult to differentiate between antibodies after vaccination and after natural infection. But in my paper, I consider only the case of exporting birds and their recommendations of the importing countries. I have an example to give in the case of France, we export poultry meat to a country like Switzerland which is free of Newcastle disease and this country does not vaccinate against Newcastle disease. And we ask that broilers, poultry meat exported to Switzerland come from flocks without antibodies against Newcastle disease, against antibodies from vaccinated bird and infected birds. And it is a program or recommendation of importing country. The requirements are decided between the importing country and the exporting country.

DR. ALEXANDER: I would like to make a comment about the use of the IVPI test as one of the major culprits using that test. I think the test shouldn't include a score of three. This has been a hangover from the use of Newcastle disease virus test where the birds clearly become paralyzed during the course of the disease. In all the hundreds of IVPI's we have done, I think we have only recorded a score of two which was for paralysis once. We usually use this to signify moribund birds and I would suggest that the maximum score in the IVPI should be two as it is in the ICPI test. I think this is quite important because early deaths which occur sometimes with relatively apathogenic viruses become artificially inflated because of a score of three on this test.

DR. NEWMAN: I would like to comment at least in terms of turkeys. I wonder if there is anybody here who has any knowledge whether a turkey breeding flock has ever been subclinically infected with influenza virus. Our suggestion would be that "it's handwriting is on the wall" — influenza comes in, egg production goes down, and you don't really need to do any serology. And unless you get into an immunization program, we wouldn't be aware that that was necessary.

DR. ALEXANDER: In 1979, one of our flocks that we isolated virus from in fact showed no clinical signs at all. And then it was only the observation of 2 to 10 of the eggs that they noticed had a peculiar white color that we went in and looked at the flock and these birds were infected and had been infected for some weeks with influenza virus.

DR. BAHL: A comment on that answer. The particular flock in question may have been positive before it came into production and the serology

was positive and you had chaulky eggs because there was some other problem on the farm.

DR. ALEXANDER: I can't offhand remember the age of the birds. But, I thought that was unlikely. Nearly all our outbreaks in that year, 1979, occurred in a very short period of about 3 weeks. And these particular birds we picked up about 3 weeks after the last one in the group of outbreaks that occurred. So I would have suspected that they had been in lay for some time before they were infected, but offhand I can't remember that.

DR. _____: Was avian influenza isolated? (cannot hear)

DR. ALEXANDER: No, serological evidence only I am afraid.

DR. BAHL: The question I have and maybe someone can put a little more light onto it—we heard today different speakers talk about the role that may or may not be played by eggs layed by flocks which are positive and egg transmission. Vertical transmission—has it ever been proven to be there even in the case of influenza or Newcastle?

DR. POMEROY: Well, I can answer for Newcastle disease.

DR. ALLAN: Thank you. I was rather hoping that our academic colleagues who have been reviewing the recombination possibilities of the influenzas would comment and give us their views on what would happen if live avirulent influenza vaccines were used on our turkey farms or possibly other ones and whether they think this might create a stable or an unstable situation. I would have thought myself that this could be in the long term an extraordinarily hazardous operation, and I would be very interested to know if the people better informed than I would share my apprehensions on this.

DR. POMEROY: Dr. Easterday, would you want to make any comment, or Dr. Hinshaw, on the pros and cons of the use of attenuated live vaccines.

DR. EASTERDAY: I think given the number of viruses that circulate already, putting one more in I can't see any complication.

DR. HINSHAW: I would differ a little bit. Certainly, live vaccines have been considered for many years for use in humans as well. And the objections, well of course, there are many studies on this. There is the worry that through genetic reassortment with the strains that are already circulating that possibly you could create one that would be worse than the one you started. However, Dr. Easterday's point is also well taken that you have an enormous number out there already that are reassorting. It is not to the virus' advantage to create one that is worse. Because when it kills its host it's not going to survive as well. So typically you will not produce one that is worse but that possibility certainly exists and I would ask Dr. Allan. I don't think we can eliminate the fact that that possibility could occur in using these vaccines. Although it is still a possibility to use live vaccines.

DR. LANG: I think it is not correct to assume that influenza viruses are

circulating in the turkey population. Certainly, not in Canada. Whenever, we have an outbreak, there is a single virus involved and that single virus disappears as soon as the flock is _____.

DR. NEWMAN: Unless we can raise turkeys in Minnesota like they do in California, we couldn't afford to use the most avirulent live vaccine. Because at the time we recombine this with live cholera vaccine, live Newcastle vaccine, hemorrhagic enteritis virus—why we have got a potent combination. So we will have to stick with the inactivated vaccines until we can rear turkeys like they do in California.

DR. BANKOWSKI: I would just like to bring up a point on this recombination again and vaccine strains. We, in California, like to produce our vaccines from the homologous strain or the strain that is producing the disease. This morning we heard that what you have is a bank of 17 different antigenic types for hemagglutinins that produce high titers, or produces high titers in eggs. I would like to know the reason why you depend upon a high titer, HA titer, rather than picking a more immunogenic strain, rather than antigenic strain for the humoral antibody.

DR. PEARSON: I can comment on the 17 strains. Dr. Price is probably the one that should comment and I don't see him. We do have 17 strains in our freezer in Ames that could be supplied to biologic companies if they desire. We would agree with you that this is the best procedures. These are not recombinants, and all of these will not necessarily produce high HA titers. We have not tested them on that. And I guess our feeling would be the same as yours that when we have an outbreak we should use an homologous strain. But these are available to biologics facilities if they want to use them.

DR. HINSHAW: I would like to comment about what I was discussing the high growing recombinants. And only a couple of these have been produced for use in the vaccine as a service because this is not our major role either. The benefits derived from high growing recombinants is that it increases the antigen content which is extremely important in whether you are going to have a vaccine that works or not. This is not the only thing to be considered. First of all, high growing recombinants alone can't do it. You may need additional purification to get the antigen level that one needs in the vaccine. I do not know the antigen levels in the ones being used. As an alternative of what I mentioned, if you cannot get a high growing recombinant, which happens, that you might be better off to resort to using a closely related strain that already has that ability. And this would have to be, of course, done by examining the virus with serological assays to see how close is the isolate from the field to the ones that you already have. If you had any doubts or if you thought it was drifted or sufficiently different, you could not use a substitute. It is always preferable to use the homologous virus. But for vaccines you do have to have that antigen; and if there is any possibility of substituting, this sometimes needs to be considered and sometimes is appropriate.

DR. BANKOWSKI: I was very pleased you brought that point up. Because if you recall, our last outbreak in California was caused by, we finally all agree, that it was an Hav6. And yet when the virus first came into the laboratory we checked it against three Hav6's which we had in California. And it only reacted with the antisera to our Hav6-Nav3 which you confirmed and so did the federal laboratory showing that there were three Hav6's in California that were related but as you said not identical. And so perhaps this adds more fuel to the fire that perhaps we ought to be thinking of a homologous rather than lab-fixed strain for vaccine production.

DR. HINSHAW: It certainly is desirable to use the homologous whenever possible. If it is such a poor grower though you are going to be in extreme difficulty in getting enough antigen to put into a vaccine to do any good. So first of all the high growing recombinants if that really hits a blank wall and you have a poor grower, then look at antigenically related strains as a possible substitute.

DR. NEWMAN: We did use the high titered recombinant and challenged it with a heterologous Hav6 in our area and found that it withstood the challenge very well, both in market birds and in breeder birds. So that to support the idea that you need antigen and that you will certainly get some cross protection with the Hav6 subtypes, our experimental data and field data would support that.

DR. HINSHAW: I would like to ask a question of Dr. Eskelund. Do you have information on the antigen content that is being used per bird or per dose? Is there information available on that aspect?

DR. ESKEKUND: Unfortunately, these vary all over the board. We have tried to pick the highest ones and the biggest problem is with an Hav6. We can almost consistently come up with antibody titers of at least 640, alot of times in the 1280's, or even 5260's. Our first experience with the Hsw1 were absolutely lousy. We obtained another isolate, a more recent isolate from Missouri, and this one worked alot better. I don't remember the actual HA titers we received but I know they were far superior; and we have used this one. During the last outbreak, we had to produce a vaccine because they wanted it yesterday. So we did go ahead and produce the minimum amount with what we had until we got other virus in. But these work strictly on trying to obtain the best HA titer we could and usually using individual harvest from two or three embryos to obtain it.

DR. BEARD: Thank you. I think some of the discussion here on vaccines and antigen content are based on some experience Virginia has had with human immunization. Problems that they have had through the years. We have had one advantage here with poultry vaccines that we are considering. The only other animal vaccine used to any extent has been the equine 1. I believe its an aqueous product. I think the human products are aqueous products. I am not sure that there has been any oil emulsion or water and oil emulsion in human products made. Am I right on that are

they aqueous vaccines primarily with no oil emulsion? Yes. So here we have an oil emulsion product that has a very good adjuvant effect in what we are using in poultry. Although the antigen content is very important, I believe you might be able to use some laboratory or manufacturers stock virus, very closely related as Virginia pointed out, and sacrifice a little bit on perhaps antigen mass if you are using oil emulsion. Granted it would be delightful to use the homologous, but we do have adjuvant in the vaccine that has been used. I think that compensates for a little of the sins of not using the homologous ones. I don't think we should lose sight of that. Hopefully, one day we can evolve to a matter of using the single radial diffusion and actually quantify the amount of antigen in our vaccines before they are mixed with adjuvant and get on a much more precise basis. Without that I guess the hemagglutination is more desirable. But I don't anticipate, Dr. Hinshaw, these commercial products being made from concentrated virus suspensions. I think they are going to be made from allantoic fluid harvest without manipulation because we have got the adjuvant that helps us get the job done in an economically feasible fashion.

DR. ESKEKUND: I might just comment that although I say we work on the HA principle, we have also tried not to sacrifice infectivity titer. We don't know how important it is, but we still work with the highest infectivity titer that we can obtain. I think the other possibility of improving these, we haven't done this with influenza, but we are working hard with our other inactivated vaccines is to obtain better adjuvants.

DR. HINSHAW: My question was more directly related to know how much even HA units per bird is being administered to get some idea of what is required to get an immune response in these animals; and how much you are going to have to have? I was talking more directly about specifically what is being used.

DR. EASTERDAY: One thing that hasn't been mentioned in the control and trying to help the individual producer in a problem is the use of antiviral compounds. Dr. Lang has published on this a few years back. Dr. Rinaldi published on the use of amantadine and given the focal from sporadic nature of influenza I think there should be some consideration into looking into the use of amantadine to help bail out those people, those producers, in these focal situations. I am not going to get into a can of worms with FDA and all that sort of thing, but amantadine is one of those compounds that is cleared for human use and I recognize the complications that go along with that.

DR. EASTERDAY: (When we inoculate with a pathogenic avian influenza

virus) that would normally kill the turkey or the chicken, we treat the birds with amantadine at the same time they are infected and have quite healthy birds that we can then obtain postinfection serum from. One more comment that relates to the question that Dr. Allan asked about, (that is) the use of these live virus vaccines (and) to answer in not quite so cavalier manner. I don't think that what Dr. Allan was asking is a problem. I wouldn't be worried about that (pathogenic recombinants). I really would be worried about the problem that Dr. Newman brought up especially in Minnesota turkeys. (avian influenza of relatively low pathogenicity yet complicated with other disease-producing organisms on the same premises)

DR. POMEROY: Our problem all started because we had a neighbor, Dr. Easterday, who isolated influenza.

(Sic) Now we've eradicated, I always use this as a good example, we've eradicated ornithosis from our turkeys in Minnesota very simply. We had a very excellent technician who knew how to run a complement-fixation (CF) test. She retired, and we haven't run a CF test since then and the disease disappeared. So in 1965, Dr. Easterday introduced us to influenza. We were making lots of diagnoses of respiratory infections in turkeys of undiagnosed etiology; that is, we were making diagnoses of tracheitis, airsacculitis, and so forth, and then he came along and confused us. So you see, we could get rid of influenza tomorrow in Minnesota. All we would have to do is just stop testing and we would not recognize the disease.

DR. ROSENWALD: Well, the only thing after your last comment I am wondering why you are using so darn much vaccine if you could get rid of it as simply as that. I haven't heard anywhere here today real good definition of what benefits you're obtaining under fairly well-controlled field conditions—where some of the birds were not vaccinated and others were from the use of the inactivated vaccine. And as Dr. Beard pointed out, the vaccine does cost money; it takes time to vaccinate. And I am just wondering how the birds are challenged, what the criteria for protection is—drop in egg production or what?

DR. POMEROY: Dr. Newman has done work on it. Dr. Bahl and I worked on it earlier. Vaccinated birds will be protected from drop in egg production. They get a serological response—we feel they are immunized, and thus, that is our criteria for using the vaccine. In the laboratory it has produced protection; and based on that fact, we take it to the field. Now no way are we going to get a man that has 100,000 turkeys on a farm to vaccinate 50 percent of them and let 50 percent go unvaccinated. If the Science and Education Administration is willing to pay the price, give us a million dollars, 2 million, or 5 million, whatever it might take to do it (test the vaccine), we'll be happy to take Uncle Sam's money—even Wisconsin would accept that (project). But I think we have to be practical

on this thing in the sense that in the laboratory the vaccine has been effective. We're satisfied with it, and no way are we going out and suggest to the industry that they ought to put another 10 cents into their birds and throw that money down the drain.

DR. NEWMAN: I guess I'd comment a little on that (issue). I thought maybe Dr. Poss might comment since they have actually used the vaccine in the face of an outbreak. There is also another (turkey) concern that has used the vaccine in an area in which an active infection was going on on the same premises. And, again, this is field data, and you have to take it for just that. But in both of these instances, the response of the user was very positive and they felt that they did get, if not elimination of infection, certainly the elimination of clinical signs and the economic burdens associated with the infection. And I heard Dr. Kumar comment on his use of the inactivated swine influenza vaccine in breeders. And, again, I am sure he'd have to indicate that he wasn't certain of the challenge subsequent to vaccination, but he certainly would agree that the infection was brought under control after vaccination. I am sure in the field situation it is really difficult to say that the vaccine worked because of its protective effect or whether because the birds were not challenged. I don't think any of us can stand up and indicate that. But the field experience does suggest and the fact that the Minnesota turkey growers now have \$50,000 worth of vaccine on hand to use in the event of an outbreak would indicate they feel that they have had some very positive effects from its use. So that's about all we can comment on, Dr. Rosenwald.

DR. LANG: We have laboratory data, however, which are contradictory to the statements made here. We have vaccinated turkeys with 7732 virus multiplied in Freund's complete adjuvant. We had HI antibody titers of 1:1230 when we challenged (these birds at the time the first turkey that got challenged) by intranasal installation with the live virus. The first bird died already at 42 days (said days may have meant hours) after, and at 80 days (said days may have meant hours) half of the turkeys were dying already with the same virus against which we had vaccinated.

REGULATORY PROBLEMS ASSOCIATED WITH AVIAN INFLUENZA

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The Animal and Plant Health Inspection Service (APHIS) is concerned about avian influenza. Because of the confusion about these viruses, we believe that it is important to have a meeting such as this Symposium to share concern, expertise, research, and opinions. As a result of this Symposium, we hope to become aware of the latest information, develop nomenclature which will be understood by everyone, develop schemes or programs which will help control the spread of these viruses, and last but not least, establish areas for needed research. This is the reason that the United States Department of Agriculture (USDA), through the Science and Education Administration and APHIS, has provided funds to the United States Animal Health Association to help with this International Symposium on Avian Influenza.

Although there are many issues that need to be resolved, the ones of paramount importance to Veterinary Services, a regulatory agency, relate to our ability and authority to protect our poultry industry from serious poultry diseases. For instance, we need to be able to describe or define specific diseases for which we can utilize manpower and funds in an effort to control or eradicate them.

At the present time as far as avian influenza is concerned, we have authority only to control fowl plague or European fowl pest. We are presently operating under a definition of fowl plague as an avian influenza having the hemagglutinin antigen avian 1 and causing high death loss or lethality in the appropriate susceptible poultry species. Our scientists have indicated to us that there are other influenzas having equal disease potential that do not have hemagglutinin antigen avian 1; and conversely, we have isolated avian influenza with this hemagglutinin on several occasions but the isolates do not cause mortality in susceptible poultry. Should we stay with a term like "fowl plague" or should we change to a general term such as influenza causing high lethality?

In regard to high lethality, what criteria do we use in establishing this evaluation? Certainly, we cannot use death loss in the originating or source flock as the sole basis for determining the lethality of an influenza outbreak. In Minnesota and elsewhere, high death losses in many cases were associated with complicated disease problems or concurrent infections with pathogenic *E. coli*, pasteurella, or some other agent. Often isolates recovered from flocks with high death losses were not capable of producing mortality in susceptible poultry under experimental conditions.

Veterinary Services as a regulatory agency needs to be able to diagnose accurately and quickly the cause of any serious disease in our poultry flocks. This variability or inconsistency of avian influenza viruses in being able to reproduce the disease in the field or laboratory complicates our diagnostic procedures and our ability to take action. A clearly defined, practical, reproducible test for pathogenicity is needed in justifying funds and manpower for an eradication program.

Our experience in Minnesota and elsewhere clearly demonstrates the ability of the influenza virus to spread from flock to flock by various means. Evidence indicates that waterfowl serve as one of the major reservoirs of many serotypes of influenzas. This fact complicates a regulatory program should migratory waterfowl become involved in the widespread distribution of highly virulent influenza viruses within the poultry industry.

We are not aware of any data that clearly indicate vertical transmission of influenza in our poultry flocks. Conversely, we have accumulated information over many years which indicates that it is probably not transmitted through the egg. Recognizing that we have had little experience with fowl plague, our tendency is to place safeguards on routine importations when fowl plague is diagnosed in a country to make sure that it doesn't get into our poultry flocks. We hope that this group will provide us with the facts and assistance in formulating a safe and practical policy to follow concerning these serious poultry diseases.

Many factors need to be considered in developing effective import-export programs to insure that dangerous poultry diseases will not be transmitted across country borders. Requirements must be uniformly applied and must be meaningful. They should reflect the capability of veterinary officials and the laboratory to provide the necessary services. Unnecessary tests add nothing to security—only to the cost of the end product. All requirements, too, should be those that are enforceable. An antibody test alone would not be of much value in the case of influenza. Also, because of the ubiquitous nature of influenza viruses, we should limit our regulatory efforts only to the highly virulent isolates of avian influenza.

Except for a few comparatively minor disease outbreaks that fortunately were restricted to a local problem, avian influenza has been essentially a turkey problem in the United States. It may be that the influenzas in the United States are not infecting chickens or perhaps are not producing a readily recognizable disease in our chicken flocks and, thereby, are not being diagnosed.

Dr. James Pearson of our National Veterinary Services Laboratories in Ames, Iowa, has given data which illustrate that many different avian influenza viruses are entering the United States and are being widely dispersed throughout the country in cage pet birds. We are also aware that waterfowl commonly shed the virus in large quantity with no ap-

parent clinical disease. In addition, serological surveys indicate that about one-fourth of our swine have antibody to an influenza which is being reported in our turkey flocks. With this amount of virus in the environment, have we just been lucky with our chicken industry?

We hope that as a result of this Symposium that we will have additional information as to the effectiveness of a homologous, inactivated vaccine for influenza and other ways of controlling or preventing avian influenza.

I'm sorry that my presentation has not provided answers but rather questions to the problem of avian influenza. We hope that as a result of this Symposium the answers to some of these issues will be available or at least we will have a common understanding of the disease and the problems associated with the disease.

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EPIDEMIOLOGICAL RELATIONSHIP OF INFLUENZA A VIRUSES IN DOMESTIC AND FERAL AVIAN SPECIES

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(ABSTRACT)

Influenza A viruses circulate in many different avian species, both domestic and feral. Recently, influenza outbreaks have become a more frequent and serious disease problem in domestic turkeys in the U. S. An important epidemiological question is — what is the source of the viruses appearing in these domestic birds? These studies have been examining whether the viruses are maintained in the turkeys themselves (or their environment) or whether they are introduced by feral birds, as ducks. In 1980, comparison of 800 virus isolates from feral ducks in Canada and in Minnesota, from sentinel ducks placed in ponds near turkey farms in Minnesota and from domestic turkeys in Minnesota illustrate the following points:

- A. antigenically indistinguishable viruses were isolated from each of these different birds — e.g., H4N2 viruses were present in feral ducks, sentinel ducks and sick turkeys at the same time,
- B. sentinel ducks rapidly acquired viruses after being placed in nature.

These studies suggest that viruses related to those which continually circulate in feral ducks are appearing in the turkeys and that transmission of these viruses can be readily accomplished in the natural setting. Disease outbreaks in turkeys often begin in range birds — a situation in which contact between turkeys and feral birds may occur. These findings provide increasing evidence that the source of the viruses appearing in the turkeys may well be feral ducks migrating through the area. To confirm this possibility, genetic comparisons of each of the eight genes of these different viruses (not just the hemagglutinin and neuraminidase surface antigens) are required; these ongoing studies are described in another session of this symposium (see *Hindshaw et al.*).

A review of the current data on influenza A viruses in feral birds, particularly ducks, underlines their significance in the epidemiology of influenza. Much of these data have been obtained from longitudinal studies on feral ducks in Canada over the past five years. Characteristics of the circulation of influenza A viruses in feral ducks include the following:

- A. High incidence of infection, e.g. viruses were recovered from as high as 60% of juvenile ducks in the Canadian studies;
- B. diversity of antigenic subtypes, including many different combinations, e.g., 27 different antigenic combinations were isolated from Canadian ducks over a 5 year period. It should be noted that ducks are the only species in nature which harbor every known

hemagglutinin and neuraminidase subtype, including those related to mammalian viruses.

- C. *Avirulence* — the isolates from Canadian ducks were all from healthy birds; experimentally infected ducks also showed no signs of disease. The avirulent nature of this virus infection in ducks is an important epidemiological factor because this allows both the virus and host to survive.
- D. *Intestinal replication* — Influenza viruses replicate to high titer in the intestinal tracts of ducks and are excreted in the feces, thus into the water where the birds live. This constitutes a very efficient mode of transmission in nature, i.e., via fecally-contaminated water supplies (fecal-oral route). This may also represent the way these birds introduce their viruses into other species as they migrate through different areas.
- E. *Genetic reassortment* — Genetic exchange between viruses occurs readily in the intestinal tract of ducks, thus generating genetically different viruses. This phenomenon may yield viruses with different biological properties — a factor important in virus survival. Another point is that antigenically indistinguishable viruses may be genetically quite different, thus, antigenic identity does not mean the viruses are the same.
- F. *Host range* — influenza A isolates from ducks can infect and replicate in several different avian species, including turkeys. In these studies, the duck viruses replicated efficiently in birds (ducks, turkeys and chickens) and mammals (ferrets, cats and pigs), but produced no disease. For example, the viruses reached high titers ($>10^6$ EID₅₀/ml of nasal wash) in the nasal passages of ferrets, similar to that of mammalian strains. These findings indicate that the host range of avian viruses is broad, supporting their epidemiological importance to both avian and mammalian species.

The above studies suggest that feral birds, particularly ducks, represent a perpetual source of viruses which may spread to other species, including domestic birds and, possibly, mammals. In view of the characteristics of influenza virus circulation in ducks, it seems likely that feral ducks serve as a source of the viruses appearing in turkeys during disease outbreaks. To fully answer the question as to whether ducks are the source of these viruses requires additional studies; however, the evidence to date supports their role in the epidemiology of influenza in domestic birds, as turkeys.