STUDIES ON PORCINE INFLUENZA VIRUSES

by

Phyllis Catharina Romijn, B.V.M.S., MSc.

Enterprise for Agriculture Research in Rio de Janeiro,
BRAZIL (PESAGRO-RIO).

This dissertation is submitted in fulfilment of the requirements for the award of the degree of Doctor of Philosophy in Veterinary Microbiology.

Department of Microbiology, University of Surrey, U.K.

1989
SUMMARY

Studies on Porcine influenza viruses.

A number of different cell cultures were examined for their susceptibility to the influenza virus A/swine/Weybridge/86(H1N1) and A/swine/Weybridge/87(H3N2). PK1 (porcine kidney) was found to be the most susceptible to the viruses, and MDCK (canine kidney), the best cell line for primary isolation. A method of infectivity assay by immunoperoxidase in microplate cultures of MDCK cells was developed which was simple enough for routine use and practically as sensitive as the egg infectivity test.

The potential risks of accidental importation of influenza infection in pig was assessed by determining the survival time of the porcine influenza virus H1N1 in pig tissues. It was found that the virus may keep its infectivity in frozen (-20°C) pig tissues for up to 15 days.

The interspecies transmission of porcine influenza viruses was studied using turkeys infected with porcine influenza isolates. Although both A/swine/Weybridge/86 and A/swine/Weybridge/87 were transmitted from infected turkeys to pigs, only A/swine/Weybridge/86(H1N1) infected turkeys presented clinical signs of disease. More than 50% of the pigs presented the virus in the nostrils and/or faeces, at
some time during the experiment, and all seroconverted. Transmission from these pigs to newly introduced turkeys was not observed, nor was seroconversion detected.

Influenza epidemiology in Brazil was investigated by serological studies using pig sera collected in different areas of that country, using human, porcine and avian isolates of influenza viruses. Highest antibody titres were found against A/Leningrad/86(H3N2) (19%) and A/Port Chalmers/73(H3N2) (17%), but not against specific porcine isolates.

Only serological evidence was found to suggest that reassortant influenza viruses occur in English pig herds. However, interspecies transmission of influenza viruses between man and pigs, and the maintenance of human strains in English pig herds was demonstrated by the isolation of two H3N2 influenza viruses very similar to A/Port Chalmers/73, present in the human population in the 1970s.
Go placidly amid the noise and haste, and remember what peace there may be in silence. As far as possible without surrender be on good terms with all persons. Speak your truth quietly and clearly; and listen to others, even the dull and ignorant they too have their story. ** Avoid loud and aggressive persons, they are vexations to the spirit. If you compare yourself with others, you may become vain and bitter; for always there will be greater and lesser persons than yourself. Enjoy your achievements as well as your plans. ** Keep interested in your own career, however humble; it is a real possession in the changing fortunes of time. Exercise caution in your business affairs; for the world is full of trickery. But let this not blind you to what virtue there is; many persons strive for high ideals; and everywhere life is full of heroism. ** Be yourself. Especially, do not feign affection. Neither be cynical about love; for in the face of all aridity and disenchantment it is perennial as the grass. ** Take kindly the counsel of the years, gracefully surrendering the things of youth. Nurture strength of spirit to shield you in sudden misfortune. But do not distress yourself with imaginings. Many fears are born of fatigue and loneliness. Beyond a wholesome discipline, be gentle with yourself. ** You are the child of the universe, no less than the trees and the stars, you have the right to be here. And whether or not it is clear to you, no doubt the universe is unfolding as it should. ** Therefore be at peace with God, whatever you conceive Him to be, and whatever your labors and aspirations, in the noisy confusion of life keep peace with your soul. ** With all its sham, drudgery and broken dreams, it is still a beautiful world. Be careful. Strive to be happy. **

Found in Old Saint Paul’s Church, Baltimore; dated 1692

(my father’s thoughts)
This work was supported by the Brazilian Government National Research Council (CNPq) grant number 20.0114/87-5-VT and English Overseas Research Students Award ORS/8740002.

I am indebted to the personnel at CVL, MAFF, especially from the Virology, Poultry, Bacteriology, Animal Production Departments and Library for cooperation in these studies.

It is a pleasure to express my gratitude to Dr. A. Barrett and other members of the University who facilitated these studies and for the advice on their presentation.

Individually, I am grateful to Mr. S. Edwards and Dr. D. Roberts for helpful discussion and comments on the manuscript.

Many thanks to Dr. D.J. Alexander for many stimulating discussions and for help during the preparation of the manuscript.

I am indebted to Mrs. R. Manvell for helpful discussions, for the many influenza virus strains and antisera supplied, and for carrying out the type specificity immunodiffusion tests.

My thanks to Keith who helped me with post-mortem procedures, Phil and Peter, with the experiments of Chapter V; Graham, Mike and David, for their helping hand whenever asked for.

I also thank Dr. P. Chakraverty and Dr. J.P. do Nascimento for supplying viruses and antisera, and for helpfull discussion. Also, my thanks to Mr. A. Douglas, and Dr. P.Chakraverty for carrying out the confirmatory antigenic analysis of two influenza virus strains isolated during these studies.

Many thanks to Miss M. Williams for technical assistance, as well as Mr. R.H. Barreras with the experiments carried out in Chapter VII and V, respectively.

I wish to express my thanks to those who have kindly supplied me with clinical material, in particular to Piabetão abatedouro de Suínos and the vets Paulo and Bauer from Brazil; Mr. Lawson, owner of the English pig farm on
which the sera for some experiments were collected, and Peter, for his assistance. My thanks to FARMERS PORK slaughterhouse, in Cambridge, U.K., in the person of livestock manager Mr. Doug Denny, who made it possible for me to collect so many pig samples.

To all those who supported me in so many ways, at home and at work, my sincere gratitude. Your warm friendship was essential.

My love to Harold, perfect assistant of all hours.
## CONTENTS

Summary ii  
Acknowledgements v  
List of Tables xiv  
List of Figures xviii  
Declaration xx  
List of abbreviations xxii  
List of virus strains xxiv  

Chapter I - Literature review 1  

1. Influenza history 1  
1.1. General 1  
1.2. Porcine influenza 4  
2. The causative agent 8  
2.1. Classification and nomenclature 8  
2.2. General properties 11  
2.2.1. Physico-chemical and biological properties 11  
2.2.2. Properties of the H and the N 13  
2.2.3. Defective interfering particles 16  
2.2.4. Abortive infection 18  
2.3. Form and structure 19  
2.3.1. General 19  
2.3.2. The core - the RNA segments 20  
- the nucleocapsid 23  
- the membrane protein 23  
2.3.3. Envelope - the lipid layer 24  
- the surface projections 24  
- the haemagglutinin 25  
- the neuraminidase 25  
- the carbohydrates 26
2.3.4. Non-structural proteins

2.4. Replication

2.4.1. Infection

2.4.2. Uncoating

2.4.3. Transcription

2.4.4. Biosynthesis of the various viral proteins
   - polymerase complex proteins
   - haemagglutinin
   - the nucleocapsid protein
   - neuraminidase
   - membrane protein
   - non-structural proteins
   - the viral lipids

2.4.5. Virus assembly

3. The disease

3.1. Pathogenicity

3.1.1. Definition

3.1.2. Pathogenicity factors
   - haemagglutinin - plaque formation and H cleavage
   - antigenic changes
   - neuraminidase
   - nucleoprotein
   - M1 protein
   - body temperature of the host and replication rate

3.2. Host range

3.3. Tropism

3.4. Culture

3.5. Spread

3.6. Clinical signs

3.6.1. Mammals

3.6.2. Birds

3.7. Histopathology
1. Materials

2. Techniques and Methods

Chapter III - Sensitivity of some cell cultures to porcine influenza viruses.

Introduction 122
Material and Methods 125
Results 137
1. Susceptibility of cell cultures to infection by porcine influenza virus. 137
1.1. The use of 96-well plates 137
1.2. The use of 6-well plates 140
2. Comparative quantitation 143
3. Immunoperoxidase test in microplate culture of MDCK cells. 148
Discussion 150

Chapter IV - Recovery of influenza viruses from fresh and frozen tissues from infected pigs.

Introduction 155
Material and Methods 158
Results 164
1. In vitro experiment 164
2. Evolution of experimental disease in vivo 167
3. Infection screening test 172
Discussion 175
Chapter V - Interspecies transmission and pathogenicity of porcine influenza viruses in turkeys and pigs.

178

Introduction

178

A - Experimental infection of turkeys with some influenza virus subtypes of porcine and avian origin.

184

Material and Methods

184

Results

187

1. Clinical signs of disease

187

2. Virus recovery

189

3. Immune response

194

B - Interspecies transmission of porcine influenza viruses in turkeys and pigs

198

Material and Methods

198

Results

202

1. Clinical signs

202

2. Virus recovery

206

3. Immune response

210

Discussion

216

Chapter VI - Serological survey of Brazilian pig herds for antibodies against influenza A virus.

219

Introduction

219

A - Non-specific haemagglutination inhibitors

225

Material and Methods

225

Results

229

Discussion

234
Chapter VII - Porcine reassortant influenza viruses in English pig herds.

Introduction

Material and Methods

Results

1. Serological studies
2. Isolation of influenza virus from nasal swabs

Discussion

Chapter VIII - General discussion

1. Goals

2. Diagnostic procedures

2.1. Cell culture susceptibility

2.2. Cytopathogenicity

3. Influenza virus in infected pig tissues

4. Interspecies transmission

5. Serological studies in Brazil

6. Reassortment studies and its implications with influenza virus origin and interspecies transmission.
List of Tables in this thesis:

1. Haemagglutinin and neuraminidase subtypes of influenza A viruses.  
2. Detection of porcine influenza virus in the various cell cultures examined, by measurement of the CPE produced by the cells, HA of culture supernatant and specific direct immunofluorescence reactivity on the cell monolayers.  
3. Detection of HA in the cell culture supernatant of the various cell cultures tested, with and without added trypsin, according to the virus strain used to infect these cells and hours after infection.  
4. Detection of porcine influenza antigen using the immunoperoxidase technique.  
5. Highest dilutions of Wey/86 infected meat suspension, kept at 4°C and -20°C which gave a positive HA result after egg inoculation.  
6. Summary of results showing the presence of influenza virus in pig tissues infected with Wey/86 virus, at the time of slaughter, as detected in various cell systems, by HA and FA tests.  
7. Persistence of virus in the tissues of a Wey/86 influenza virus infected pig (no 222) on storage.  
8. Weight of turkeys (in Kg) infected with influenza virus strains.  
9. Isolation of H1N1 virus from cloacal and tracheal
swabs collected from turkeys during experiment I, following egg passage and detection by HA.

10. Isolation of H3N2 virus from cloacal and tracheal swabs collected from turkeys during experiment II, following egg passage and detection by HA.

11. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 1A (using virus strain H1N1).

12. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 1B (using virus strain H3N2).

13. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 2A (using virus strain H1N1).

14. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 2B (using virus strain H3N2).

15. Presence of non-specific HA in test and specific antisera.

16. RDE titrations using the method as recommended by the WHO.

17. HI activity present in normal pig sera against influenza strains Wey/86(H1N1), Dck/77(H1N2) and Wey/87(H3N2) when using kaolin or RDE to remove non-specific inhibitors.

18. Reciprocal of HI titre of specific pig antisera
before and after treatment with RDE or kaolin.

19. Cross-reaction between porcine and human influenza A viruses in HA tests.

20. Prevalence of positive antibody responses against influenza virus in pig herds of various farms in Brazil.

21. Percentage of positive antibody responses against influenza virus in pig sera of various farms in Brazil.

22. General mean of antibody levels detected in pig sera against the various virus strains tested.

23. Screening of pig farms for the presence of H1 antibodies against influenza virus subtypes (porcine H1N1 and H3N2, human USSR/77, type B, type C) in England.

24. Detection of specific antibodies against various influenza virus types and subtypes in pig sera originated from different areas in England.

25. Animals presenting antibody levels equal to or higher than 1/40 HI against influenza strains Wey/86, Wey/87 and dck/77.

26. Results of the serological screening test carried out on sera collected from pigs originated from farm 73.

27. Results of HI and NI tests carried out on influenza isolates obtained from nasal swabs of pigs reared on farm 73.

28. HI and NI tests carried out at WHO World Influenza
**List of Figures in this thesis:**

2. Structure and the eight molecules of negative stranded RNA of the influenza virus types A and B.  
5. Schematic presentation of the NI test.  
8. Plaque formation of Wey/86 and Wey/87 in MDCK cell culture with and without added trypsin.  
9. Growth curves of porcine influenza viruses in cell cultures with and without added trypsin.  
10. Temperature range observed in pigs experimentally infected with Wey/86 (H1N1), during a 10 day observation period.  
11. Weight gain during a 10 day period following experimental infection of pigs with Wey/86 influenza virus strain subtype H1N1.  
12. Temperature of turkeys following infection with porcine and avian strains, subtype H1N1.
13. Results of HI tests on sera from turkeys of experiment I and II - presence of influenza virus specific antibodies. 196
14. Temperature monitoring of pigs during the first 6 days of experiment 1. 204
15. Mean antibody titre detected in animals exposed to the porcine H1N1 strain (experiment 1A). 211
16. Mean antibody titre detected in animals exposed to the porcine H1N1 strain (experiment 2A). 212
17. Mean antibody titre detected in animals exposed to the porcine H3N2 strain (experiment 1B). 213
18. Mean antibody titre detected in animals exposed to the porcine H3N2 strain (experiment 2B). 214
19. Geographical distribution of antibody titres against the different influenza virus strains found in pig herds in Brazil, expressed in percentages. 243
20. Geographical location of the farms where the sera used in the studies were collected. 244
21. Analysis of highest antibody levels detected in pig sera according to geographical area of origin, against the various viruses tested. 247
22. Location of pig farms from which sera were collected in the survey for reassortant influenza viruses. 258
23. Photograph of the IDD test plate showing the identity of the two influenza viruses isolated from pigs from farm 73. 269
Declaration

I hereby declare that this thesis has been composed by myself and has not been used in any previous application for a degree. All the work has been carried out by myself except some of the experiments with turkeys in Chapter V, which were performed in collaboration with Dr. R. Hammer-Barreras, the initial HI titrations in Chapter VII, which were performed by Miss M. Williams, and the final typing of the influenza virus strains isolated, as mentioned in Chapter VII, which were carried out by Mr. A. Douglas and Dr. P. Chakraverty.

Phyllis C Romijn
Abreviations used in this thesis

B (cells) - bursa-equivalent lymphocytes
CEF - chick embryo fibroblast
CF - complement fixation
CFT - complement fixation test
CPE - cytopathic effect
CRBCs - chicken red blood cells
CVL - Central Veterinary Laboratory
DI - defective interfering
EID₅₀ - 50% egg infective dose
ELISA - enzyme linked immuno absorbent assay
FA - fluorescent antibody
FITC - fluorescein isothiocyanate
H - haemagglutinin
H₁ and H₂ - subunits of the H
HA - haemagglutination, haemagglutinin activity
HAU - haemagglutinating units
HEPA - high efficiency particulate (filter)
HEPES - N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid
HI - haemagglutination inhibition
IDD - immuno double diffusion
Ig - Immunoglobulin
IgG, IgM and IgA - different types of immunoglobulins
IM - intramuscularly
IPX - immunoperoxidase
ISCOM - immunostimulating complex of virus membrane proteins
M₁ - membrane or matrix (protein)
M2 and M3 - protein subunits encoded by the same RNA segment that encodes M1

MDBK - Madin and Darby bovine kidney

MDCK - Madin and Darby canine kidney

MEM - minimal essential medium

mRNA - messenger RNA

N - neuraminidase

NA - neuraminidase activity

NANA - N-acetyl neuraminic acid

NI - neuraminidase inhibition

NP - nucleoprotein

NS1 and NS2 - non-structural (proteins) encoded by one of the influenza RNA segments.

P - polymerase

PA - acid polymerase protein

PB1 and PB2 - basic polymerase proteins

PBS - phosphate buffered saline

PK1 - porcine kidney

PPK - primary porcine kidney

RDE - receptor destroying enzyme

RNA - ribonucleic acid

RNP - ribonucleoprotein

SPF - specific pathogen free

SRH - single radial haemolysis

SRID - single radial immuno diffusion

T (cells) - thymus-dependent lymphocytes
TBA - thiobarbituric acid
TCID$_{50}$ - 50% tissue culture infective dose
U.K. - United Kingdom
U.S.A. - United States of America
U.S.S.R. - Union of Soviet Socialist Republics
WHO - World Health Organization
Virus strains used in these studies and their abbreviations

A/Brazil/11/78-PR/8/32(H1N1) - Braz/78
A/duck/Hong Kong/196/77(H1N2) - Dck/77
A/England/42/72(H3N2) - Eng/72
A/FM/1/47(H1N1) - FM/47
A/Hong Kong/1/68(H3N2) - HK/68
A/Leningrad/360/86(H3N2) - Lenin/86
A/Philippines/2/82(H3N2) - Phil/82
A/Port Chalmers/1/73(H3N2) - PC/73
A/Singapore/1/57(H2N2) - Sing/57
A/turkey/England/69(H3N2) - turk/69
A/turkey/England/250/79(H1N1) - turk/79
A/swine/Suffolk/1/88(H3N2) - Suf/1/88
A/swine/Suffolk/2/88(H3N2) - Suf/2/88
A/swine/Weybridge/117316/86(H1N1) - Wey/86
A/swine/Weybridge/163266/87(H3N2) - Wey/87
A/USSR/0098/77(H3N2) - USSR/77
A/Victoria/3/75(H3N2) - Vic/75
B/England/21/68 - influenza B
C/Glasgow/85 - influenza C
CHAPTER I

1. INFLUENZA HISTORY

1.1. General

Influenza is a respiratory disease caused by one of the three distinct immunological types of influenza virus known as A, B and C (Webster and Kawaoka, 1988).

Influenza A viruses naturally infect humans, pigs, horses, seals (Murphy and Webster, 1985), whales (Hinshaw and others, 1986), mink (Klingeborn and others, 1985) and a great variety of birds. Influenza B viruses have been reported only in humans (Murphy and Webster, 1985). Influenza C has been isolated from humans (Taylor, 1949) and pigs (Yuanji and others, 1983).

The disease, now called influenza, has been recognized for many centuries in man, with explosive seasonal epidemics spreading rapidly and widely over large areas, only to disappear as suddenly a few weeks after onset (Alexander, 1982a).

Porcine influenza was described for the first time in the United States of America (U.S.A.) during the human influenza epidemic of 1918-1919. According to articles of that time, they occurred as repeated illnesses directly after acute influenza outbreaks in man, especially those
caring for piggeries (Koen, 1928). Some years later, in 1930, Shope isolated the causative agent, the virus of porcine influenza (Shope, 1931) three years before the first isolation of influenza virus from humans (Smith and others, 1933). Variants of the type A virus were reported, and four years later, in 1940, a second type of influenza virus, influenza B, was isolated from humans during a moderate epidemic in New York (Review by Grilli, 1981). The haemagglutinating property of the influenza virus was discovered in 1941, and enabled diagnosis of the disease to be carried out in small laboratories (McClelland and Hare, 1941). The influenza virus of subtype H1N1 (HSw1N1) remained in pig populations, particularly in the USA, and had not been reported as being epizootic for several years, although it was slowly spread and sporadically diagnosed in Europe. Reports of virus isolation in Great Britain (Blakemore and Gledhill, 1941) and the Union of Soviet Socialist Republics (U.S.S.R.) (Harnach and others, 1950) during that period were not considered reliable as a retrospective serological survey carried out by Kaplan and Payne in 1959 demonstrated the presence of the specific antibodies against the virus only in the U.S.A., Federal Republic of Germany and Czechoslovakia, out of a total of 33 countries tested. In 1955 it was demonstrated that a virus which had been isolated and described in 1901 (Centanni and Savonuzzi, cited by Stubbs, 1984; Wilson and Valenti; Lode and Gruber,
cited by Alexander, 1986a) as a filtrable agent which caused a disease in chickens, known as "fowl plague", was a type A influenza virus (Schafer, 1955). In that same year the virus was also isolated from horses for the first time (Sovinova and others, 1958).

After the 1918 pandemic, antigenic shift (i.e., the first major change in subtype) occurred in 1957, and a new strain, called "Asiatic" appeared, a H2N2 subtype virus. Following the appearance of H2N2 in man, in 1957, the same virus was described in pigs (Martineau and Charette, 1986). In 1957 the World Health Organization (WHO) started to encourage and coordinate work on the epidemiology of animal influenza viruses, particularly in their relationship to human influenza (Kaplan, 1980). The H2N2 virus, with some antigenic drift (small changes), remained in the human population until 1968, when another antigenic shift resulted in the Hong Kong H3N2 virus emerging in both humans and animals (Martineau and Charette, 1986). After the 1968 human pandemic, Kundin (1970) reported the presence of human Hong Kong virus in pigs.

The findings that reassortment occurred in live animals, and that influenza virus infection of wild birds was common, sometimes simultaneously with multiple strains, led to considerable speculation in the 1970s about the origins of influenza. The strong relationship of porcine influenza virus with subtypes isolated from other animal
species was demonstrated several times (Butterfield and others, 1978b; Hinshaw and others, 1978a). Evidence of interspecies infection accumulated: Smith (1977); Bachmann (1983); Martineau and Charette (1986).

1.2. Porcine influenza

The occurrence of porcine influenza epizootics associated with classical porcine influenza virus was first demonstrated in Northern Italy (Nardelli and others, 1978) and Japan (Yamane and others, 1979a; Satsuta and others, 1981) with evidence of direct transmission by breeding animals in the U.S.A.. In 1979, the same virus, but more virulent, was detected in Western Europe. Outbreaks of influenza in pigs were thereafter described in Belgium (Biront and others, 1980; Vandeputte and others, 1980; Pensaert and others, 1981); Federal Republic of Germany (Muller and others, 1981; Ottis and others, 1981; Witte and others, 1981); France (Gourreau and others, 1980), Holland (Pensaert and others, 1981; Masurel and others, 1983), Austria (Burki and others, 1985) and Spain (Castro and others, 1988).

It has been recognized, from serological evidence, that pigs in the United Kingdom (U.K.) have been infected with subtype H3N2 since 1968 (Roberts and others, 1987) but outbreaks were only occasionally diagnosed. These H3N2 virus
strains continue to persist in the pig population as shown by monitoring of pig herds in Great Britain (Roberts and others, 1987).

Porcine influenza in East Anglia (U.K.) was reported by MacKintosh in 1987. The origin remains unknown. Tofts (1987) reported an outbreak of pig influenza in Somerset in April 1986. "Typical" clinical signs (see Section 3 of this Chapter) were observed although sometimes the disease was subclinical. According to Barlow (1987), the origin of this porcine influenza outbreak (Wisconsin strain H1N1) in the U.K. may have been imported live pigs from Denmark during 1985. The virus was not isolated from British herds until April 1986. Roberts and others (1987) described a 1986 outbreak of acute respiratory disease from which an influenza A virus of the H1N1 subtype was isolated. This H1N1 virus, designated A/swine/Weybridge/117316/86, was very similar to European influenza A viruses. Evidence of clinical disease by H3N2 in England was only obtained in 1987, when Pritchard and others (1987) studied an influenza outbreak among pigs in Suffolk and isolated the virus, observing a seroconversion rate of 70% among the animals sampled. Afterwards, Wibberley and others (1988) characterized the isolate, which was very similar to H3N2 strains circulating in the human population in the 1970’s. The authors recovered an influenza A virus (H3N2) from an outbreak of influenza among pigs in East Anglia that was similar but not identical to some other human and pig
H3N2 strains.

The pig influenza outbreak that occurred in February 1987 in Suffolk was an example of the variable clinical severity of the disease in pigs, appearing to depend largely on intercurrent disease factors, secondary bacterial infection and stress.

The current status of influenza in pigs in Great Britain is characterized by a complex aetiology involving influenza A H1N1 and H3N2 viruses. (See Section 6 of this Chapter).

To date, no influenza outbreaks in pig herds in Brazil have been reported.

Collaborative studies on H1N1 isolates from different countries throughout the world indicate that at least two distinct antigenic variants of these viruses are currently circulating in pigs (Scholtissek and others, 1983; Hinshaw and others, 1984).

The recent H1N1 virus (HSw1N1) that had caused problems in pigs in European countries (Vandeputte and others, 1980; Pensaert and others, 1981) produced clinical signs similar to those described in pigs in the U.S.A., but the haemagglutinin of European isolates was quite different from that of American isolates. In both continents the isolated virus was similar to the A/New Jersey (HSw1N1) variant
(Scholtissek and others, 1983), although European porcine influenza is more like H1N1 viruses isolated from ducks, and different antigenically and genetically from those found in the U.S.A..
2. THE CAUSATIVE AGENT

2.1. Classification and nomenclature

Animal viruses have been separated into 14 groups by Melnick (1982). The Orthomyxoviruses were originally designated myxoviruses, together with the paramyxoviruses. They were classified as medium sized viruses containing single-stranded RNA and essential lipids, and exhibiting helical symmetry. The family of Orthomyxoviridae contains only influenza viruses and it was suggested by Fenner (1976) that it should be divided into two genera, one containing type A and B, and the other type C. Since they share no common antigens, the three types are properly regarded as species (Webster and Kawaoka, 1988). Influenza A viruses are further divided into subtypes, all of which share a common nucleoprotein (NP) and membrane (M1) proteins but differ in either their haemagglutinin (H) or neuraminidase (N). So far 13 subtypes of H and 9 of N have been described in birds, animals or man (Table 1) (Hinshaw and Webster, 1982; Murphy and Webster, 1985).

Several systems of nomenclature for influenza viruses have been used, and continuing re-evaluation of the taxonomy of influenza virus will be required as more is learned about
Table 1. Haemagglutinin and neuraminidase subtypes of influenza A viruses (according to Murphy and Webster, 1985).

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>human</th>
<th>pig</th>
<th>horse</th>
<th>bird</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemagglutinin</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1(H0, H1, Hsw1) PR/8/34 Sw/Ia/15/30</td>
<td>-</td>
<td>Dk/Alb/35/76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2(H2) Sing/1/57</td>
<td>-</td>
<td>Dk/Ger/1215/73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3(H3,Hav7,Heq7) HK/1/68 Sw/Taiwan/70 Eq/Miami/1/63 Dk/Ukr/1/63</td>
<td>-</td>
<td>Ty/Mass/3740/65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H4(Hav4)</td>
<td>-</td>
<td>-</td>
<td>Eq/Prague/1/56 FPV/Dutch/27</td>
<td></td>
</tr>
<tr>
<td>H5(Hav5)</td>
<td>-</td>
<td>Ty/Ont/6118/68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H6(Hav6)</td>
<td>-</td>
<td>Ty/Wis/1/66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H7(Heq1,Hav1)</td>
<td>-</td>
<td>Ck/Ger/N/49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H8(Hav8)</td>
<td>-</td>
<td>Dk/Cz/56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9(Hav9)</td>
<td>-</td>
<td>Ty/Austral/1/72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H10(Hav2)</td>
<td>-</td>
<td>Eq/Prague/1/56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H11(Hav3)</td>
<td>-</td>
<td>FPV/Dutch/27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H12(Hav10)</td>
<td>-</td>
<td>Eq/Miami/1/63 Dk/Ukr/1/63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H13</td>
<td>-</td>
<td>Dk/Mem/546/74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1(N1)</td>
<td>PR/8/34 Sw/Ia/15/30</td>
<td>-</td>
<td>Ck/Scot/59</td>
<td></td>
</tr>
<tr>
<td>N2(N2) Sing/1/57Sw/Taiwan/70</td>
<td>-</td>
<td>Ty/Mass/3740/65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N3(Nav2-3)</td>
<td>-</td>
<td>Tern/S.A./61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N4(Nav4)</td>
<td>-</td>
<td>Ty/Ont/6118/68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N5(Nav5)</td>
<td>-</td>
<td>Sh/Austral/1/72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N6(Nav1)</td>
<td>-</td>
<td>Dk/Cz/56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N7(Neq1)</td>
<td>-</td>
<td>Eq/Prague/1/56 FPV/Dutch/27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N8(Neq2)</td>
<td>-</td>
<td>Eq/Miami/1/63 Dk/Ukr/1/63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N9(Nav6)</td>
<td>-</td>
<td>Dk/Mem/546/74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The first isolates from that species and afterwards used as reference strains of influenza viruses.
** Current subtype designation
*** Previous subtype designation
the antigenic relationships among the H and the N subtypes.

The revised system of nomenclature consists of two parts: a) a type and strain designation; and b) for influenza A viruses, a description of the antigenic specificity of the surface antigens (H and N). In 1980 the WHO (Melnick, 1982) recommended that the identification and clear description of influenza virus subtypes, particularly within type A, was essential for dealing with the antigenic variability of these viruses and the periodic emergence of new strains against which population immunity is low or absent. It was recommended that the following revised system should be used universally (WHO, 1980):

- type and strain designation (for all three types, the strain designation includes information on the antigenic type of the virus (based on the antigenic specificity of the nucleoprotein) as A, B and C; the host of origin (for strains isolated from non-human species); geographical origin; strain number; year of isolation.

- description of the antigenic specificity of the surface antigens, the haemagglutinin (H) and the neuraminidase (N) (in parentheses).

Example: A/swine/Weybridge/117316/86(H1N1) defines an influenza A virus isolated from a pig at Weybridge, strain number 117316, in 1986.

In the cases where influenza A strains isolated from
different host species are of a common subtype designation, it does not necessarily imply that these viruses are transmitted naturally from one species to the other.

Disease in pigs by influenza A viruses has been found to be caused by viruses with H1N1 and H3N2 surface antigens (Roberts and others, 1987). A reassortment between these two subtypes has also been found (Arikawa and others, 1981). The H1N1 and H3N2 subtypes are considered immunologically distinct from each other, and H1N2 a reassortant between the other two. They are all closely related to other avian and human influenza viruses. Other type A influenza viruses have been isolated from pigs, with or without production of disease (see Section 6 in this Chapter).

2.2. General properties

2.2.1. Physico-chemical and biological activities

Influenza virus has a unique spectrum of biological activities among animal viruses, such as selective affinity for epithelial cells, strain specific haemagglutination, neuraminidase activity (influenza viruses A and B), and their replication characteristics, accounting for multiplicity reactivation (Henle and Liu, 1951), incomplete virus formation (Von Magnus, 1954), sensitivity to actinomycin D (Barry and others, 1962), a noninfectious
nucleic acid, and high rates of genetic reassortment (Simpson and Hirst, 1961; Easterday and Beard, 1984). In particular, the segmentation of the Orthomyxoviruses genome is a major feature which accounts for many properties of the virus such as the occurrence of pandemics - see Section 6 in this Chapter.

Replication of mammalian influenza viruses is optimal at 33 to 35°C, although cold-adapted and temperature-resistant variants have been produced experimentally in laboratories, as well as isolated in nature (Odagiri and others, 1984). Several avian influenza viruses replicate efficiently at 40 to 42 °C. This property may distinguish between mammalian and avian viruses and also determine the host species in which the virus is able to replicate (Murakami and others, 1988).

Infectivity of virus preparations is lost following heating at 56°C for 30 minutes (Hirst, 1948), after ultraviolet irradiation (Cane and others, 1987), sonic vibration and treatment with formaldehyde, detergents, oxidizing agents, ether and long-chain fatty acids (Hoyle and others, 1961; Laver, 1963).

Maximum stability of biologic properties is maintained between pH 6.5 and 7.9 (Shelokov and others, 1958; Lang and others, 1968). Purified preparations can be held at 4°C for a month, and suspensions stored at -70°C maintain their titre for many months (Easterday and Beard, 1984). The
infectivity of the virus is maintained in faeces for 30 days at 4°C (Webster and others, 1978) and 7 days at 20°C in lake water (Hinshaw and others, 1979).

2.2.2. Properties of the H and N

The H is responsible for binding virus particles to cells by interaction with sialic acid residues on the cell surface (Hay and Skehel, 1982), and is also involved with the pH dependent fusion of the virus with the cell membrane (see Section 2.4 in this Chapter). Epitopes of the H proteins are responsible for the induction of neutralizing antibodies in the sera of infected or vaccinated animals.

The N facilitates the spread of the virus from cell to cell. This is mediated by the hydrolysis of terminal N-acetylneuraminic acid from the specific glycoprotein receptors for the virus H on the cell surface (Gottshalk, 1966; Palese and others, 1974). It is also involved in the fusion process, which permits penetration of the virus into the cell (Fig.1) (Huang and others, 1980). However, this picture has been complicated by the recent identification of H activity (HA) associated with the N9 protein (Laver and others, 1984).
Fig.1. Cooperative action of H and N in influenza penetration by membrane fusion (after the proposed mechanism of Huang and others, 1980). Attachment of the virus to cells is initiated through the binding of the viral antireceptor (H₁) to the sialic acid receptor molecule of the cell membrane. Then N makes a closer contact between the membranes, by releasing the binding between H₁ and the primary receptor, and unmasking a second receptor, so that the hydrophobic segment of H₂ can interact with this second receptor of the cellular membrane and fusion occurs.

\[ \oplus = H_1 \]

\[ \neg \rightarrow = H_2 \]
2.2.3. Defective interfering particles

A lighter and noninfective particle has been found to occur among influenza viruses, very similar to defective interfering (DI) virus particles found in other viruses. DI viruses have been characterized by their deleted genomes, inability to replicate in the absence of a helper (standard) virus, and their ability to interfere with infectious virus replication while being selectively amplified (Janda and others, 1979; Nayak and others, 1985; Barrett and Dimmock, 1986). They may suppress the cytopathic effect of standard (infectious) virus and aid in initiating persistently infected cultures in vitro (Cane and others, 1987).

The first observation of DI influenza virus particles was made by Friedewald and Pickels (1944) and by Von Magnus (1954); the latter describing the conditions under which "fully active" and "incomplete" virus (as they were termed at that time) were produced. High multiplicity passages of influenza viruses caused the appearance of DI particles which differed from the fully active particles by having a lower sedimentation constant, lack of infectivity and capacity to interfere with and inhibit the propagation of the infective virus. Schlesinger (1950) and Choppin and Pons (1970) showed that the loss of the largest piece of viral ribonucleic acid (RNA), and the increase in the amount of small RNA are involved in the production of incomplete viruses, as well as partial host cell dependence. According
to Janda and others (1979), each DI virus possesses a characteristic RNA pattern indicating the formation of different types of DI viruses. Their results also suggested that DI viruses are already present in the clonal stock virus prepared at a very low multiplicity from a single clone, and that subsequent passages caused the amplification of these DI RNA segments. They also tried to explain the different size of plaque formation in a single clone by the formation of DI particles at different stages of plaque formation. A single DI virus particle may inhibit infectious centre formation by standard virus and this virus may be replaced almost entirely by DI virus after four undiluted passages.

Currently, it is generally accepted that DI influenza particles are generated by high multiplicity passage in permissive cells, facilitate the establishment of persistent infection in cell cultures and may, therefore, be involved in latency (Webster and others, 1982). They contain new small RNA molecules (which are absent from standard virus) which are generated predominantly by massive internal deletion from the three polymerase (P) genes (Moss and Brownlee, 1981; Fields and others, 1981; Nayak and others, 1985).

It has been proposed by Bean and others (1985) and Hope-Simpson and Golubev (1987) that during epidemics the virus is multiplying at a very high rate, rapidly producing
DI particles which lead the virus into a state of persistent infection that ultimately leads to latency. The small amount of infective virus circulating would then correspond to a low multiplicity infection, with a concomitant reduction in the number of DI particles. When almost no DI particles are present, the virus would again become pathogenic. Subsequently, however, Kawaoka and others (1986) and Webster and others (1986) found that the change in virulence of the strain studied by Bean and others (1985) was not due to the interference of DI particles, but a point mutation at the H molecule, associated with its cleavability.

Other particles, such as noninfectious and non-interfering particles (defective-noninterfering particles) has been found and described in influenza virus preparations (Nayak and others, 1985).

2.2.4. Abortive infection

In some cells infected with influenza A viruses, mature virus particle formation does not occur. This abortive infection is defined as the capacity of a cell line to produce influenza viruses with the same haemadsorbing and haemagglutinating capacity with a low yield (one per cent) of infectious virus (Sugiura, 1972). A failure of assembly of the virion was found by Lerner and Hodge (1969) as being the cause of accumulation of synthesized segments in non
permissive host cells, such as HeLa cells. Their data demonstrated that all viral RNA segments are synthesized, but they accumulate in the cell and no infectious virus is liberated. Choppin and Pons (1970) found that a qualitative change of viral RNA could induce abortive infection in some cell lines, for example, HeLa. Sugiura (1972) observed that virus particles produced in abortively infected cell contained a full complement of RNA segments when analyzed by gel electrophoresis. Also, ribonucleoproteins (RNPs) were similar in molecular weight in both abortive and productive cell systems. They concluded that the cause of the abortive infection in some cells probably resided in steps after the formation of RNPs. Lohmeyer and others (1979) found that in some abortively infected cell lines, smaller amounts of M1 protein were detected, and suggested (because of its role in virus assembly and budding from the infected cells) this as being the possible cause of the absence of free infectious virus in abortive infection.

Abortive infection may be relevant in the analysis of host range.

2.3. Form and structure

2.3.1. General

Historically, studies on influenza virus have been at the forefront of work on general virus structure. The
influenza virus was one of the first to be studied in the electron microscope (Taylor and others, 1943), and it was with influenza virus that assembly by budding from the cell membrane was first demonstrated (Murphy and Bang, 1952; Compans and others, 1970a).

Early evidence showed that influenza virus may be found in spherical, slightly elongated, pleomorphic, or long filamentous forms (Elford and others, 1936; Taylor and others, 1943; Compans and Dimmock, 1969; Choppin and Compans, 1975; Wrigley, 1979). The diameter of the majority of virions within a preparation is approximately 100 nm (Murphy and Webster, 1985).

The morphology and arrangement of components in the influenza virion (Fig. 2.1) were reviewed and described by Webster and Kawaoka, 1988). It is composed of a core and an envelope. The core contains the RNA segments and the nucleocapsid, surrounded by the M1 protein, which is located beneath the lipid bilayer of the envelope. From the envelope two distinct surface glycoprotein units, the H and N, protrude as spikes.

2.3.2. The core

The RNA segments

Orthomyxoviruses of the types A and B have a genome comprised of eight molecules of linear negative sense stranded RNA (Fig. 2.2). Type C viruses possess 7 segments
Fig. 2. Structure (Fig. 2.1) and the eight molecules of negative sense stranded RNA (Fig. 2.2) of the influenza virus types A and B. The influenza virus is composed of a core (containing the RNA segments and the nucleocapsid) surrounded by the M1 protein, and the envelope (composed of a lipid bilayer in which the two glycoproteins H and N are embedded). Eight molecules of single-stranded RNA comprised the influenza virus A and B genome. They differ in size, some producing one (PB2, PB1, PA, NP, H, N), others 2 (NS) or 3 (M) proteins.
The RNA segments are closely associated with the nucleocapsid protein (NP) to form the RNP complex.

The nucleocapsid

In the core of the virus particle, the nucleocapsid consist of the RNP complex (McCauley, 1987) and three large polymerase associated proteins (PB2, PB1 and PA) (Klenk and Rott, 1988). The three P protein genes are encoded by the three largest RNA segments respectively and supply all the enzymatic machinery for viral RNA synthesis (Kingsbury, 1985). They are capable of transcribing complementary messenger (m) RNA molecules from the genome RNA template (McCauley and Mahy, 1983). The NP is composed of a single species of nonglycosylated phosphoprotein. The degree of phosphorylation varies according to the virus strain and host species infected and is also a determinant of host range (Kistner and others, 1985). This RNP is the type-specific internal antigen of influenza viruses used to classify the viruses into types A, B and C.

The matrix protein

Within the lipid envelope, and surrounding the core of the virus particle lies the M1 protein, so called to distinguish it from the M2 and M3 proteins (see Section 2.4 in this Chapter). The M1 protein is a type-specific antigen, and used in influenza typing, principally because of the
peculiar resistance of its antigenicity to treatments such as boiling which can consequently be used to remove contaminating antigens, either cellular or viral, from the serological test system (Hay and Skehel, 1982). The matrix is about 6 nm thick, comprising about 30 per cent of the total protein of the virus.

2.3.3. Envelope

In this review, the viral envelope refers to that structure which encloses the non-glycosylated proteins and the viral RNA. It is composed of the viral lipid and the glycoproteins which make up the surface projections: the H and the N.

The lipid layer

The lipid is located as a layer on the surface of the virus particle at the base of the surface projections. The lipid is derived from the plasma membrane of the host cell and has properties related to the species of host cell (Schild, 1972). It is the only antigen found to be common to all three types of influenza viruses (Schoyen and others, 1966).

The surface projections

The surface projections that cover the surface of the virus particle are of two distinct kinds, one associated
with the H and the other with the NA (Laver and Valentine, 1969; Lazarowitz and others, 1971; Schulze, 1975). The exact mechanism of attachment of the surface projections to the viral membrane is still under study. Both the H and N have hydrophobic bases that are involved in hydrophobic interactions with the viral lipids which are present in the form of a bilayer (Murphy and Webster, 1985).

The haemagglutinin

The H is the major glycoprotein component of the virus membrane amounting to approximately 25 per cent of the virus protein, compared with about 5 per cent for the N (Schulze, 1973). It is a trimer of identical sub-units each consisting of two polypeptide chains H₁ and H₂ (Fig. 2.1) (Wiley and others, 1981). These subunits are attached by their hydrophobic ends to the lipid layer of the envelope. Their hydrophilic ends protrude from the surface of the virus into the surrounding aqueous medium, and are the location of biological activity (Wilson and others, 1981). The host cell receptor pockets are situated on this distal part of the molecule, one on each subunit. They also contain the antigenic sites. To date, four major sites have been defined on each subunit (Wiley, 1985).

The neuraminidase

The N of influenza A and B virus constitutes the second virus-specific antigen on the surface of the influenza virus
particle, and is immunologically unrelated to the H subunits. N subunits have a hydrophobic tail, associated with the lipid layer of the virus, and a hydrophilic head, which carries the enzymatic activity and protrudes from the virus particle. It has a mushroom-like appearance in the electron microscope (Haslam and others, 1970b), and the molecule appears to consist of a peripheral box of 8 x 8 x 4 nm which is located on top of a narrow stem about 10 nm long (Hay and Skehel, 1982). The catalytic site of the N is situated at the top of the head of each subunit, so each N contains four active sites, just as each H trimer contains three receptor binding sites (Laver and Valentine, 1969; Wrigley and others, 1973; Murphy and Webster, 1985).

The carbohydrates

The carbohydrate is a host specific antigen and as such its structure depends upon the origin of the host cell and upon the virus strain, which also determines the number and type of glycosylation sites (Matsumoto and others, 1983). About 20 per cent of the surface area of the H is covered by carbohydrate. Its function is not well understood.

2.3.4. Non-structural proteins

In infected cells, two proteins (M2 and M3) are found,
encoded from the same gene segment as the M1 protein. M2 is expressed on the surface of infected cells (Lamb and Choppin, 1981). The function of M3 is not fully understood (see Section 2.4 in this Chapter). Two further nonstructural (NS) proteins are synthesized (Wrigley, 1979). NS1 is a phosphoprotein associated with inhibition of host cell protein synthesis, and has a function in viral RNA synthesis (Skehel, 1973). It has also been proposed that it is involved in the stability of the H, and also in the function of the M1 protein in budding and infectious particle production (Koenecke and others, 1981). NS2 accumulates in the cytoplasm, its function being unknown (Mahy and others, 1980).

2.4. Replication of influenza viruses

2.4.1. Infection

Infection of influenza virus is initiated by the attachment or adsorption of the virus to cells through the binding of the virus H (antireceptor) to the sialic acid receptor molecules of the cell membrane. The specificity of the H for a particular receptor can vary (Rogers and others, 1983). As already mentioned, N cooperates in unmasking a receptor for the hydrophobic N-terminus of the H₂ (Fig.1 and Fig.3) (Rott, 1982; Rott and Scholtissek, 1982).
Fig. 3. Schematic representation of influenza virus replication.

After attachment of the viral particle to the cell, fusion of the viral and cell membranes occur, and the virus penetrates the cell. The activity of the lysosome, and the endosome containing the virus causes the liberation of the nucleocapsid into the perinuclear area. The RNA segments contained in the nucleocapsid penetrate the nucleus, where much of the replication occurs. Viral proteins are produced and liberated into the cell cytoplasm. H and N are transported to the cell membrane where they are inserted. RNP assembles in the nucleus, is transported to the cytoplasm, and associates with the M1 protein. This core is involved by the budding cell membrane, which then folds out and pinches off.
**Cell**

**nucleus**

Virion Enzymes

(−) Strand Parental RNA

(+) Strand RNA

(−) Strand Progeny RNA

Progeny Virus

Pbil, Pbi, Ppa

ns

m

np
2.4.2. Uncoating

The viruses are then engulfed into endosomal vesicles by receptor-mediated endocytosis (Matlin and others, 1981), and the viral genome transferred into the cell cytoplasm by membrane fusion induced by a reduction of pH. The low pH of the lysosome promotes fusion of the virus membrane with the lysosomal membrane, and viral RNA is released into the cytoplasm (Bukrinskaya and Zhdanov, 1984) (Fig.3). Subviral particles accumulate in the perinuclear area and apparently interact with the nuclear membrane. This interaction leads to the liberation of the cores from the M1 protein and the entry of eight RNP's (one for each RNA segment) into the cell nucleus (Fig.3) (Huang and others, 1981).

2.4.3. Transcription

After uncoating, the single-stranded virion RNA is transcribed into the complementary RNA, which is the first product of replicative RNA synthesis of all influenza virus genome RNA segments (Hay and others, 1982). The positive-stranded antigenomes produced in the first stage of replication contain all the genetic information present in each of the genomic RNA segments in complementary form. These antigenomic RNA segments, in turn, act as templates for the production of faithful copies of the genome segments (Fig.3) (Krug and others, 1984). Of the 8 molecules so produced, 6 are monocistronic mRNAs which are translated directly into the proteins representing H, N, NP, and the
various components of the viral polymerase. The other 2 cRNA molecules (M and NS) undergo splicing, yielding 3 and 2 mRNAs, respectively, which are translated in different reading frames. In other words, the 8 viral RNA molecules code for 11 proteins: 7 structural and 3 or 4 nonstructural.

2.4.4. Biosynthesis of the various viral proteins

Polymerase complex proteins

The 3 largest RNA segments encode for PB2, PB1 and PA, respectively. It is thought that they are synthesized in the nucleus (Murphy and Webster, 1985).

Haemagglutinin

Kreil (1981) demonstrated that the biosynthesis of the H is encoded by RNA segment 4 and involves translation at membrane-bound ribosomes, cotranslational modifications, and transport to the plasma membrane of the host cell through the Golgi apparatus (Klenk and Rott, 1988). The initial product of H-specific mRNA translation contains an amino terminal hydrophobic signal peptide which varies in length for different H. It contains between 14 and 17 amino acids and although of restricted amino acid composition, differs in sequence considerably from H to H. The initial translation product, after insertion into the membrane of
the rough endoplasmic reticulum is subsequently removed to form the precursor molecule H. The H must have a structural feature that, after insertion of the molecule into the membrane, promotes active transport to the cell surface. The first post-translational modification undergone by the H is sequential glycosylation in the rough endoplasmic reticulum and smooth internal membranes (Stanley and others, 1973; Klenk and others, 1974). These viruses are not infectious, even though they can haemagglutinate (Lazarovitz and others, 1973), but they can be rendered infective by proteolysis as a consequence of which the H₁ and H₂ components are generated (Fig.4) (Hay and Skehel, 1982). Proteolytic cleavage may take place a) on smooth internal membranes b) at the plasma membrane (Compans, 1973; Hay, 1974; Klenk and others, 1974) by host-specific proteolytic enzymes (Lazarowitz and others, 1973) or c) in extracellular environments such as the respiratory tract. This activity can be inhibited by protease inhibitors (Klenk and Rott, 1973). The extent of the cleavage depends on the virus strain, the host cell, and the presence or absence of serum in the medium (Lazarowitz and others, 1971, 1973; Klenk and Rott, 1973; Stanley and others, 1973).

By proteolysis, an N-terminal signal peptide is cleaved off, with the removal of one or more intervening amino acids (Fig.4). Webster and others (1982), studying the H, found that cysteine residues and certain other amino acids are conserved in all sequences, indicating that the 13 H
Fig. 4. Diagramatic representation of haemagglutinin polypeptides. The H is synthesized as a single polypeptide from which an N-terminal signal peptide is cleaved off. To become infectious, a latter cleavage produces the subunits $H_1$ and $H_2$, by the removal of one or more intervening amino acids, depending on the subtype. The two subunits remain linked by a single disulfide bond. The H is anchored into the lipid layer by a sequence of hydrophobic amino acids near the C-terminus of $H_2$. No cleavage is known to occur within the N.
haemagglutinin

neuraminidase
subtypes evolved from a common ancestor and share a common basic structure. The H of some influenza A viruses is proteolytically cleaved in all host cell systems analysed, whereas the H of other strains is activated only in a few host cells. From the H of the serotypes H2 and H3 a single arginine is removed, and for these only trypsin or trypsin-like enzymes activate the H (Klenk and others, 1975). For the H of an avian influenza subtype (H7) which is cleaved in all hosts, a basic peptide containing several arginine and lysine residues is eliminated (Fig.4). Influenza A virus can grow in cell cultures with low protease activity. The virus recovered has uncleaved H, and is noninfectious, though capable of attaching to host cells or erythrocytes (Lazarowitz and Choppin, 1975). The relevance of H cleavage in pathogenicity and epidemiology of influenza viruses will be considered in Section 3 in this Chapter.

The nucleocapsid protein

The NP, encoded by RNA segment 5, is involved in virus maturation. According to Taylor and others (1969, 1970), Becht (1971) and Krug (1972), the NP is synthesized in the cytoplasm and transported into the nucleus, where it accumulates, helically surround the RNA segments holding them together (Russell and Edington, 1985) and later spreads to the cytoplasm. Under von Magnus conditions the NP does not leave the nucleus (Rott and Scholtissek, 1963) (see Section 2.2 in this Chapter).
Neuraminidase

Little is known about the biosynthesis of the second glycoprotein of the viral envelope, encoded by RNA segment 6. It is suggested that the neuraminidase is glycosylated and transported in a similar way to the H (Kreil, 1981). No post-translational cleavage of the N polypeptide occurs (Brand and Skehel, 1972).

Membrane protein

The M1 protein is found on smooth membranes and plasma membranes of the infected cells (Lazarowitz and others, 1971; Compans, 1973, Klenk and others, 1974). Together with M2 and M3, it is encoded by RNA segment 7 (Lamb and others, 1981). The M proteins of various type A influenza viruses are serologically closely related (Rott and Scholtissek, 1982). This protein is thought to be essential for virus assembly by initiation of budding (Klenk and Rott, 1988). Its biosynthesis is thought to occur in the nucleus (Murphy and Webster, 1985).

Non-structural proteins

The NS proteins are encoded by the smallest RNA segment (Murphy and Webster, 1985). NS1 is produced early in infection and accumulates in the nucleus. NS2 is made later in infection and detected predominantly in the cytoplasm (Murphy and Webster, 1985).
The viral lipids

Virus does not specifically direct lipid synthesis in the host cell (Kates and others, 1961). Synthesis of lipids found in the virion occurs by the normal cellular biosynthetic process, and the viral envelope is formed by incorporation of lipids from the host cell plasma membrane (Wiley, 1985).

2.4.5. Virus assembly

Assembly of the RNP involves assembly of viral RNA and NP and takes place within the cell nucleus. After it has accumulated, it is then transported into the cytoplasm (Krug, 1972). Assembly of the envelope is a multistep process. Viral envelope proteins are incorporated into areas of the cell membrane (Compans and Choppin, 1975). The M1 protein is the bridge between the core and envelope, by associating with the RNP and the cytoplasmic side of the cell membrane, in interaction with the cytoplasmic domain of the glycoproteins (Roizman, 1985), forming an electron-dense layer. The RNP then binds specifically to the M1 protein, and the process of budding occurs by an outfolding and pinching off of a segment of the membrane, enclosing the associated RNP and M1 protein (Wiley, 1985).

It is unclear whether there is a mechanism by which a full complement of segments is incorporated into each virion (Hay and Skehel, 1982), or that a random selection of
segments occurs, and that the total number of segments included in the average virion is large enough to afford a reasonable probability that a significant fraction of virions produced will have at least one copy of every virus gene (Compans and others, 1970b).

When two influenza viruses infect a cell, their replicating genome segments can mix freely and progeny with various assortments of genes from both parents are produced. This interchange of influenza virus RNA segments is referred to as genetic reassortment and is of considerable importance in the epidemiology of the virus (see Section 6 in this Chapter). Also incomplete genomes may be generated at high multiplicity infections or reassortments (Hay and Skehel, 1982).
3. THE DISEASE

3.1. Pathogenicity

3.1.1. Definition

Pathogenicity is defined as the quality of producing, or the ability to produce, pathological changes or disease (Dorland, 1985). At present it is of more relevance to distinguish between influenza viruses of high and low pathogenicity, than to identify the particular subtype, serotype and variety (Alexander, 1986b) because pathogenicity involves the interaction of a number of factors, both viral and host derived. An exchange of any RNA segment between strains can modify pathogenicity and it has been shown that optimal constellation of all RNA segments or their respective gene products is required for the production of highly pathogenic virus strains (Rott and others, 1979; Scholtissek and others, 1979; Bonin and Scholtissek, 1983; Tian and others, 1985).

3.1.2. Pathogenicity factors

It has been found that the genes coding for H, N, NP and M1 have an influence on the pathogenicity of influenza virus (Klenk and Rott, 1988).
Haemagglutinin
Plaque formation and H cleavage

It has been found that viruses that produce plaques in cell culture in the absence of trypsin, are more virulent than viruses that do. Also, other strains exhibit a number of intermediate stages of plaque forming capacities. Plaque forming ability is correlated with cleavage of the H protein into $H_1$ and $H_2$ in tissue culture, induced by the presence of proteases. The extent of H cleavage has been shown to be host dependent and to correlate with the cytopathic effect (CPE) produced (Lazarowitz and others, 1973; Stanley and others, 1973). This property is exchangeable to some reassortants of influenza A serotypes, but not all (Simpson and Hirst, 1961). Bosch and others (1982) suggested that the plaque forming capacity could be associated with a broadening of the host range of the virus so that it could infect and destroy all cells in a certain cell population. Thus, the susceptibility of the H to trypsin cleavage is an important determinant of pathogenicity (Ogawa and Ueda, 1981; Alexander, 1982b) and is based upon the variable composition in basic amino acids near the cleavage site (Richardson and others, 1980; Rott, 1982; Bosch and others, 1982) (see Section 2.4 in this Chapter). Bosch and others (1979) were able to demonstrate that highly pathogenic avian influenza viruses had additional inserts of basic amino acids at the cleavage site compared with viruses of low
virulence for chickens. This enabled the highly pathogenic viruses to be cleaved by a wide range of host proteases compared with restriction to cleavage by trypsin-like proteases for viruses of low pathogenicity.

Some bacteria are capable of exacerbating the infectivity of an influenza virus by producing a trypsin-like enzyme that cleaves the H. Tashiro and others (1987) demonstrated that some strains of *Staphylococcus aureus* secrete proteases that activate the infectivity of some influenza A viruses by cleaving the uncleaved H into H₁ and H₂. Co-infection of the mentioned bacteria with influenza A viruses in this way enables the development of severe disease by permitting the virus to undergo multiple replication cycles in host cells that do not possess the protease themselves.

**Antigenic changes**

Changes on the hydrophilic portion of the H molecule, resulting in different virus specificity for binding sites on the cell surface, may occur following reassortment during mixed infection or from genetic mutations. This offers the potential for changes in pathogenicity or virulence (McCauley, 1987). The susceptibility to cleavage of the H can also be selected by adaptation of the virus to a novel host (Rott and others, 1984).
Neuraminidase

The N may act as a virulence factor by enzymatically removing sialic acid residues from the viral H, making the H susceptible to the proteolytic cleavage which is necessary for viral infectivity (Nakajima and Sugiura, 1980; Sugiura and Ueda, 1980).

Nucleoprotein

The genetic determinants of host range were studied by Kistner and Scholtissek (1984), Scholtissek and others (1985) and Kistner and others (1985). They suggested that specific cellular protein phosphokinases are also responsible for the host range of influenza viruses. They demonstrated that the tryptic phosphopeptide pattern of the nucleoprotein of different influenza A viruses is strain-specific and depends on the host cell. Earlier studies (Scholtissek and others, 1978, 1979; Vallbracht and others, 1979; Bonin and Scholtissek, 1983) had already demonstrated that the host range of a virus could be changed by reassortment, using a virus with an inadequate phosphorylation of its NP and replacing it for a functional NP. The authors suggested that specific cellular protein phosphokinases are involved in virus replication and that these may determine host range and cell tropism by site specific phosphorylation of viral phosphoproteins.
M1 protein

The M1 protein appears to play an important role in virus pathogenicity, since its underproduction leads to a decrease in budding of mature virus particles and may be responsible for abortive infection (Lohmeyer and others, 1979).

Body temperature of the host and replication rate

It was suggested by Alexander and others (1981) that the rate of replication may partly determine the level of pathogenicity of a virus, as well as the proportion of infectious particles produced. Consequently, body temperature of the host may have an important role as a selective character (Van Hoyningen-Huene and Scholtissek, 1983; Dohner and others, 1984), due to the kinetics of thermal inactivation of different influenza virus strains, which range between 36°C and 50°C. Klenk (1973) observed that influenza viruses kept at 25°C or less accumulate uncleaved H in the cell, with no production of H₁ and H₂. At a temperature of 37°C the amount of uncleaved H in the cell decreased and H₁ and H₂ were detected.

Several other factors must be considered and defined in any in vivo assessment of pathogenicity, such as age of infected animal, route of infection, and clinical signs (Alexander, 1988a).

43
3.2. Host range

The host range characteristics are unpredictable because the spread of the virus requires the proper gene combination for virulence, host range and tissue tropism (Scholtissek and others, 1979). Therefore, the ability of a virus to replicate in a particular host tissue depends on successful interactions between cellular proteins and most of the viral gene products.

The historical literature on influenza frequently refers to outbreaks of illness in lower animals at the time of epidemics in man, and pigs have frequently been implicated as reservoir hosts for human influenza viruses (review by Bachmann, 1983).

Avian influenza A virus was found to infect several mammalian species experimentally; some of these viruses survived in pig populations and were transmitted among them (Hinshaw and others, 1981c). Replication of the avian viruses occurred only in the respiratory tract of mammals, whereas, in birds, they also replicated in the intestinal tract (see Section 6 in this Chapter).

The chick embryo is a highly susceptible host, and ten-day-old chick embryos can be infected with all influenza viruses via the allantoic or amniotic cavity. Five to eleven-day-old embryonated fowls' eggs are ideal for influenza virus replication because at that age proteolytic activity is present in the chorioallantoic fluid of the eggs.
Influenza virus can attach to, penetrate, and initiate infection in a wide range of cells, both in terms of animal species and kinds of tissues or organs. In the majority of host systems, however, it results in the production of non-infectious particles, and sometimes abortive infection. RNAs (both complementary and virion) and proteins are synthesized but very little infectious virus is produced (Schlesinger, 1950; Isaacs and Fulton, 1953; Henle and others, 1955; Lerner and Hodge, 1969; Haslam and others, 1970a; Choppin and Pons, 1970; Sugiura, 1972).

Host range limitations do occur, and potentially pathogenic virus strains can be generated and maintained in a particular species without disease and then suddenly manifest themselves as pathogenic agents when introduced into a different species (Rott and others, 1986). This is the case with many influenza viruses which infect free-living birds without causing disease. Eventually, these viruses can spread to other birds or mammals, with variable pathogenicity (Klingeborn and others, 1985; Hinshaw and others, 1986; Gibson, 1987). Variations in receptor specificity due to structural alterations at the receptor binding site of the H, as already mentioned, may permit virus adaption to another host (Klenk and Rott, 1988).
3.3. Tropism

Specific cell and host tissue tropism is mediated by the presence of specific receptors for the virus on the cell surface and presence of trypsin-like enzymes (Klenk and Rott, 1988).

In acute virus diseases, the infecting virus has to rapidly reach a tissue in which it can multiply to high titre. In this way, it can overcome host defence mechanisms and spread to the target organ in which the viral pathogenicity is expressed. The replication of non-pathogenic strains of influenza virus is restricted to the respiratory and intestinal tracts, whereas pathogenic viruses can be recovered from many other organs of the host (Bosch and others, 1979). Virulent influenza virus isolates replicate outside the respiratory tract and may be enterotropic (ducks), neurotropic (seal influenza) or pantropic (fowl plague) (Klenk and Rott, 1988).

Tissue tropism has been studied by Naeve and others (1983), who showed that some viruses which were able to replicate in the intestine had a H antigenically distinguishable from that of viruses which could not. The mutation responsible for cell tropism seems to occur in the H1. Only H1 with a serine to glycine mutation at amino acid position 228 along with a glutamine at position 226, exhibited avian enterotropism (Rogers and others, 1983).

The sites of replication of influenza A viruses in ferrets and pigs were studied by Kawaoka and others (1987).
Most porcine, equine and avian influenza A viruses tested were recovered from both the intestinal and respiratory tracts of experimentally infected ferrets, whereas most of the human influenza viruses studied were only recovered from the respiratory tract. This indicates, as already mentioned, that there are differences in tissue tropism by influenza A viruses in different animals and that both viral and host factors determine the tissue tropism of influenza viruses in mammals. It would appear that the ability of influenza viruses to replicate in the lungs is determined by temperature, while replication in the intestinal tract is dependent on the pH (Webster and Kawaoka, 1988). The intestinal route serves as a major mechanism for virus transmission from and between birds, by shedding the virus in their faeces (Alexander, 1983).

3.4. Culture

The most frequently used host system for the isolation of influenza viruses is the fowl’s embryo, as it will tolerate inoculation of supernatants from faeces or tissue homogenates and supports replication of all subtypes of virus (Burnet, 1940). The virus has been grown in several tissues cultures from many animal species, including human embryonic lung or kidney (Mogabgab and others, 1956; Palmer and others, 1975, Dowdle and Schild, 1975) and adult monkey

Alternative culture systems employed for studies of influenza viruses include primary organ cultures (Tyrrell and Hoorn, 1965) and the allantois-on-shell system (Finter and Armitage, 1957).

Generally speaking, most viral infections are self-limiting. In a number of circumstances, however, some influenza viruses may persist or become latent in the host. Some experimental evidence suggests the existence of low-grade chronic infection of cultured cells with myxovirus (Holmes and Choppin, 1966; Gavrilov and others, 1972; Wilkinson and Borland, 1972; Osipova and others, 1980; Kaufman and Fields, 1985). Clinical manifestations or viral reactivation may occur at a time remote from the initial infection. Persistent infections can be defined as those in which infectious virus can be reproducibly and continuously recovered from the host well past the usual period of illness. There may be overt, mild, or no clinical disease as a result of persistent infection. Latent infections are those in which the virus remains within the host in a cryptic form. Infectious virus is only detected intermittently, usually in association with clinical recurrences of disease (Kauffman and Fields, 1985).

Cytopathogenic effects have not often been used as
evidence of influenza virus infection in cell cultures. These effects (reviewed by Pereira, 1961) are extremely variable for different virus strains and different cell cultures. Only Kopp and others (1968) reported on the presence of inclusion bodies in influenza virus infected fowl embryo fibroblasts.

3.5. Spread

Successful experimental routes of infection include aerosol, intranasal, intrasinus, intratracheal, oral, conjunctival, intramuscular, intraperitoneal, intravenous, cloacal and intracranial administration of the virus (Narayan and others, 1969).

In humans and pigs, most influenza A virus infections are probably transmitted by droplets formed during coughing or sneezing, and also by direct contact (Murphy and Webster, 1985). The virus may also spread by indirect contact, as well as via fluids in/on which the virus can survive long enough to find a new susceptible host. Accidental transmission of the virus through farm personnel, flies, equipment, food, air, are all very likely to occur (Bean and others, 1985). The possibility of virus transmission through water can not be excluded (Alexander, 1983). Almost all wild birds may excrete the virus in high concentrations in the faeces. The virus, remaining in dried fomites or in
infected carcasses for up to 2 weeks, and in lakes at low temperatures (4°C or lower), may spread between wild birds, aided by aquatic birds or by colonial nesting. The infected birds can then introduce the virus into poultry flocks or pig herds.

Vertical transmission of the influenza virus has always been controversial (Narayan and others, 1969; Brown and others, 1980; Lang and others, 1968). Mensik (1959) found that transmission of the virus through the placenta was possible, as influenza virus was isolated from lungs and other organs of an infected sow’s offspring. Clinically, Muller and others (1981) reported on abortion at the end of the gestation period of sows during a severe influenza outbreak among pigs in Hannover, Germany. Laboratory studies permitted the isolation of different strains of influenza virus (H3N2 and H1N1) from the fetuses and stillbirths (Gourreau and others, 1985). Vertical transmission of influenza virus in poultry may be possible, as the virus has been detected in eggs laid by experimentally infected birds (Bean and others, 1985). Eggs may also be contaminated and infected after they have been laid (D.J. Alexander, personal communication).
3.6. Clinical signs

Pathogenicity differences among the influenza viruses result in the production of a wide spectrum of clinical diseases that range in severity from fatal systemic to mild, sometimes inapparent respiratory disease (Miller and others, 1973). Severity of the disease is also determined by the host species infected (Alexander and others, 1978; Homme and Easterday, 1970; Alexander and others, 1986b), and in part by such factors as host age and sex, virus dose, environment and concurrent infections with other pathogens (Easterday and Beard, 1984). Many of these host and viral factors interact, to determine the outcome of infection and the capacity of viruses to produce disease.

3.6.1. Mammals

The clinical signs of influenza infection in mammals are generally associated with its location in the epithelial cells of the respiratory tract.

In pigs they are similar to those found in other mammals, and include decreased activity and feed consumption, nasal discharge, sneezing, coughing, fever, laboured breathing, roughening of the hair and sometimes conjunctivitis after an incubation period of 1 to 3 days (Shope, 1931; Nayak and others, 1965a; Tofts, 1987). Small enlargements of submandibular and cervical lymph nodes can be felt in a minority of cases. Recovery occurs in about 3-5
days, the herd being back to normal in 14 days. The respiratory tract may be severely affected, and being very contagious, the virus spreads rapidly among the herd to animals of all ages (Dea and others, 1980). Morbidity is high, but mortality is low. Fatal cases have been reported during outbreaks (Dea and others, 1980). Although no clinical disease may remain among the recovered pigs, if new animals are introduced to the herd, they can become infected soon after arrival.

3.6.2. Birds

In birds, disease signs also vary. Typical clinical signs of highly pathogenic avian influenza in chickens or turkeys include decreased egg production, respiratory signs, rales, excessive lacrimation, sinusitis, cyanosis of unfeathered skin especially of the combs and wattles, edema of head and face, ruffled feathers, diarrhoea, and nervous disorders (Easterday and Beard, 1984).

Nonpathogenic avian influenza viruses may be produced in the epithelial cells of the respiratory tract and the intestine of birds without inducing signs of disease. Virus may be shed in high concentration in the faeces (Slemons and Easterday, 1978; Webster and others, 1978; Kida and others, 1980).
3.7. Histopathology

It is difficult to arrive at a clear picture of the respiratory pathology in influenza, since it is frequently complicated by secondary bacterial effects.

Severe damage to the epithelium of the respiratory tract is the main feature in influenza virus infections (Hers and Mulder, 1961). This results in an epithelial wound, which facilitates secondary infection by bacterial respiratory pathogens. In the acute stage of the disease the ciliated epithelium degenerates and desquamates (Askanazy, 1919; Schmidtmann, 1920), and within 48 hours after infection, the nasal respiratory epithelium can be already largely destroyed, leaving only the germinative basement membrane (Hotz and Bang, 1957; Martin and others, 1959; Nayak and others, 1965b; Heath, 1973).

Significant pathological changes in other organs have not been consistently observed among infected mammals, although haemorrhagic, necrotic, congestive and transudative changes are characteristically observed in infections of birds by highly virulent avian viruses. Haemorrhagic changes are often severe in the oviducts and intestines. Sometimes, despite the high titre of virus, there are no lesions in the lung (Rowan, 1962). Encephalitis (perivascular lymphoid cuffing, vascular-glial and neuronal degeneration) may develop in the cerebrum and cerebellum. Alteration in myocardial tissue has also been observed in
some of the highly virulent virus infections (Polasa and others, 1984).

After about five days of illness regeneration of the epithelium begins, but complete resolution of the epithelial necrosis can take up to a month. Regeneration begins rapidly from the basement layer, and repair may proceed, through stages of stratified squamous, hyperplastic columnar and the return of relatively normal ciliated columnar cells excreting mucus (Francis and Stuart-Harris, 1938). As recovery progresses, the mucous membrane is repaired and the underlying inflammatory changes resolve. Small collections of mononuclear cells can persist for several weeks (Walsh and others, 1961).

Experimental influenza infections in pigs produced respiratory signs such as sneezing, coughing and dyspnoea. Pulmonary lesions were infrequent and limited to the cardiac and apical lobes. Histologically, interstitial pneumonia may be seen, with massive infiltration of the interalveolar spaces by mononuclear cells. Lymphocyte infiltration can be found around the bronchial tree, and sometimes a discrete hyperplasia of the lymphoid tissue (Vannier and others, 1985).

It has been shown that the distribution and progress of porcine influenza infection varies with the inoculum concentration (Nayak and others, 1965a). Other factors include droplet size of inoculum, method of inoculation and
virulence, and adaptation of the virus.

3.8. Immunology

Mutation and reassortment of individual segments of the influenza genome create a genetically heterogeneous virus population which is seen by the immunological defence system of the organism as new foreign antigens consisting of virus particles with new surface structures and with altered biological properties. At the same time, the host immune system acts selectively, leading to the selection of new antigenic components.

An immunological response has been demonstrated to each of the protein components of the virus (Schild and others, 1975), but only those to H and N are associated with resistance to infection (Askonas and others, 1982). Thus, the important proteins as far as B cell immunity to influenza is concerned are the surface H and N (Laver and Kilbourne, 1966; Hobson and others, 1972; Virelizier and others, 1976; Laver and others, 1976; Potter and Oxford, 1979). The main immunogenic target is encoded by the \( \text{H}_1 \) protein and immunoglobulin (Ig) M and IgA antibodies to H may exert its effect by preventing the attachment of virus to cells (Ada and Jones, 1986).

Individuals with haemagglutination-inhibition (HI) antibody in their sera are less likely than nonimmune
individuals to become infected during influenza epidemics, as demonstrated by a lower proportion showing four-fold or greater antibody rise (Davenport and others, 1969; Miller and others, 1973).

Immunization with purified isolated N is associated with reduction of pulmonary virus titre and less extensive lung lesions after challenge with virus containing the same N (Schulman and others, 1968).

Experimental animals which have been immunized with N and have potent anti-H antibodies can still be infected with the virus. Clinical signs of illness, however, do not appear because spread of newly formed virus in the host organism is prevented. Antibody to N may bind the virus to the cell surface by bridging antigenic sites on virus particles to similar sites on the cell membrane (Murphy and others, 1972; Couch and others, 1974; Rott and others, 1974; Dowdle and others, 1976). Limited replication, with the production of the whole spectrum of virus antigens, results in solid protective immunity and full prevention of a secondary infection (Schulman and others, 1968; Rott and others, 1974).

Antigenically the N is strain specific and displays antigenic variation within a subtype and major antigenic differences between subtypes (Hay and Skehel, 1982).

When evaluating the host's antibody response, one must take into account prior infection by influenza virus and the
extent of antigenic variation of the infecting virus.

The immune response evoked by influenza infection comprises both humoral and different cellular reactions. Humoral immunity is important in protection (Portnoy and others, 1973; Virelizier and others, 1976), IgG antibody being the protective factor. Because influenza virus is principally an extravascular attack on a superficial tissue, the influence of circulating antibody in preventing injury to the respiratory epithelium depends on the diffusion of these antibodies into the nasal secretions. These combine with virus and prevent its pathological action (Francis, 1941; Murphy and Webster, 1985).

Specific cellular immunity was observed by Feinstone and others (1969), with the participation of cytotoxic thymus-dependent lymphocytes (T-cells) (recognition of NP gene antigens, common to all influenza A viruses), by killer activity (towards virus infected cells) and delayed-type hypersensitivity. Fleischner and others (1985) demonstrated that T-cells also recognize non-transmembranous virus proteins. They consider that certain epitopes of the viral NP, H and probably M1 protein are expressed on the surface by means of active presentation by the infected cell, even in abortive infections. T-cells also regulate the production of anti-H antibody by either enhancing or suppressing bursa-equivalent lymphocytes (B memory cells) (Virelizier and others, 1974).
Lymphocytes mediating influenza virus-specific immune functions have been isolated from the blood and lower respiratory tract secretions of infected individuals (Jurgensen and others, 1973).

Endogenic interferon secretion was studied by Gresser and others (1976). Influenza viruses are efficient inducers of interferon, and are sensitive to their antiviral properties (Hill and others, 1972; Murphy and others, 1973). It has been detected during the acute stages of illness in the upper respiratory tract secretions and in sera of influenza virus-infected individuals (Murphy and others, 1973). According to Murphy and Webster (1985), however, clinical efficacy of interferon has not been proven.

There is evidence that macrophages are capable of inactivating the virus, and the protective role of the Kupffer cells of the liver was mentioned by Virelizier (1983).

Protection against influenza probably involves to some extent all the components of the immune system. Unfortunately, these immune mechanisms are circumvented by the unique capacity of influenza viruses for periodic major antigenic change, and to a lesser extent are compromised by less extensive alterations in antigenic structure (see Section 6 in this Chapter).
4. DIAGNOSIS

4.1. Virus identification

Direct detection of virus in clinical samples can be achieved through electron microscopy, by the use of histological stains or by demonstrating the presence of HA agents (Murphy and Webster, 1985).

The 9 to 11-day-old chick embryo is the most suitable, easily available and reliable host system for the isolation of influenza viruses, following intra-amniotic and/or intra-allantoic inoculation. Cell cultures may also be used for the isolation of influenza viruses, often with trypsin being added to the medium, as a conditioning factor for the cleavage of the H (Murphy and Webster, 1985).

The allantois on shell technique (shell fragments, with adherent chorio allantoic membrane, suspended in synthetic medium) is also useful for isolation, growth and assay of these viruses (Webster and Laver, 1975).

Laboratory animals are rarely used, and because of the considerable variation between species, the natural host is used as often as possible for laboratory studies.

Methods for identification of influenza viruses are described in standard technique manuals (Beare, 1980).

Early growth of Orthomyxoviruses can be recognized by haemadsorption of erythrocytes onto cell culture monolayer (Vogel and Shelokov, 1957).
Virus production in eggs as well as the release of virus from infected cells may be followed by testing the culture fluids for virus products, employing tests for biological activity or by assays of influenza-specific antigens. The most frequently used method of following the course of virus release is the HA test (Hirst, 1941), which takes advantage of the haemagglutinating property of the virus. The test was standardized by Miller and Stanley (1944), and the microtitre system developed later by Sever (1962) and Hierholzer and others (1969).

When a HA agent is isolated after enrichment in embryonated eggs or tissue culture, the new isolate must be distinguished from all other agents that exhibit HA, such as Newcastle disease viruses and other paramyxoviruses. Also, infections by Chlamydia, Mycoplasma and bacteria in susceptible species must be ruled out by virological and serological methods.

The identification of the virus isolated from samples can be achieved by immuno-double-diffusion (IDD), single radial immuno diffusion (SRID) or complement fixation (CF) tests, and the samples separated into groups A, B or C (Palmer and others, 1975; Schild and Dowdle, 1975).

New influenza subtypes must be tested in immunodiffusion assays with influenza A antinucleoprotein or antimatrix protein antisera.

The type-specific CF test (Lief and Henle, 1959) has
traditionally been used for NP protein identification. The IDD test was recommended as a replacement for the CF test (Dowdle and others, 1974). It does not permit quantitation of the antigen. The SRID test of Mancini and others (1965) may be useful for assay of NP and M proteins.

Subsequently the antigenic nature of the surface antigens should be determined, by the HI, neuraminidase inhibition (NI), IDD, SRID, single radial haemolysis (SRH) or CF tests (Palmer and others, 1975; Schild and others, 1975).

The specific inhibition of HA and NA permit subtype identification (Dowdle and others, 1974). A panel of antisera that is monospecific for each of the 13 H influenza subtypes is used for the HI test, and antisera to each of the 9 N types are used in NI tests.

As an alternative to erythrocyte agglutination tests, SRID and SRH has been used to assay H antigen (Schild, 1970; Schild and others, 1976). They are extremely sensitive in detecting differences between strains.

The WHO has recommended that N subtypes be distinguished on the basis of the NI and IDD test results (WHO, 1980; Schild and others, 1980), since the IDD test is broadly reactive and may demonstrate antigenic relationships not apparent by other methods.
4.2. Serology

The study of the circulation of porcine influenza within the pig population relies mainly on the detection of antibodies. The assay normally used for this purpose has been the HI test (Palmer and others, 1975), although other workers (Schild and others, 1972) maintain that the SRID test has the advantages of rapidity and simplicity.

Serological methods are very useful when virus shedding is brief and of low titre as is often the case with respiratory viruses, whereas the serological response to infection is quite high and quick. For serological diagnosis of influenza by pigs, it is best to obtain paired acute and convalescent samples of sera, in which a four-fold or higher increase in specific antibody may be considered evidence of active infection. The presence of maternal and vaccinal antibodies can confuse the diagnosis as the former can be detected for up to 4 months after birth, and inhibit the production of natural antibody during an infection; vaccines may induce antibodies similar to those of natural infection (Hunneman and Van Oirschot, 1987).

The serum of many species contains inhibitory substances that may interfere with the specificity of HI tests and other tests. The presence of non-specific inhibitory substances in normal serum from several species was described by Hirst (1942), McCrea (1946), Burnet and
Stone (1947), Davenport and Minuse (1964), among others. Further studies (Francis, 1947; Smith and others, 1951; Sampaio and Isaacs, 1953; Cohen and others, 1963) showed that there are three types of inhibitors, termed "Francis inhibitor" or "Alpha inhibitor" (thermostable), "Chu inhibitor" or "Beta inhibitor" (thermolabile), and "Gamma inhibitor". The inhibitors are usually glycoproteins found in the serum (Palmer and others, 1975). These inhibitors have to be inactivated when assessing antibody patterns in immune sera. Different laboratories use different methods for this purpose (Kaplan and Payne, 1959).

McCrea (1946) showed that heat treatment (62°C for 15 to 20 minutes) destroyed the non-specific inhibition in rabbit serum, by denaturation of the globulin responsible for this effect.

Non-specific inhibitors were also studied by Coleman and Dowdle (1969). They tested sera from monkey, goat, chicken, human, rabbit, ferret, guinea-pig and horse, by treating them all with heat, trypsin, periodate, receptor destroying enzyme (RDE) and kaolin, to determine the most effective way of removing non-specific inhibitors. They concluded that the presence and reactivity of influenza H inhibitors in animal sera are complex and unpredictable. In their opinion, empirical findings should be taken into account in the interpretation of HI results.

In a similar experiment, Ananthanarayan and Paniker
(1960) could not find a single technique that was suitable for all animal species sera tested, and in their opinion the qualitative and quantitative differences of the inhibitors are responsible for this.

Combinations of heat, N, trypsin and periodate treatment have been used also. The most commonly used treatments are potassium periodate and RDE (Schild and Dowdle, 1975).

With some sera, particularly guinea pig sera, kaolin is the only effective treatment for removal of inhibitors.

Since these treatments may also reduce the level of specific antibody, the procedure used must be designed for the specific virus strain and type of serum under investigation.

In addition to non-specific inhibitors of HA, some non-chicken sera contain nonviral substances that agglutinate the chicken erythrocytes used in the HI test. This HA must be removed by pre-treating the serum with chicken erythrocytes (Nakamura and Easterday, 1967).

The detection of type-specific antibodies is assayed by the CF and IDD tests. The CF test, with soluble antigen, commonly prepared from infected chorio-allantoic membrane is type-specific, essentially not affected by non-specific inhibitors and may be conducted with antigen from any of a number of strains of the same type.

Immunofluorescent procedures have been used in various
studies on porcine influenza (Hers, 1962; Pensaert and others, 1986) but this technique has not been used routinely in influenza serology.

Subtype specific antibodies to influenza viruses may be detected by a number of laboratory tests involving different techniques.

The IDD test has been used by Schild and others (1971).

HI is the best known and most used test for antibodies to the H of influenza. This test, as described by Hierholzer and others (1969) (used throughout this dissertation) is considered to have a reproducibility between 84 and 96 per cent, when evaluated statistically.

The HA reaction is inhibited by antibodies specific for the viral H and also by a number of soluble glycoproteins found in the serum and other body fluids, as already described. HI due to either specific antibody or to non-specific inhibitors is quantitated by mixing a standard amount of virus with dilutions of the serum to be tested. Erythrocytes from a variety of species, used as an indicator of free virus, are then added and titres are determined from the highest dilution of serum which prevents agglutination.

HA may also be inhibited by the specific activity of anti-N or anti-host antibodies.

Complete inhibition can best be determined by slanting the test plate and observing the teardrop shape of nonagglutinated cells streaming at the same rate as the cell
controls.

The advantage of the HI test is that it can be performed with a variety of erythrocyte species over a wide range of pH concentrations. The temperature at which it is performed is not critical, except with influenza type C, which must be tested at 4°C.

For the HI test, the virus HA antigen is adjusted to contain four HA units (HAU) in a specified volume.

The NA reaction, using standard methods (Aymard-Henry and others, 1973; Takatsy and Barb, 1979) or lectins (Luther and others, 1983) is used for the identification of N subtypes of influenza viruses.

Aymard-Henry and others (1973) described the NI test as performed by the WHO World Influenza Centre, London, England, and the WHO International Influenza Centre for the Americas, Atlanta, Ga., U.S.A., and recommended to the laboratories in the WHO influenza programme.

In short, the steps involved in the assay of neuraminidase activity comprise the release of free N-acetyl neuraminic acid from the fetuin substrate by the action of neuraminidase, the conversion of N-acetyl neuraminic acid to beta-formyl pyruvic acid by periodate oxidation; the formation of chromophore by thiobarbituric acid and the extraction of chromophore into organic solvent for spectrophotometric analysis (Fig.5). For the NI test, the
Fig. 5. Schematic presentation of the neuraminidase inhibition test.

Free N-acetyl neuraminic acid is released from the fetuin substrate by the action of neuraminidase.
Periodate oxidation converts N-acetyl neuraminic acid into beta-formyl pyruvic acid and a chromophore is formed by the action of thiobarbituric acid. This chromophore, after extraction into acid butanol, may be measured by spectophotometry, the optical density at 540 nm found corresponding to the NA present.
Viral neuraminidase

Fetuin substrate

Free N-acetyl neuraminic acid

Periodate oxidation

Beta-formyl pyruvic acid

Thio barbituric acid

Chromophore

Extraction into acid butanol

Optical density neuraminidase activity
standard N dose is incubated with serial dilutions of normal and test sera; the inhibitory effect of serum on neuraminidase activity is determined and the NI titre is calculated (Palmer and others, 1975).

The level of inhibition by antibody has been found to be independent of substrate concentrations (Fazekas de St.Groth, 1963). Van Deusen and others (1983) reported on a simplification of the NI test, and described an adaptation of the macro NI test to a microtitre plate method that can be used for antigenic classification of viral N and detection of antibodies to these antigens. Kilbourne and others (1968); Holston and Dowdle (1973) and Smith (1977) reported on a neuraminidase-haemagglutination-inhibition test that specifically detected antibodies to the neuraminidase, with a high degree of reproducibility and relative specificity.

A micro method based on Warren's colour reaction for influenza NA and NI assays was described by Takatsy and Barb (1979). They developed a simple test for large-scale investigations. This micro-method permitted the titration of 60 serum samples in much less time than other assays, saving reagents and equipment.

SRID techniques offer a simple and rapid procedure for assaying sera for antibody to the surface or internal antigens of influenza viruses. One advantage is that standardized immunoplates prepared in a single centre may be
used for serological surveys in many countries, thus eliminating variations from laboratory to laboratory which are inevitable in the case with HI and CF tests. An additional advantage is that very small volumes of serum or even whole blood obtained by venopuncture may be used in surveys (Palmer and others, 1975).

Schild and others (1972) described a quantitative SRID test which is a sensitive, rapid and convenient method of estimating antibodies to the H and N of the influenza virus. Intact influenza virus particles are incorporated into an agarose gel and in layers of this, circular wells are cut to accommodate the antisera. Zones of opalescence surrounding the wells with antibodies to the H or N of the virus incorporated into the gel may be detected within 4 hours. Zones produced by anti-H antibodies are dense and sharply defined, whereas those produced by anti-N antibodies are less intense and with less defined margins. The zones are stable and their diameters were constant in size when the plates were stored by immersion for several weeks in phosphate buffered saline. Non-specific inhibitors do not interfere with the specificity of the test, and even whole blood samples can be used for antibody assay in this test. The test depends upon an increase in the light scattered by the virus after primary attachment of antibody and is independent of secondary agglutination or precipitation (Schild and others, 1976). The prepared plates of agarose
gel containing virus can be stored at 4°C for up to 6 months before use, giving reproducible results.

Immunoprecipitation methods for the detection and immunological comparison of the H of different influenza A viruses were described by Schild (1970).

Single radial haemolysis is a technique which was first used in 1965 to detect antibodies of a specific allotype directed against anti-red blood cell antibodies (Weiler and others, 1965). Since then it has been used widely to detect antibodies against a number of antigens including influenza (Russell and others, 1975; Callow and Beare, 1976; Schild and others, 1976; Ogawa and others, 1978; Chakraverty, 1980).

An haemadsorption immunosorbent technique was developed by Van der Logt and others (1984), to detect specific IgA or IgM response after infection with influenza A and B viruses.

Sato and others (1984) developed a stabilized modification of the single radial complement fixation test for detecting influenza antibodies.

The enzyme linked immuno absorbent assay (ELISA) has been widely used to detect antibodies against human influenza viruses and their specific antigen. Denyer and others (1984) applied ELISA to porcine influenza serology and
virus detection.

An ELISA for the detection of IgG and IgM antibodies against influenza A and B virus was described by Koskinen and others, (1987).

A problem in the use of ELISA for influenza antigen and antibody detection is the variable nature of the virus itself. Different subtypes will give different results, and for each new subtype that is found new plates must be produced. Further, no indication of the biological properties of the virus, such as pathogenicity, are given by this test.

Techniques for assay of influenza antibodies in nasal secretions are similar to those described for sera, except that IgA antibodies do not fix complement.

Considering the large variety of influenza viruses isolated until now, and the potential of many others to be created by reassortment, a more objective classification measuring the pathogenicity of the isolates is under study by several authors. Pathogenicity indexes has been set up for avian influenza viruses (Alexander, 1986b; Senne and others, 1986; Karunakaran, 1988) but not yet for mammalian influenza viruses.
5. TREATMENT AND PROPHYLAXIS

Knowledge of genetic and environmental influences on influenza is inadequate, and there is no specific therapy for influenza, treatment being essentially symptomatic and palliative. Antibiotics and other antibacterial agents do not affect the viral infection, but may sometimes be used to prevent complications such as bacteriological coinfection. Specific control measures include the use of antiviral drugs and vaccination. General prophylactic measures in mammals are mainly based on preventing the introduction of influenza viruses of wild aquatic birds into domestic pig herds and poultry (see Section 3.5 in this Chapter).

5.1. Antivirals

Many authors have claimed success with antiviral substances against influenza infections (Barry and others, 1962; Conti and Portincasa, 1984; Manolova and others, 1984; Aso and others, 1984).

Due to the nuclear step in the replication of influenza virus, increasing concentrations of Actinomycin D produce a progressive decrease in yield of influenza virus. From studies of Barry and others (1962) and Barry (1964) it appeared that Actinomycin D was blocking the production of virus specific RNA.
Amantadine hydrochloride (1-adamantanamide hydrochloride) and its analogue rimantadine (methyl-1-adamantanemethyl amine hydrochloride) have antiviral properties against all subtypes of influenza A virus (Wingfield and others, 1969; Lang and others, 1970; Zlydnikov and others, 1981) but not against influenza B or C (Hayden and others, 1980). Also, under certain conditions simulating natural transmission of virus amantadine and rimantadine-resistant viruses can arise and be transmitted to other animals in contact (Lang and others, 1970). Amantadine is believed to inhibit influenza virus replication by interfering with uncoating and transcription by the polymerase (Skehel and others, 1982; Indulen and others, 1984), although the effect is dose-dependent (Hay and Zambon, 1984).

Antiviral activity of oxydiazin for influenza A and B viruses was found by Gagov and others (1984). The antiviral activity of ribavirin for influenza has only been established in vitro. It can have a prophylactic or therapeutic effect (Cohen and others, 1976; Magnussen and others, 1977).

Despite the in vitro susceptibility of the virus to interferon, there is no evidence of clinical efficacy of interferon therapy in humans, pigs or birds (Murphy and Webster, 1985). According to Tashiro and others (1987), protease inhibitors may have a therapeutic effect in influenza infected animals, by interfering with H cleavage.
5.2. Vaccines

The variable epidemiological behaviour of influenza causes immense problems particularly in the field of prevention. In general natural infection is considered to produce a more effective and broader immunity than inactivated virus vaccines (McLaren and others, 1974). Vaccines against influenza are at present only partially effective in preventing the disease and do not limit the broad spread of influenza in epidemics and pandemics. Also, different animals respond to a different extent to the various antigens when immunised with the same preparation of isolated H subunits (Webster and Laver, 1975). Even so, vaccination is the current principal means of controlling influenza. Based on the knowledge of strain variation and the variation in antibody patterns of the different age groups in the population, vaccines are prepared that contain a pool of antigens, including those known to have been prominent in epidemic strains of influenza virus. It is essential to incorporate in the vaccine any new antigens that have appeared by antigenic variation (Buonavoglia and others, 1987; Haesebrouck and others, 1987).

Recently, Haesebrouck and others (1987) have reported on the successful use of human vaccines to protect pigs against porcine influenza, and currently, commercial porcine influenza vaccines are available in several West European
countries. In general, increasing the dose of vaccine increases the level of anti-viral antibody achieved and the administration route has a little influence on the immunity produced (Murphy and Webster, 1985). Outbreaks of influenza were least frequent in areas with high vaccine utilisation, suggesting that the vaccine had lead to increased herd immunity.

Oil in water emulsion vaccines, containing H1N1 and H3N2 strains proved to be protective for pigs (Kuiper, 1988), and have been in use in most of the european pig-rising countries in recent years.

Experimentally, vaccines have been produced in Echerichia coli plasmids, by insertion of a complementary DNA coding for the H gene into the plasmid (Arnon, 1980). A more promising approach seems to be the Immunostimulating Complex of Virus Membrane Proteins (ISCOM), a novel structure for antigenic presentation of membrane proteins from enveloped viruses (Morein and others, 1984).

Maternal antibodies are present in the colostrum of sows, at the same level as in the serum at time of farrowing and can be detected in the suckling piglets up to four months after birth, giving some protection against influenza virus (Wilkinson, 1983).

It is not possible to forecast minimal protective serum antibody titres, when various virulent virus strains are concerned. Where short-term protection is concerned, a titre
of 1:32 or higher is considered sufficient (Meiklejohn and others, 1952; Hobson and others, 1972) to prevent H1N1 influenza viral replication in the respiratory tract. This was not the case with H3N2 subtypes, where much higher HI titres were necessary for protection (Haesebrouck and others, 1987).
6. EPIDEMIOLOGY

Epidemiology is the science of cause, distribution and frequency of disease (Dorland, 1985; Thrusfield, 1986). In the case of influenza this involves the distribution, transmission, and antigenic variation of the causative agent. Aspects of the distribution of the virus have been dealt with in Section 1, and transmission in Section 3. This section will deal mainly with variations observed between these viruses, and the role these play in the epidemiology of the disease.

For many years, influenza in man was diagnosed principally on epidemiological grounds, rather than by aetiology or clinical course. The disease occurred in rapidly spreading epidemics, appearing in small, focal outbreaks, and spreading irregularly in pandemic form. Its origin has been questioned for decades, and although nearly 400 years have passed since the first clinical description of influenza, theories for its epidemiology are still conflicting, and the explanation for the origin of new subtypes is still the subject of much debate (Murphy and Webster, 1985). The processes proposed to be involved in the epidemiology of the disease may all have been operating at different times and may still be at work.

Understanding the epidemiological behaviour of influenza can only be achieved by intensive and continued international surveillance (Thrusfield, 1986). The number of
influenza strains recognized as antigenic variants has increased in recent years, but this may be only an apparent increase resulting from examination of a greater number of strains and improved techniques and reagents.

6.1. Theories for the origin of pathogenic influenza virus

Influenza has been a constant target for mysticism, hypothesis, theory, and philosophical speculation. There are at present three main explanations for the origin of the pandemic strains: a) an influenza virus is transmitted to other animals or man and acquires the capacity to cause disease in this second host (anthropozoonosis); b) The existing strains undergo multiple rapid changes by mutation to give rise to surface antigens that are unlike earlier strains; and c) an influenza virus undergoes genetic reassortment with another influenza virus and acquires a completely new surface protein while retaining the capacity to cause disease in the parent host.

In early times, causal links were proposed with the stars, the weather and poisonous gases from swamps. The idea that cometary material might carry biological and pre-biological material which could affect terrestrial biology is sustained by Hoyle and others (1986).

The effect of season has never been well understood, however, it has been observed that epidemics occur more
frequently in summer monsoons in the tropics than in other periods, even though temperatures throughout the year vary by only a few degrees. In temperate and colder climates, peaks of influenza activity have nearly always occurred in winter. It is possible that sunspots and season affects wild bird migration and indirectly causes the spread of influenza virus strains along the migration routes (Yagodinskii and others, 1977).

6.1.1. Influenza as an anthropozoonosis

A hypothesis, based on the prevalence of subtypes and its repetition in cycles, states that the influenza virus, when eliminated from a population with specific antibodies, survives in other populations until the immunity of the first population decreases and permits the reintroduction of the virus (Beare and others, 1971; Webster and others, 1977b; Easterday and Beard, 1984).

a) Serological studies

Until 1970 most of the information on the relationships between human and animal influenza viruses was based on serological studies (Easterday and others, 1977).

Antibodies to porcine influenza in sera of humans were found by Andrewes and others (1935), showing that human beings in England and America had been exposed to an
influenza virus serologically similar to, or identical with, the porcine influenza virus. Kaplan (1982) reviewed evidence for the animal origin of human influenza epidemics and pandemics. Both he and Tillon and others (1980) believed that pigs could be incriminated in the 1918-1919 pandemic, by acting as reservoirs. The very close relationship between the influenza A viruses causing the human pandemic in 1918 and those affecting pigs from 1930 (Davenport and others, 1953) strongly suggested that transmission took place at that time between the two hosts.

Antibodies against avian influenza virus Chicken/Scotland/1/59 (H5N1) were found in humans (Tumova and others, 1968) raising the possibility that an avian virus could have been involved in the human pandemic of 1918, and that pigs acquired the infection from man later on (Easterday, 1970).

The presence of antibodies against influenza viruses of porcine, equine, canine, and avian origin have also been demonstrated several times in human sera (Kluska and others, 1961; Masurel and Mulder, 1966; Davenport and others, 1969; Kasel and others, 1969; Schnurrenberger and others, 1970; Hinshaw and others, 1983). Similarly, the presence of antibodies to human influenza virus in pig sera has been demonstrated by numerous authors (Kaplan and Payne, 1959; Kundin, 1970; Styk and others, 1971b; Harkness and others, 1972; Satsuta and others, 1981; Ottis and others,
1982; Miwa and others, 1986), but the absence of viral isolates made it difficult to confirm the involvement of animals in human epidemics and vice-versa (Schild and Stuart-Harris, 1965; Tumova and others, 1968; Davenport and others, 1969) until the 1970's when large numbers of viruses were recovered from different animal species, each species being possibly involved in human influenza epidemiology.

b) Virus strains relationships

The first demonstration of the existence of antigenic relationships between the surface antigens of type A influenza viruses isolated from different species was between the human virus A/Singapore/1/57(H2N2) and an influenza virus A strain isolated from North American turkeys. Pereira and others (1967) showed that these viruses contained antigenically identical N but distinct H antigens. Since this finding numerous other antigenic relationships have been observed.

c) Evidence of interspecies transmission

The association of the disease in animals with human influenza epidemics has been confirmed several times. A number of historical accounts of the disease mention the coincidence of influenza-like diseases in animals,
immediately preceding or accompanying influenza epidemics in man (Andrewes and others, 1935; Laidlaw, 1935; Schild and Stuart-Harris, 1965; Masurel and Mulder, 1966; Tumova and Pereira, 1968; Kaplan, 1969; Pereira, 1969; Tumova and Easterday, 1969; Beare and others, 1971; Styk and others, 1971b; Webster and others, 1971; Harkness and others, 1972; Tumova and Schild, 1972; Beveridge, 1977; Ehrengut and others, 1980; Schild and others, 1980).

The outbreak of influenza among seals (Lang and others, 1981; Webster and others, 1981a and c) provided the first confirmation of the hypothesis of interspecies transmission from birds to mammals associated with disease production. By competitive hybridization assays with the RNA segments of this influenza virus, Webster and others (1981b) demonstrated that all eight segments were most closely related to recent avian isolates.

Human to animal transmission

The presence of an influenza variant strain in the pig population of a country coincident with the current epidemic strain infecting the human population has been described frequently (Webster and others, 1977a and b; Aymard and others, 1980; Hannoun and Gourreau, 1980; Tillon and others, 1980; Nerome and others, 1981, 1982; Haesebrouck and others, 1985; Roberts and others, 1987). The persistence of human influenza virus in pig populations has been studied
by several authors. Tumova and others (1980a and b) presented evidence that influenza A (H3N2) viruses were brought into pig herds by diseased people during human epidemics, and that the viruses could persist in the pig herds for at least two years. It was found that pigs are susceptible to infection with several different variants of the H1N1 and H3N2 subtypes of influenza A virus. These include the H1N1 viruses of classical porcine influenza, the H1N1 viruses antigenically similar to avian isolates, and H1N1 and H3N2 viruses similar to strains from humans.

Animal to human transmission

The role played by influenza viruses from mammals and birds in the origin of some pandemic human strains was emphasized by Hinshaw and others (1979b).

Frequently, wild birds are seropositive to human and domestic mammal as well as avian strains of influenza virus (Kovalchuk-Ivanyuk and others, 1975; Sakstelskaja, 1975). Also, an increase of antibodies against influenza virus was observed in wild birds at the same time, or immediately after the active circulation of the same subtype in the Asian continent (Sakstelskaja, 1975). Influenza A viruses have also been frequently isolated from apparently healthy birds (Hinshaw and others, 1978b; Ottis and Bachmann, 1980;
Sinnecker and others, 1983a). Many such strains are closely related to others, of human or animal origin, in the antigenic structure of their internal ribonucleoprotein. Pensaert and others (1981) suggested that influenza viruses, persisting in birds, could infect mammals. Other workers put forward similar theories (Rosenberger and others, 1974; Sakstelkaja, 1975; Hinshaw and others, 1981a and Witte and others, 1981), some observing that epizootics occurred mainly during the migration period of birds to the continent. Migration of birds provides the ideal opportunity for influenza viruses to spread to other species by aerosols and the faecal-oral route. Many of the influenza viruses of ducks multiply in the cells lining the intestinal tract without producing any signs of disease, and are shed in high concentrations into water (Webster and others, 1977b; 1978). These viruses will remain viable for at least four weeks in water at 4°C. Since avian strains may replicate readily in mammalian cells and in mammals (Sinnecker and others, 1983a), there is a distinct possibility that influenza viruses are transmitted between wild birds and domestic birds and eventually to mammals including man.

Beare and others (1980), studying Hsw1N1 influenza viruses in man, concluded that the H1N1 viruses recovered from humans from the influenza outbreak at Fort Dix, New Jersey, U.S.A. (Weekly Epidemiological Record, 1976), and from recent single human infections (Smith and others, 1976; Thompson and others, 1976; O’Brien and others, 1977), were
wholly derived from enzootic porcine viruses that underwent limited human adaptation through man-to-man passage. Later, De Jong and others (1986) also reported on infection of man with porcine influenza-like viruses.

**Pig and bird intertransmission**

Porcine influenza viruses have frequently been isolated from birds, and *vice-versa* (Butterfield and others, 1978a; Yamane and others, 1979b; Hannoun and Devaux, 1980; Ottis and Bachmann, 1980; Mohan and others, 1981; Alexander and Spackman, 1981; Pensaert and others, 1981; Kida and others, 1988).

Several outbreaks of influenza have been reported among turkeys in Europe and the U.S.A. (Alexander, 1982a). Studies by Hinshaw and others (1983) demonstrated that some H1N1 influenza viruses isolated from domestic turkeys in the U.S.A. were genetically closely related to those from pig, which offers the theoretical possibility of transmission of avian influenza viruses to pigs and *vice-versa*. Aymard and others (1985) concluded that transfer of influenza H1N1 viruses occurs between pigs and turkeys and that antigenically similar viruses cause outbreaks of disease in both species. The transfer of H1N1 influenza viruses from pigs to turkeys was suggested from field evidence (Pomeroy, 1981) of avian influenza from 1964 to 1981 in the U.S.A.;
however persistence of avian influenza A viruses in turkeys following natural infection was not well defined at that time.

Homme and Easterday (1970) analysed the effects of influenza A/turkey/Wisconsin/1966 (H9N2) virus in ring-necked pheasants, mallard ducks, Canada geese, and domestic geese, by virus isolation and HI tests. They concluded that an inapparent carrier state could exist, and that turkeys might remain infected for a considerable period, shedding virus intermittently during this time.

Experimental interspecies transmission of influenza virus was achieved several times: human isolates to rats and guinea-pigs (Stuart-Harris, 1937), human isolates to chick embryo (Burnet, 1940), human isolates to pigs (Kundin and Easterday, 1972), avian isolates to mammals (Hinshaw and others, 1981c), human isolates to mice (Polasa and others, 1984), human isolates to cats (Paniker and Nair, 1970), pig isolates to cattle (Lopes and Woods, 1987), avian isolates to mice (Otsuki and others, 1987).

In summary, transmission of influenza viruses between animals and humans does occur, principally pig and human (Mayr, 1980), and probably turkey to pig to human (Ottis and Bachmann, 1980); animals (pigs, birds and possible other species) may be reservoirs of influenza viruses and there exists a genetic relationship between human and animal influenza virus types (Bachmann, 1983). It is possible that
the presence of the virus in each animal acts as a reservoir of infection for another.

The above theory has been criticized by Hope-Simpson and Golubev (1987). They have argued that it does not explain the world-wide spread of the virus in a very short period of time, nor does it explain the abrupt disappearance of a predecessor serotype. Nonetheless, it is remarkable that the progression of epidemics is closely associated with the intensification of international contact, either among humans or animals through import and export. Burnet (1979) suggested that influenza infections of humans probably did not occur until sufficiently large aggregations of people existed to maintain the cycle of influenza transmission. Since the embryonated egg, used world-wide for isolation purposes, may be strain selective (Shortridge, 1981), it is possible that the reported isolation frequencies and antigenic combinations do not necessarily represent the actual occurrence of the virus. Also, the isolation of an influenza virus from a diseased herd or flock is normally taken as proof of cause of disease, even though several other agents may be coinfected with the influenza virus playing a secondary role.
6.1.2. Virus reactivation

Alternative explanation for the origin of influenza viruses is that an old virus remained hidden and unchanged in a frozen state in the colder areas of the world, or preserved in an animal reservoir, or even retained in an integrated form in the genetic material of humans or animals, or as a persistent infection. In the latter situation, the lytic virus must change so that it no longer injures the host, or the host cell must change so, that it can support virus replication without being destroyed.

Hope-Simpson (1979) suggested that influenza may remain as a latent human infection, producing asymptomatic carriers who shed mutant viruses which are capable of escaping specific immunity in the individual and their contacts, following various environmental seasonal stimuli, such as sunspots.

Hope-Simpson and Golubev (1987) suggested that influenza A virus is reactivated periodically in carriers, by seasonally mediated stimuli such as variations in solar radiation. These virus particles encounter the host immunity they themselves produced. This favours selection of mutants, by small antigenic variation and the disappearance of predecessor types.

According to Wallace (1977, 1979) pigs can harbour latent H1N1 influenza A virus, while Mensik (1962) and Nakamura and others (1972) have demonstrated that sows
latently infected with influenza virus can transmit the virus to their offspring. Gourreau and others (1985) isolated influenza virus from aborted pig foetuses and stillborn piglets, and Mensik (1962) demonstrated symptomless latent infection of the offspring in infected sows. Many bird species, specially ducks, also harbour influenza virus, the virus replicating in these hosts without causing illness (Sinnecker and others, 1982). Jakab and others (1983) have demonstrated the persistence of high concentrations of influenza viral antigen for more than a year in the alveolar cells of mice, although infectious virus disappeared within 10 days.

The subtype H3N2 of influenza A viruses isolated from pigs during this decade has only sporadically been associated with clinical manifestations (Ottis and others, 1982; Haesebrouck and others, 1985; Buonavoglia and others, 1987, Castro and others, 1988). In the past, this subtype has been isolated or serologically diagnosed in apparently healthy slaughter pigs (Shortridge and others, 1977; Fontaine and others, 1983). The isolation of six H3N2 influenza strains closely related to human A influenza virus strains, from both healthy and diseased pigs in Italy, emphasizes yet again the possibilities of interspecies transmission and the existence of asymptomatic carriers (Buonavoglia and others, 1987).
6.1.3. Antigenic variation

Several types of antigenic variations have been observed among influenza viruses, all having particular effects on its epidemiology. Antigenic variation among influenza viruses in man since 1933 has been reviewed by Webster and Laver (1975). The H1N1 influenza virus isolated in 1933 shifted into H1N1. Then, the H2N2 strain appeared in 1957, with totally different H and N from the preceding influenza virus. The Hong Kong strain, which appeared in 1968, had only a major change in the H (H3N2). The fact that the 1957 H2N2 strain had totally different external antigens explained the severity of the pandemic, as no immunity to either of the external antigens was present in the human population.

Studies suggest that minor epidemics occur as a result of selection of variants arising from the preceding pandemic virus (antigenic drift), and pandemics occur upon the sudden emergence of a virus with antigens new to all, or part, of the population affected (antigenic shift) (Beare and others, 1971).

Both drift and shift have been observed in influenza A viruses, but only antigenic drift has been detected in influenza B viruses, while influenza virus C has been much less studied, and has rarely been isolated. Pereira (1969) and Beare and others (1971) suggested that the lack of major antigenic shift in type B influenza viruses may be a
consequence of the absence of such influenza viruses in lower animals and birds.

Drift

Point mutations in a gene, leading to the accumulation of amino acid sequence changes that alter the antigenic sites such that they are no longer recognized by the host's immune system, account for antigenic drift (Burnet, 1956). Mutations in the conserved parts presumably lead to loss of function and may be lethal. Mutation in a variable part might not interfere severely with function, but possibly with the antigenic properties of the virus. The process has been considered as a response to immunological pressure by the host.

The phenomenon of antigenic drift has been demonstrated under natural conditions (Schulman and Kilbourne, 1969; Meier-Ewert and others, 1970) and in the laboratory (Archetti and Horsfall, 1950; Hamre and others, 1958; Laver and Webster, 1968), by using immunological selection against the earlier strain. It was observed among influenza viruses A and B by Davenport (1977). Chakraverty (1978) demonstrated that antigenic variation also occurs among type C influenza viruses. Drift is most pronounced in human influenza A strains, although antigenic variation of porcine influenza A virus was detected by Meier-Ewert and others (1970). Analysis with monoclonal antibodies indicates that minor
antigenic heterogeneity is detectable among different influenza isolates at any time (Webster and others, 1981b).

**Shift**

Sudden and radical changes in the composition of the virion RNA genome, involving mutation or the reassortment of different segments of RNA, each of which represent a gene, or thousands of nucleotides in sequence, are called antigenic shifts. When a new H and N combination is created, no immunity will be present in the population to this "new" virus, and pandemics will occur (Dea and others, 1980).

**Mutation**

One explanation for the production of pandemics due to antigenic change could be that a virus of human, other mammal or avian origin became infectious for humans or other animals by mutation. Burnet and Lind (1951b) studying reassortants from mixed influenza virus infections, suggested that new influenza strains could arise by mutation from one or other strain. Such an explanation was accepted up to 1957 (Burnet and White, 1972). The discovery that the Asian strain (H2N2) is totally unrelated to other strains of influenza viruses isolated until that date, changed the concept that a simple mutation could have caused such an extensive antigenic change. Consequently, the idea
developed that Asian influenza may have emerged from some animal reservoir (Burnet and White, 1972), as previously discussed in this Chapter (Section 6.1.1).

Reassortment

Since the RNA genome is segmented, genetic reassortment can occur by reconstitution of infective particles from the pool of replicating units, produced by double infection of a single cell by virus particles of different strains of influenza A viruses (Burnet and Lind, 1951b; Webster and others, 1973; Webster and Laver, 1975).

When no relationship could be found between the H of the H2N2 and the H3N2 isolated from human epidemics, two possible theories were proposed to explain the origin of the new H (H3): 1) mutation of the previous H or 2) reassortment of the H2N2 virus with animal influenza viruses. The possibility of mutation was discounted as there was no similarity between the amino acid sequence of the polypeptides of the old and new H proteins. Evidence was obtained implicating an animal or avian influenza virus as the possible progenitor of the Hong Kong strain of human influenza (Coleman and others, 1968; Masurel, 1968; Kaplan, 1969; Zakstelskaja and others, 1969; Tumova and Easterday, 1969; Kasel and others, 1969; Laver and Webster, 1973).

It also seems that the Asian subtype (H2N2) originated in a similar manner, that is, by a reassortment of animal H and N genes, with the gene controlling the ability to infect
humans being coded by a human subtype (Webster and others, 1977a; Scholtissek and others, 1978).

Several other cross reactions have been demonstrated among H and N antigens of human, animal and avian viruses (Kilbourne, 1973). Reports by Hirst and Pons (1973); Webster and Laver (1975); Stuart-Harris and Schild (1976); Yamane and others (1978) all established that under certain conditions, where some different influenza A viruses co-exist and co-infect a single host, genetic reassortment between the infecting agents frequently occurs.

The idea has been supported by the finding that genetic reassortment between avian, animal and human influenza viruses could be obtained not only in vivo under conditions of natural transmission but also in vitro in the laboratory (Burnet and Lind, 1951a; Tumova and Pereira, 1968; Easterday and others, 1969; Webster and Laver, 1975).

Webster and Laver (1975) have stressed that not all new influenza viruses of lower mammals, birds, and humans have arisen by this mechanism, and that this is only one of the methods by which new viruses can arise.

Simultaneous infections of pigs by more than one influenza strain are frequent, and in this way, conditions may exist for the production of reassortants. The pig may act as a producer of mutant viruses, that infect other species. The possible frequency of genetic variations is directly related to the incidence of infection, which is
currently very high on intensive rearing pig farms (Pensaert and Haesebrouck, 1986, 1987). No preventive measures are taken against subclinical influenza infections, because they do not represent an apparent economical danger to the farmer.

Reassortment may also occur in free-living birds, associated with mixed infections in the same bird (Sakstelskaja, 1975). As the virus may be avirulent for the birds themselves, a reservoir could be created which perpetuates the viruses without endangering the host.

Reassortants between H3N2 and H1N1 viruses in pigs in Japan have been reported (Sugimura and others, 1980; Nerome and others, 1983). New virus strains possessing H1N2 antigens appeared in Japan in 1978 and 1980 (Yasuhara and others, 1983) and it appears that genetic reassortment has taken place between porcine influenza virus H1N1 and the Hong Kong influenza virus H3N2. In 1981, Arikawa and others reported serological evidence indicating the occurrence of natural genetic reassortment between Hsw1 and N2 antigens derived from Hsw1N1 and H3N2 viruses respectively, in Japanese pigs. Genetic reassortment was also observed by Mancini and others (1985) when analysing biochemical variations observed in influenza A H3N2 viruses isolated from pigs in Italy. Their findings suggested the persistence of the virus in pigs, as some of the isolates
were more related to H3N2 strains occurring before 1968.

It is noteworthy that new antigenic variants usually arise in the zone between northern Australia, Oceania and south-west Asia. Historical data collected by several authors were reviewed by Noble (1982). Many of the recent epidemics first appeared in China, where the population lives in close contact with their domestic animals, especially pigs and ducks. The Pearl River delta of southern China provides a large reservoir for influenza viruses, due to the large number of duck farms, the eating habits of these ducks, the apparent apathogenicity of the virus for this species and the presence of innumerable wild migratory birds that overwinter in the delta area (Shortridge, 1981). This densely populated, intensively farmed area of Southern China adjacent to Hong Kong has been considered by several authors as an influenza epicentre, providing an ideal ecosystem for events such as interchange of viruses between host species to occur (Shortridge and Stuart-Harris, 1982).

6.2. Molecular analysis and epidemiology

Confirmation of the simultaneous occurrence of the different proposed mechanisms of influenza virus variation has been possible by molecular analysis of virus isolates and the use of specific antibodies produced against them. Molecular epidemiology offers insights into the genetic
relatedness of viruses, whether or not they come from the same source and the genetic changes associated with the alteration of biological properties such as virulence. The degree of sequence homology between the genes of viruses may determine their origin, and explain how they acquired their virulence.

6.2.1. Common origin

To establish that viruses are from the same origin, all genes and gene products should be analysed and compared, using polyclonal and monoclonal antibodies, RNA-RNA competitive hybridization and/or nucleotide/amino acid sequence analysis. The results of Hinshaw and others (1983) and Kida and others (1988) suggest that turkeys and ducks, as well as pigs are potential sources of influenza for other species. Influenza A H1N1 isolates from turkeys were very similar to viruses typically detected in pigs. Three monoclonal antibodies that recognize different antigenic determinants on H1 strains were shown to recognise these determinants of recent turkey, human and porcine viruses but not by the duck viruses. A high degree of nucleotide and amino acid sequence homology has been found between the avian H7, seal H7 and equine H7 (Gibson, 1987), suggesting a common progenitor.
6.2.2. Mutation

Mutation rates can vary for the different genetic markers within the same influenza subtype, and those which mutate frequently have been termed "hot spots". If these spots code for virulence and antigenic determinants in the virion, virulence and antigenicity may change frequently. Other changes are not often observed (Thrusfield, 1986). Burnet and Lind (1951a and b) described a recombination of influenza viruses, and reported that only combinations capable of surviving and growing in the conditions provided are isolated. Changes altering the external H antigen are important. Since this protein elicits protective immunity the appearance of an influenza virus with a different H in the population will result in insufficient immunity and a new epidemic may occur (Pensaert and Haesebrouck, 1987). Sequencing and structural studies of the H of a number of influenza A viruses revealed that over a 10 year period the H gene accumulated a total nucleotide change of approximately five per cent (Palese and others, 1984). Location of the amino acid substitutions which accompany antigenic change has been described (Wiley and others, 1981). McCauley (1987) has proposed that one change at each of the three antigenic sites is necessary to produce a virus capable of causing widespread disease.

Antigenic variation also occurs in the N of influenza viruses (Kendal and Kiley, 1973) with an isolation frequency similar to, but independent of, that of the H (Schulman and
Kilbourne, 1969; Laver and others, 1982). Studies have revealed a considerable number of antigenic relationships between the neuraminidases of influenza viruses of human and non-human origin (Schild and others, 1969).

The nucleotide sequence coding for the M1 protein is highly conserved (Webster and others, 1982), as is that of the NP (Kistner and others, 1985; Scholtissek and others, 1985). The non-structural protein (NS1) encoded by influenza virus is also considered to be antigenically highly stable within type A strains, although Brown and others (1984) found that several antigenically variant NS1 proteins were present in avian strains. Conclusions from many studies now suggest that the genomes of influenza viruses undergo sequential mutations as the viruses are passaged in the host population (Palese and others, 1984). It has been demonstrated that glycosylation of amino acid 13 of the H may alter virulence (Kawaoka and others, 1987) by allowing only trypsin cleavability of the H, despite the presence of basic amino acids. Clearly such a situation may arise from a single point mutation of the gene. The accumulation of point mutations may also lead to virulence.

It is generally accepted that antigenic changes are brought about by the effect of the presence of antiviral antibody (Laver and Webster, 1968). After infection the immunity of persistently infected individuals suppresses the emergence of the original infecting virus but allows the
emergence of variants. Thus, apparent disappearance of the preceding strains can be explained in terms of self-annihilation (Webster and Laver, 1975).

Experimentally, a mixture of an influenza virus strain with homologous antibodies of low avidity, inoculated into embryonated eggs, may produce mutants that are not neutralized by these antibodies (Dea and others, 1980). The variants selected mimic the variants appearing in nature after infection of a previously immunized host. Such variants have single amino acid substitutions in the $H_1$ polypeptide chain. Laver and Webster (1968) isolated antigenic variants of influenza viruses by passage in the presence of sub-neutralizing concentrations of antiviral antibody, and peptide maps of the haemagglutinin protein of such variants indicated that one or two tryptic peptides differed.

During identification of influenza A and B virus strains for diagnostic purposes, De Jong and others (1984) found the antigenic reactivity of the majority of the strains to change occasionally on passage under laboratory conditions, even in the absence of immune pressure. Also, when virus grown in embryonated eggs was compared with monkey kidney cell-grown virus from the same strain, differences were observed. It has also been shown that influenza A and B viruses replicating in mammalian cells are more akin in the antigenic structure of the $H$ to natural epidemic viruses. Differential receptor binding properties
can explain the varying affinities of virus populations for egg or mammalian cells (Oxford, 1987).

6.2.3. Reassortment

When two different influenza viruses infect a single cell, their genomes may be mixed and different strains produced. This is called genetic reassortment. Only surface antigen reassortment will be detected during serological surveys, but each segment of the RNA genome can be reassorted, either individually, or together with some other segments (Palese and others, 1984; Webster and Kawaoka, 1988).

The use of chick embryonated eggs in which to grow influenza virus may have led to the erroneous conclusion that egg grown influenza viruses are closely related in their antigenic and biologic properties to natural epidemic influenza virus. Selection may take place. Many antigenic variants of influenza viruses can exist in even a cloned pool of infective allantoic fluid, and reassortment among these viruses can not be excluded.

Shortridge and others (1987) used monoclonal antibodies to the H, N and NP of human H3N2 influenza viruses to analyse isolates of virus from pigs and birds. Their studies indicate that some of the porcine H3N2 viruses in China might be reassortants that obtained some gene segments from
avian influenza viruses and others from human H3N2 viruses. Some of the H3N2 influenza virus analysed from pigs possessed NPs that were antigenically closely related to NPs from avian sources. None of the viruses could be matched exactly with H3N2 viruses from human or avian sources. Marker rescue and other experiments have shown that the 1982 isolates from pigs may possess NP genes derived from avian viruses (Scholtissek and others, 1985). This finding would suggest interspecies transmission of avian viruses and genetic reassortment between H3N2 and H1N1 influenza viruses in pigs.

6.2.4. Interspecies transmission

All factors influencing host range may affect interspecies transmission, however mutations and reassortments are thought to be the most important.

The host cell type can affect the antigenic make-up and replication of influenza viruses, and consequently its transmissibility. The viral phenotype is determined in part by the host cell which provides, amongst other factors, enzymes necessary for the synthesis of the carbohydrates and the glycosylation of virus-coded polypeptides. Because the envelope of influenza virus and related viruses is derived from host cell plasma membrane during budding, the chemical composition of lipids and glycolipids of the envelope closely resembles that of the plasma membrane (Frommhagen
and others, 1959; Klenk and Choppin, 1970). According to Klenk and Choppin (1970) some cellular glycoproteins, although host antigens, become incorporated in the viral envelope as constituents of the virion. Biological differences were observed in the plasma membranes, such as differing sensitivity to virus-induced cell fusion and immune cytolysis, and large differences in the amount of infective virus produced. The viral H is involved in host range selection by determining to which cell surface receptor the virus will attach (Holmes and Choppin, 1966), and in what range of cells newly replicated H is cleaved (McCauley, 1987). Also, the extent to which the H polypeptide is cleaved into H₁ and H₂ exerts a strong selection pressure for the preferential appearance of certain reassortants. Host range may be determined by a single gene (Almond, 1977; Buckler-White and others, 1986) or different host cells determining different subpopulations of the virus (Schild and others, 1983; Patterson and Oxford, 1986). Also, a functional cooperation may exist among some of the RNA segments, those genes coding for the polymerase complex seemingly playing an important role in host range (Scholtissek and others, 1979; Rott and others, 1979; Bonin and Scholtissek, 1983; Tian and others, 1985). The importance of the NP in the determination of host specificity was examined by Scholtissek and others (1985). Their observations were compatible with the idea that human
H3N2 strains might not be able to infect birds directly, nor avian strains infect humans, without prior reassortment in pigs. Pigs seemed to be tolerant towards the multiplication of both human and avian influenza viruses. In the example that they studied, the species specificity was apparently determined by the NP gene or its gene product. They suggested that the host control of influenza virus replication may operate through specific phosphorylation of the NP.

Even in the absence of anti-viral antibodies, a certain influenza virus subtype may fail to spread, due to its lack of specific pathogenicity for the infected host (Beare and others, 1980). This is believed to have occurred during the Fort Dix outbreak (Bachmann, 1983). Certain reassortants multiplying in one host cell might be unable to grow in another cell type, in which the parent strains are able to multiply and vice-versa. Isolation of influenza viruses could not be made from the trachea or cloaca of ducks infected with any of the human or porcine viruses used by Scholtissek and others (1985), but avian viruses could be isolated for up to 18 days post-inoculation from both sites. Nevertheless, antibodies to both human and porcine viruses were detected in the serum of the ducks inoculated with these viruses suggesting that virus replication had occurred.

6.2.5. Strain resurgence

The H1N1 virus strain reappeared in humans in the
1970s. Its origin is unknown, although all genes are closely related to those of viruses isolated from man in 1950 (McCauley, 1987). Theoretically, it must have been present in a host, without causing disease, or arisen by mutation or reassortment in the same way as it arose in 1950. Another possibility is that it escaped from a laboratory.

6.3. Conclusion

Recent molecular studies of influenza viruses have made it clear that the enormous variety of virus strains currently found have evolved from a common origin, by point mutations, reassortment and the selection provoked by host availability and immune status of the host.

Until now, no new porcine influenza virus strain has been isolated that proved to be dangerous for man. Because of the short lifespan of domesticated animals such as pigs and birds, the co-circulation of different strains is likely to play a more important role in variation than is gradual antigenic drift (Hinshaw and others, 1981d). Even so, porcine influenza strains must be considered as potential zoonotic agents. As porcine influenza does not represent a major economic burden to farmers, there are few efforts to eliminate this virus by vaccination or other means, and its presence in apparently healthy herds represents a risk for other susceptible populations (Barlow, 1987).
7. AIMS OF THESE STUDIES

Influenza viruses are responsible for diseases in animals of economic importance by affecting the respiratory tract and sometimes other organs; these viral infections result in significant morbidity and mortality, and consequent financial losses every year throughout the world.

In addition to its economic importance, the anthropozoonotic aspect of influenza has received attention in regard to the several human pandemics that have affected the world, sometimes with a high mortality rate. Studies on the origin of pandemic influenza have been in progress since 1930, when a new respiratory disease in pigs was considered very closely related to human influenza (Koen, 1928). During the following half century, many theories have arisen to explain the persistence of the virus and its sudden appearance producing pandemics in humans.

Furthermore, influenza viruses have proved increasingly useful as models of viral infection for laboratory study. The last 20 years have witnessed an accelerating advance in understanding of the structure, replication, and immunology of the virus that has been due, in part, to the application of techniques widely used in molecular biology, together with the development of specialized systems and techniques for the study of influenza virus.

The present work is mainly concerned with the
epidemiology of influenza in pigs, although some work involving avian strains and the interrelationships between birds and pigs has also been undertaken.

Research has been carried out with the aim of improving routine diagnosis of influenza virus, using the immunoperoxidase technique in microplates. This will permit the analysis of larger numbers of materials during surveillance exercises.

Influenza A viruses infect humans, pigs, horses, seals, whales and a variety of domestic and wild birds (Murphy and Webster, 1985). Representatives of each of the known subtypes of influenza A viruses have been isolated from feral water birds (Hinshaw and Webster, 1982). Interspecies transmission, as well as possibilities of reassortant production have been investigated, by studying influenza infection in cell culture systems, in pigs and turkeys, and by a search for particular antigenic variants (H1N2) in English pig herds.

In addition to the above, the special interest of the author in the epidemiology of porcine influenza in Brazil led to the studies presented in this thesis. Pig meat production is becoming increasingly intensive in Brazil, as the consumer market expands. As in the poultry industry, herd health control must be rigorous to ensure that major outbreaks of disease are avoided, and in this situation, zoonoses are of special concern.
Respiratory disorders are very common in economic pig units because of the intensive rearing and close contact of the animals. The problem is especially important during the fattening phase. Economic losses are of considerable concern and this is one of the justifications for continued research on the topic.

An understanding of the epidemiological behaviour of influenza can only be achieved by intensive, and continued, international surveillance. Included in such surveillance is the identification of the antigenic characteristics of influenza viruses currently prevalent in human and animal hosts, and the estimation of the level of population immunity. Therefore, a chapter on serological evidence for influenza virus infection in pigs in Brazil has been included in this thesis.

Objectives

The studies include work on porcine influenza in the United Kingdom and Brazil, and should provide a basis for future studies on the subject.

The objectives of the research on influenza virus described in this thesis were:
1. To develop improved diagnostic methods.
2. To determine the risk of spread through pig meat and other tissues.
3. To investigate the role of interspecies transmission, with special emphasis on pigs and turkeys.
4. To analyze seroepidemiological influenza in pig herds in Brazil.

5. To determine the possible presence of reassortant influenza strains in British pig herds.
CHAPTER II

GENERAL MATERIAL AND METHODS

1. Materials

Alsever’s solution (anticoagulant) - 2.05 g glucose (C₆H₁₂O₆), 0.8 g anhydrous tri-sodium citrate and 0.42 g sodium chloride (NaCl) were mixed in 100 ml destilled water. The pH was adjusted to 6.1 with 10% citric acid solution. The solution was sterilized through a 0.22 μm membrane and kept at 4°C. 2 ml was used for each 6 ml of blood collected.

Antibiotics - the antibiotics routinely added to cell culture media constituted of 100 IU/ml penicillin (Glaxo), 100 μg/ml streptomycin (Evans) and 25 IU/ml mycostatin (Squibb).

Complement Fixation Test diluent tablets - 1 tablet is dissolved in 100 ml of distilled water. A litre of this diluent contains 0.575 g barbitone, 8.5 g NaCl, 0.168 g magnesium chloride (MgCl₂), 0.028 g calcium chloride (CaCl₂) and 0.185 g soluble barbitone. The final pH is 7.2.

Fetuin - this was obtained ready for use from Poultry Unit, Central Veterinary Laboratory, Weybridge, Surrey (CVL).
Kaolin suspension preparation - a 25% kaolin suspension was made in borate buffer (pH9.0) as follows. 83.5 ml of solution A (12.37 g boric acid + 100 ml 1 N sodium hydroxide (NaOH) made up to 1 litre with H2O) was added to 16.5 ml of solution B (0.1 N HCl) and 25 g Kaolin and 0.85 g NaCl.

Phosphate buffered saline (PBS) pH 5.9 - 7.02 g of dibasic sodium phosphate (Na2HPO4) in 100 ml distilled water were mixed slowly into 7.89 g monobasic sodium phosphate (NaH2PO4) 2H2O in 300 ml distilled water, until the pH of 5.9 was reached. The volume was then completed to 500 ml by adding distilled water.

PBS pH 7.2 - 40 ml of solution A (5.48 g Na2HPO4 + 1.575 g NaH2PO4.2H2O in 200 ml H2O) was added to 60 ml of solution B (8.5 g NaCl in 60 ml H2O) and the volume completed to 1 litre with 900 ml H2O. The pH was adjusted to 7.2.

PBS pH 7.4 - 160 g NaCl, 4g potassium chloride (KCl), 28.8 g Na2HPO4 2H2O, 100ml 0.2% phenol red, 4 g dipotassium phosphate (KH2PO4), 2 g MgCl2 6 H2O, 2 g CaCl2 6H2O in 20 litres of distilled water, final pH 7.4.
Sodium arsenite solution - the amount of 2 g of sodium arsenite were added to 100 ml of 0.5N acetacetic acid (HCl), to obtain a 2% solution.

Sodium periodate solution (0.025M sodium periodate (NaIO₄) in 0.125N sulfuric acid (H₂SO₄)) - in 500 ml of an H₂SO₄ solution (1.75 ml in 500ml H₂O), 2.67 g NaIO₄ were dissolved. The solution was kept at 4°C until used.

Thiobarbituric acid solution (TBA) - the amount of 7.2 g of TBA was added to 400 ml of H₂O and put on a magnetic stirrer. NaOH 3N was added until all TBA was dissolved and the pH reached 9.0, when the solution was ready, after making the final volume up to 500 ml with distilled water.

Virus strains - all virus strains used in these studies were obtained from research laboratories or isolated from pigs from English herds. With the exception of A/Brazil/11/78-PR/8/32, received from Dr. J.P. do Nascimento from Instituto Osvaldo Cruz, Rio de Janeiro, Brazil, and strains A/duck/Hong Kong/196/77, A/Port Chalmers/1/73, A/swine/Weybridge/117316/86 and A/swine/Weybridge/163266/87, obtained from stock kept by Mrs. R. Marvell and Miss M. Williams at CVL, all other strains were kindly supplied by Dr. P. Chakraverty, from Central Public Health Laboratory, Colindale, London, UK. Strains A/swine/Suffolk/1/88 and A/swine/Suffolk/2/88 were isolated from a pig farm in Suffolk, UK.

113
2. Techniques and Methods

Antisera production – all antisera used in the studies, if not stated otherwise, were produced in adult specific pathogen-free (SPF) chickens, as follows. 12 week old SPF chickens were inoculated intramuscularly with 0.7 ml of infective allantoic fluid containing the desired influenza virus \(10^5\) egg infective dose (EID)\(_{50}/0.2\) ml. Boosters were given by the same route after 14 and 28 days of the first inoculation. The birds were bled out after 42 days if the specific antibody level (HI) was equal or above 1/256. Otherwise, another booster was given and antibody level checked again after 14 days. The collected blood was kept at 35°C for 3 hours and the serum separated, if necessary by centrifugation (500 g for 15 minutes). The serum was inactivated by heating at 56°C for 30 minutes, distributed in aliquots of 1 ml and kept at -20°C until used.

Antigen purification – purified antigens to be used in the SRID and IDD tests were produced by the technique recommended by Palmer and others (1975). Large amounts of freshly harvested, infective allantoic fluid of fowls' eggs were centrifuged at 1700 g at 4°C for 20 minutes to deposite gross egg components. The supernatant was centrifuged at 22,500 g (HI-spin 21 centrifuge, MSE) for 60 minutes at 4°C to pellet the virus. The pellet was ressuspended in ten
times its volume in PBS pH 7.4 and purified by passing this suspension through a 30-55% w/w sucrose (BDH) gradient (AH 627 swing-out buckets rotor, Sorvall ultracentrifuge) at 83,100 g for 60 minutes at 4°C. The virus band was collected by perforating the bottom of the plastic centrifuge tube and harvesting the bands in aliquots of 4 ml. The concentrated virus was pelleted again and washed twice (22,500 g for 60 minutes at 4°C) and the pellet, after resuspension in ten times its volume in PBS pH 7.4, used in the SRID and IDD tests.

Chicken red blood cell (CRBC) collection and preparation - adult white Leghorn chickens were bleed intracardiacally and 6 ml of blood collected in Alsever’s solution. The red blood cells were pelleted by centrifugation (500 g for 15 minutes), washed twice in CFT diluent, and then diluted to the required concentration in CFT diluent, 10% if for treatment of sera, 1% if for use in HA and HI tests.

HA test - the standard HA microtechnique was always used, as recommended by Palmer and others (1975), as follows. Doubling dilutions of the virus were made with CFT diluent in U bottomed microplates. To this, one volume of CFT was added, followed by one volume of 1% CRBCs, to each well. After mixing, the cells were allowed to settle at room temperature for 30 minutes, and the results were read according to the patterns of the sedimented cells. One HAU was considered the reciprocal of the last dilution showing
HA. In all HI tests, 4 HAUs of the virus were used.

**HI test** - HI titres of sera were determined using a standard microtitration procedure and 4 HAU of virus, as described by Palmer and others (1975).

Using U shaped wells microplates, serial 2-fold dilutions of the sera were made by placing 25 µl of complement fixation test (CFT) diluent in all wells of the plate, except the first one of each test series. The same amount of serum was then placed in the first and second well of each test series and passages made, taking the same volumes (25 µl) out of the second well and putting it into the third and so on, up to the last well of that row. Known positive and negative control sera were included in each test. An equal volume of 4 HAU of virus was added to the diluted sera and allowed to react for 20 minutes. The virus-serum mixtures were gently mixed and 25 µl of 1% fresh, washed CRBCs in CFT diluent added to all wells. The plates were agitated, incubated overnight at 4°C and the results read the following day. HI titres were recorded as the reciprocal of the highest initial dilution of serum which gave partial or complete agglutination.

**Protein estimation of antigen preparations** - the protein content of the influenza antigen containing suspensions was measured using the Microprotein Determination Kit (Sigma),
which detects the tryptophan and tyrosine content of protein. First, the samples were diluted and mixed with the biuret reagent. After allowing to react for 10 minutes at room temperature, the Folin and Ciocalteu’s phenol reagent was added. The colour developed after 30 minutes, and was read on a colorimeter at 700 nm wavelength and the absorbance values compared with a calibration curve, prepared using standard protein concentrations. The protein concentration of the samples was estimated according to this calibration curve.

**Spot NI test** - as used routinely at the CVL has been used throughout these experiments. Basically, it includes the following steps: Antisera to cover all neuraminidase subtypes were diluted in 0.1M phosphate buffer pH 5.9 1/10, and all test viruses in the same buffer 1/20. 100 µl of the diluted virus was placed into each test tube, using one tube for each antisera. To this, 100 µl of sera was added, and left to react for 20 minutes at room temperature. 50 ul fetuin in 250 µl phosphate buffer was added to each tube, including the substrate blanks. The tubes were left at 37°C overnight.

Released N-acetyl neuraminic acid (NANA) was estimated by adding 200 µl of sodium periodate solution (0.025M sodium periodate in 0.125N H₂SO₄) to all tubes and placing the tubes at 37°C for 30 minutes. Then, 200 µl sodium arsenite solution (2% sodium arsenite in 0.5N HCl) was added to all
tubes (brown colour develops and fades). 2 ml thiobarbituric acid solution (0.1M thiobarbituric acid pH 9.0) was added to all tubes, and these placed in boiling water for 7 or more minutes, when a red colour should appear in those tubes where the neuraminidase antibodies did not inhibit the neuraminidase activity.

**Spot NA and NI micro tests** - The NA and NI micro tests were performed as used routinely at CVL, and adapted to microplates, by using the same reagents as in the macro test in corresponding smaller amounts. The micro NI test was performed in white polystyrene 96-well plates (Flow laboratories). 20 μl amounts of a 1/2 dilution of virus was added to the wells, followed by 20 μl of antisera to the nine reference Ns (hyperimmune undiluted chicken sera) and a negative serum from a SPF chicken. The content of the wells was mixed by hand. Each plate was then covered with adhesive tape to prevent evaporation and incubated at room temperature for 20 minutes. 60 μl of fetuin (diluted 1:6 in PBS) was added to each well, including a well with only 40 μl of PBS pH 5.9 for fetuin and blank control, and mixed for 10 seconds. The plates were incubated overnight at 37°C, covered with adhesive tape. 40 μl of sodium periodate (0.025 M sodium periodate in 0.125 N H₂SO₄) was added to each well, and incubated at 37°C for 30 minutes. 40 μl of sodium arsenite (2% sodium arsenite in 0.5 N HCl) was added to each well and mixed thoroughly until the dark brown
colour that first developed had disappeared. The content of all wells was transferred to small 2 ml tubes containing 400 µl of thiobarbituric acid (0.1 M thiobarbituric acid pH 9.0). These tubes were placed in a boiling waterbath for 10 minutes, or until a dark red colour developed in the tubes containing only virus. The inhibition was evaluated visually, considering that no inhibition took place in the dark red coloured tubes, and inhibition in those tubes where colour was reduced or absent, indicating that the virus possessed an N that reacted with the known positive antiserum.

The presence of antibodies to specific Ns was detected using the same procedure except that the sera to be examined were included in the test with known viruses and homologous controls.

**Treatment of sera** - to avoid non-specific reactions in the HI tests, all serum samples, if not stated otherwise, were heat inactivated and treated with Kaolin and CRBCs.

**Heat inactivation** - all sera were heated at 56°C for 30 minutes before use in any test, to inactivate complement and removal of non-specific HA inhibitors.

**Kaolin treatment** - 0.1 ml of serum was added to 0.8 ml of 25% kaolin in borate buffer pH 9.0 and incubated overnight at 4°C. The next day the kaolin mixture was spun down (500 g for 10 minutes), and treated with CRBCs.
**CRBCs treatment** - 0.9 ml of kaolin treated serum was added to 0.1 ml of a 10% CRBC suspension. After incubation of the mixture at 37°C for 60 minutes, it was pelleted (500 g for 10 minutes), and the supernatant serum used in the test (now at a dilution of 1/10). This dilution was the starting dilution used at the HI tests.

**Virus growth** - all virus strains used in these studies were grown in 9-day-old embryonated fowls' eggs using the allantoic sac route, and by inoculating 0.2 ml of a 10^{-3} dilution of the virus with a 1 ml hypodermic syringe and a 21g x 11/2" needle. After incubation for 96 hours at 33°C (according to technique of Wibberley and others, 1988), the eggs were chilled for several hours, the allantoic fluids harvested, and their EID_{50} calculated. All virus strains were diluted in order to contain approximately 10^5 EID_{50}/0.2 ml of inoculum, then tested for sterility and stored at -70°C. Virus so prepared was used as stock virus.

**Virus HA titrations** - these were performed to detect HA agents and the influenza virus HA titre, and before and after HI tests.

**Virus titration by EID_{50}** - groups of 3 embryonated eggs were inoculated intrallanticiocally with 0.2 ml volumes of 10-fold dilutions of the influenza strain, starting at the dilution of 10^{-2}. The eggs were incubated at 33°C. Deaths
before 24 hours post-inoculation were discarted, and after that kept at 4°C. After 5 days incubation, all eggs were chilled for several hours and their allantoic fluid harvested and tested for HA. The EID$_{50}$ was calculated using the allantoic fluids giving the highest positive HA, and according to the formula of Spaerman-Kärber (Kärber, 1931).
CHAPTER III

SENSITIVITY OF SOME CELL CULTURES TO PORCINE INFLUENZA VIRUSES

Introduction

After the findings of Burnet (1940), that influenza virus could be propagated in cells lining the allantoic and amniotic cavities of the developing fowl embryo, and the discovery of the haemagglutinating properties of the influenza virus particle (Hirst, 1941), the search began for other cell systems in which the virus was able to replicate (Green and others, 1957). Sugiura and others (1962) used monkey kidney and HeLa cell cultures to study the intracellular development of human influenza virus antigens. Organ explants from infected animals were used by Blaskovic and others (1970) in an attempt to isolate the porcine influenza virus present in those tissues. Nath and Minocha (1977) obtained successful replication of equine and porcine influenza viruses in Madin and Darby canine kidney (MDCK) cell cultures. Various kidney cultures were used successfully by Frank and others (1979) to isolate and titrate human influenza virus. Yamagishi and others (1981) tested African green monkey kidney (Vero), canine kidney, bovine kidney, African green monkey kidney, porcine kidney, and porcine embryo kidney cells for their susceptibility to
equine influenza virus with the best results with porcine embryo kidney cells.

Behaviour in cell cultures is a property that may permit the characterisation of new viruses and their classification into interrelated groups (Pereira, 1961). The site of replication and mechanism of maturation and release of the virus from cells all contribute to the cytopathic effect (CPE) and the overall degenerative picture produced by the virus. Negroni and Tyrrell (1959) described the morphological changes in primary epithelial cell cultures after infection with influenza A viruses. Changes varied according to the virus strain and cell culture used, cytopathic effects being more extensive when no serum was used in the cultures. Changes ranged from the production of small vacuoles to disintegration of the cell membrane. According to Pereira (1961), the lesions caused by influenza viruses are purely degenerative, consisting of cytoplasmic granulation and vacuolation, nuclear pyknosis, cellular contraction, and disintegration. Pette and Teufel (1966) observed CPE in calf and chicken embryo primary kidney cell cultures after infection with equine influenza virus, as did Yamagishi and others (1981) in various other cell cultures. Alexander and others (1981) tested several influenza isolates in MDCK cells for infectivity and plaque-forming ability, concluding that these cells are adequate for influenza virus replication, although presenting different size of plaque formation according to
the virus strain under test and when trypsin was incorporated into the medium.

With the exception of Alexander and others (1981b) and Yamagishi and others (1981), all of the above mentioned authors used tubes, flasks, or bottles for the cultivation of the cells.

The lack of ready availability and cost of specific pathogen free (SPF) embryonated eggs prompted the search for alternative culture systems for use in studies of the influenza viruses. In the present study, four cell lines and two primary cell cultures of epithelial and fibroblast morphology were tested for susceptibility to Weybridge strains of porcine influenza virus (H1N1 and H3N2). Namely, with epithelial-like morphology: Madin and Darby canine kidney cells (MDCK), Madin and Darby bovine kidney cells (MDBK), and porcine kidney cells (PK1); and with fibroblast-like morphology: chick embryo fibroblasts (CEF), primary porcine kidney cells (PPK) and African green monkey kidney (Vero). Flat-bottomed multiwell microplates were chosen as the support for the cell culture monolayer as they would be advantageous for large surveys of porcine influenza virus in animal excretions and/or tissues. Also they are a cheaper and less laborious substitute for chick embryo inoculation.

The presence of virus was detected by CPE, plaque formation, haemadsorption of red blood cells to infected cell monolayers, haemagglutinating capacity of cell culture
fluids, specific immunofluorescence in the infected monolayer, and also by an immunoperoxidase technique.

This chapter describes these observations and results of developing techniques for the detection of antigen by immunofluorescent and immunoperoxidase (IPX) assays developed in microplates.

Material and methods

Virus strains - A/swine/Weybridge/117316/86 (H1N1) (Wey/86) and A/swine/Weybridge/163266/87 (H3N2) (Wey/87) strains of influenza A virus were used throughout this study, after 5 passages in the allantoic fluid of embryonated eggs. The seed virus contained $10^5$ EID$_{50}$/0.2 ml (see Section 2 in Chapter II).

Cell cultures

MDCK (CCL 34, Flow), MDBK (Madin and Darby, 1958) and Vero (CCL 81, Flow) - these cell cultures were grown in Eagle's minimum essential medium (MEM) (Gibco) supplemented with 10% fetal calf serum (Flow) and antibiotics. PK.1 - this porcine kidney cell line was grown in medium 199 (Flow) supplemented with 10% fetal calf serum, 5% lactalbumin hydrolysate (Flow), 3% sodium bicarbonate, 1% L-glutamine (Flow) and antibiotics.
CEF - chick embryo fibroblasts were grown in MEM (Gibco) supplemented with 10% fetal calf serum, 0.67% sodium bicarbonate (at 7.5%) and antibiotics.

PPK - primary porcine kidney cells were grown in Hanks based medium (LYH - Flow) supplemented with 10% calf serum, 10% lactalbumin, 0.5% sodium bicarbonate and antibiotics.

To all media 2% of N-2-hydroxyethyl piperazine-N-2-ethanesulphonic acid (HEPES) (Flow) and 1% sodium bicarbonate (at 7.5%) was added when cultivating cells in microplates.

Antibiotics - see Section 1 in Chapter II.

Cell cultures were prepared in flat-bottomed multiwell microplates by seeding each well with approximately $2.5 \times 10^3$ cells suspended in 0.1 ml of medium (96 well plates), or $1.25 \times 10^5$ cells suspended in 5 ml (6 well plates).

The sealed plates were incubated in an atmosphere of 5% CO$_2$ in air at 33°C for up to 7 days, and unless stated were infected when the cell monolayers reached confluence.

Infectivity assay in 96 well microplates - serial two-fold dilution series of the $10^5$ EID$_{50}/0.2$ ml of the virus were made in culture medium containing 10 μg/ml trypsin but no serum. After washing the wells with culture medium, 0.1 ml of each dilution was inoculated into 4 to 8 wells of the microplate containing the various cell cultures. During the incubation period at 33°C, the cultures were observed daily.
for CPE. After 7 days, the cell culture media were removed and tested for HA. The cells themselves were observed for the presence of virus by a micro fluorescent antibody technique.

The plates used for IPX assay were tested on the same day using the same reagents for all, to minimise technical error. Accordingly, each day the cells of one infected plate were fixed, dried and stored at -20°C in a plastic bag, until all were ready for testing.

**Infectivity assay in 6 well plates** - serial ten-fold dilutions series (10⁻² up to 10⁻⁶) of 10⁵ EID₅₀/0.2 ml of the virus were made in culture medium without serum and 0.2 ml inoculated into 8 wells of the plates that had previously been washed with culture medium. After 60 minutes at 33°C, the plates were washed twice with medium without serum, and the cultures overlaid in duplicate (Fig.6) with 5 ml (a) agar overlay (MEM without serum containing 2.2 mg/ml of NaHCO₃ and 0.6% Noble Agar (Sigma) with trypsin (10 μg/ml crystalline trypsin (Merck)), (b) agar overlay containing no trypsin, (c) culture medium containing 10 μg/ml trypsin, (d) culture medium without trypsin. Mock infected wells were used as controls. The plates were sealed and incubated for 5 days at 33°C. At 6, 12, 24, 36, 48 and 72 hours post-inoculation, samples from
Fig. 6. Experimental lay-out of infectivity assay using 6-well cell culture plates.

Each cell culture was plated in 8 6-well trays. After confluence, the cell monolayers were infected with various dilutions of each virus. Mock infected controls were included. Wells were overlaid with medium, such that each virus dilution had a replicate agar overlay with and without trypsin, and culture medium with and without trypsin.

(A) - wells used for plaque-formation

(B) - wells used for HA testing and haemadsorption test.

(C) - wells used for neutralization test and CPE observations.

* exponents of reciprocal $\log_{10}$ virus dilutions

contr. = mock infected wells
<table>
<thead>
<tr>
<th>Virus Dilutions</th>
<th>Wey/86</th>
<th>Wey/87</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- trypsin</td>
<td>+ trypsin</td>
</tr>
<tr>
<td>2</td>
<td>○ ○ ○</td>
<td>○ ○ ○</td>
</tr>
<tr>
<td>3</td>
<td>○ ○ ○</td>
<td>○ ○ ○</td>
</tr>
<tr>
<td>4</td>
<td>○ ○ ○</td>
<td>○ ○ ○</td>
</tr>
<tr>
<td>5</td>
<td>○ ○ ○</td>
<td>○ ○ ○</td>
</tr>
<tr>
<td>6</td>
<td>○ ○ ○</td>
<td>○ ○ ○</td>
</tr>
<tr>
<td>cont.</td>
<td>○ ○ ○</td>
<td>○ ○ ○</td>
</tr>
</tbody>
</table>

With only medium and with agar overlay.
the plates containing plain medium with and without trypsin were taken and assayed for HA. In addition, 0.5 ml of the medium was stored at -70°C for inoculation into embryonated eggs and titration of infectivity. The same plates were used for the haemadsorption and neutralization tests and observed for cytopathic effect. After these tests, the plates were washed, fixed, and stained, to confirm the extent of CPE. The plates with agar overlay were examined for the presence of plaque-formation (see later in this Section).

**Haemadsorption technique** - at the end of the incubation period, supernatant fluids of cultures containing infected and control cells which had been incubated without replacement of medium were removed from wells. 0.2 ml of a 0.5% suspension of washed citrated fowl erythrocytes were added to each well and left to absorb for 1 minute. The wells were washed with culture medium and the medium replaced. Clumps of agglutinated red blood cells adhering to the tissue indicated the presence of H on the cell surface.

**Neutralisation test** - inhibition of haemadsorption was obtained when specific antisera was added to the cell cultures prior to their treatment with erythrocytes.

**Cytopathic effect** - infected and non-infected cells in the 96-well plates were compared each day of incubation, and differences between them analysed. The 50% endpoint (TCID\(_{50}\))
was calculated by the Spearman-Kärber method (Kärber, 1931).

**HA titrations** - see Section 2 in Chapter II.

**Immunofluorescence** - the standard technique for direct immunofluorescence was adapted to microplates as follows. Following removal of the cell culture medium, cell monolayers were fixed in each well by adding 100 µl of a 60% acetone - 40% methanol dilution for 5 minutes. The procedure was repeated twice, and the plates left to dry on the bench. Using a microplate washer, or manually, the plates were washed 3 times with TRIS buffer (0.01 M tris-HCl pH 8.7 with 0.14 M NaCl). The conjugate, prepared according to the method of Nairn (1976), was applied to all wells, using 50 µl/well (96 well plates) of the optimal dilution, and left for 30 minutes at 37°C. The plates were washed again 3 times with TRIS buffer, and dried by tapping them on paper towel. After this, the plates were read, inverted, under an U.V. microscope, using a 50 Watt high pressure mercury lamp (WOTAN) as a light source.

**Conjugation of specific porcine influenza virus antibodies with fluorescein isocyanate (FITC)** - briefly, the following method was used (Nairn, 1976): Crystalline Na₂SO₄ and hyperimmune serum, in a final concentration of 14%, were stirred together for 2 hours at room temperature, and then centrifuged at 2000 x g for 20 minutes. The supernatant was
discarded, and the precipitate dissolved in 0.1 M PBS pH 7.2 to original volume. This procedure was repeated three times, after the last of which the precipitate was resuspended in only half of its original volume of distilled water. This was dialysed against slowly running water, and as soon as a precipitate began to form, it was taken out and put into a dialyzing bath of 0.1 M PBS pH 7.2 at 4°C overnight. The following day, the protein concentration was measured and brought to 1% (dilution in PBS). 0.5 M carbonate-bicarbonate buffer pH 9.0 was added in the proportion of 1 volume buffer for every 2 volumes of protein solution. Stirring constantly, fluorescein isothiocyanate (FITC) isomer 1 crystals (BBL) were added directly to the protein solution (1 mg FITC : 60 mg protein). This was left stirring overnight at 4°C. The mixture was dialysed overnight at 4°C against 0.01 M tris-HCl pH 8.7 with 0.14 M NaCl. This same buffer was used to equilibrate and pack a Sephadex G-25 (medium) column. The column bed volume was about double the conjugate volume. The conjugate was passed through this column and washed through with buffer. The green band was collected in fractions. A DEAE-cellulose column (Whatman DE-11) was equilibrated and packed in the same buffer, with a bed volume equal to the conjugate volume. The conjugate was passed through this column and washed out. The green eluate was collected fraction-wise. The two peaks (0.14 M and 0.28 M NaCl) were pooled, as they were both satisfactory. Merthiolate was added to a final
concentration of 1:10,000, and the conjugate filtered through a 450 nm Millipore membrane.

The conjugate was titrated in a fluorescent microscope against porcine influenza antigen and negative controls, to find the optimum working dilution of 1:16. Aliquots of 0.1 ml of the conjugate were stored at -20°C.

Specificity test - non-infected (mock infected) cultures were treated in the same way as infected cultures, and the results compared.

Plaque formation testing technique - 6-well plates with agar overlay were used for plaque formation evaluation (Fig. 6). After 5 days at 35°C, the plates with agar overlay were flooded with 60% acetone - 40% methanol for 10 minutes, inverted and tapped on paper towel to remove the overlay, again flooded with fixing mixture and allowed to dry. 0.05 % Crystal Violet in PBS was added to each well in sufficient amounts to cover the monolayer and left for 20 minutes. The plates were inverted and left to dry for plaque counting.

IPX test - MDCK cells cultivated as described were infected at the same time of their passage to 96 well microplates, using 4 HAU of Wey/86 and 2-fold dilutions, 8 replicates per dilution. In this way, one single plate contained 11 columns of virus dilutions, starting at 1/2 up to 1/2048, and one column of non-infected cells, to serve as
a control (Fig. 7).

After 3, 4, 5, 6 and 7 days of incubation at 33°C, the medium was decanted and the plates rinsed with physiological saline (8.85 g NaCl/litre H₂O, pH 7.2). The cells were fixed onto the plate using a mixture of 60% acetone and 40% methanol, for 10 minutes. After draining, the plates were left to dry on the bench. The plates were washed again with 100 μl physiological saline for 5 minutes, drained, and then 50 μl 1/10 or 1/20 specific antiserum (HI titre 1/40, Weybridge strain) were added to alternative rows, and left at 25°C for 15 minutes. After washing twice with 100 μl warm wash fluid (30°C), (8.85 g NaCl + 5 ml Tween 80 + 1 litre H₂O, pH 7.6), 50 μl peroxidase conjugate was added, using rabbit anti-chicken conjugate (prepared at CVL) in a diluent of 29.5 g NaCl + 5 ml Tween 80 + 1 litre H₂O, pH 7.6. Dilutions of 1/400, in the first and second rows; 1/300, third and fourth rows; 1/200, fifth and sixth rows; and 1/100, seventh and eight rows were used. This conjugate was left on the plates for 15 minutes, at 25°C, and then the free conjugate was removed by washing the plates twice with warm wash fluid. Then 50 μl of substrate (0.3 ml of stock (0.1 g 3-amine-9-ethylcarbazole in 15 ml N,N-dimethyl formamide) plus 5 ml acetic/acetic buffer (0.36 ml glacial acetic + 1.16 g sodium acetate + 400 ml H₂O, pH 5.0), activated with 5 μl of 30% H₂O₂, was added to all wells and left for 15 minutes under the lamps. The staining reaction
Fig. 7. Diagrammatic lay-out of microtitre plates for IPX technique efficiency test for influenza virus detection.

Four HAU of the influenza virus strain were diluted, in 8 replicates, starting at 1/2 up to 1/2048. One column consisting only of diluent was included as negative control. Antiserum dilutions of 1/10 and 1/20 were added to alternative rows, and the various dilutions of peroxidase conjugate were added to each consecutive row, so that each pair of antiserum dilutions received a different peroxidase conjugate dilution.
reciprocal of virus dilution (WEY/86)

2  4  8  16  32  64  128  256  512  1024  2048

neg cont.

10
20
30
40
50
60
70
80
90
100
200
300
400

reciprocal antiserum dilutions

reciprocal peroxidase conjugate
was stopped after that by decanting the substrate and adding 100 μl of wash fluid to all wells. The plates then could be read under an inverted microscope.

Antisera production - antisera against the virus strains used were produced in chickens, as described in Section 2 in Chapter II.

Results

1. Susceptibility of cell cultures to infection by porcine influenza virus

1.1. The use of 96-well plates

The presence of influenza virus in the 96 well microplates was detected by quantitative measurements of "free" H in the medium (HA test), and by qualitative virus antigen detection in the cell monolayers by an immunofluorescence technique (for this experiment considered "cell-associated" virus). The susceptibility to the porcine influenza virus strains of the several cell cultures grown on microplates were also compared by means of the CPE detected. The results are presented in Table 2.

All infected cell cultures showed CPE, with the
Table 2: Detection of porcine influenza virus in the various cell cultures examined, after 7 days incubation, by measurement of the CPE produced by the cells, HA of culture supernatant and specific direct immunofluorescence reactivity (FA) on the cell monolayers.

<table>
<thead>
<tr>
<th>Cell cultures</th>
<th>VERO</th>
<th>MDCK</th>
<th>PPK</th>
<th>PK1</th>
<th>CEF</th>
<th>MDBK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i</td>
<td>ni</td>
<td>i</td>
<td>ni</td>
<td>i</td>
<td>ni</td>
</tr>
<tr>
<td>CPE</td>
<td>0.75*</td>
<td>-</td>
<td>0.45</td>
<td>-</td>
<td>0.45</td>
<td>-</td>
</tr>
<tr>
<td>HA**</td>
<td>1.05</td>
<td>-</td>
<td>0.75</td>
<td>-</td>
<td>0.45</td>
<td>-</td>
</tr>
<tr>
<td>FA***</td>
<td>3.07</td>
<td>-</td>
<td>1.83</td>
<td>-</td>
<td>1.95</td>
<td>-</td>
</tr>
</tbody>
</table>

i = infected cultures  
ni = non-infected cultures

* All values are reciprocal log \(\text{log}_{10}\) exponents of the means of the last wells with virus dilutions demonstrating virus activity using the different techniques.

** HA was detected in cell culture medium.

*** FA was detected on cell culture monolayer, and refers to influenza virus antigen present on the cell membrane.

- = the presence of virus was not detectable by HA using undiluted cell culture medium, or by FA technique.
exception of the CEF and MDBK. The CPE was characterised by the retraction of the cytoplasm, producing a web formation of adjoining cells connected to each other, and subsequently cells floating in the cell media. Rounding and aggregation was only observed with the infected PPK cells. In general, CPE was observed earlier in PPK cells, and the resultant degeneration was more pronounced and extensive than in other cells. The culture fluid of each well of the infectivity titration was tested for H using the mean of wells showing a positive reaction as proof of virus replication. After 7 days incubation, free virus was only detected by HA up to a dilution of $10^{-1.12}$ in PK1 and less in other cells. A significant rise in free H in the cell culture supernatant was not seen until between 12 and 24 hours after infection, when the onset of the CPE was first observed. Infectivity of HA positive cell culture supernatants for embryonated eggs was tested at the end of the incubation period, and proved that the virus detected was infectious. Cell associated specific antigen was detected by FA technique in much higher dilutions, with variations for each type of cell culture. The results indicated that virus infectivity determined by HA was generally higher than that determined by CPE, with the exception of the CPE present in PK1 cells.

Cell cultures showing CPE had the disadvantage that the cell monolayer came off after a period of time, sometimes not permitting accurate FA testing. These cell cultures had to be tested before total destruction of the cell
monolayer, that is, about 4 to 5 days post-inoculation. A correlation could be found between intensity of degeneration of cell monolayers and either HA and/or FA techniques.

In this experiment, of all the cells tested, PK1 cells were the most susceptible to the influenza viruses.

1.2. The use of 6-well plates

Vero, PK1, MDBK and MDCK cell cultures were also cultivated in 6-well plates, infected with the same virus strains in cell cultures with and without the addition of trypsin. Virus replication was assessed using CPE observations, haemadsorption, neutralization tests, and HA and infectivity of the harvested cell culture supernatant following passage in embryonated fowls' eggs. Also, the ability of plaque formation of the virus strain was tested.

Vero cells were not suitable for influenza virus plaque formation, since the cells were no longer viable under the experimental protocol after 3 days incubation. Nonetheless, it could be observed that the influenza virus was able to replicate in these cells, as determined by the increasing HA titres (Table 3). Also the wells with agar overlay containing trypsin showed a general CPE, which was not evident in the wells where no trypsin had been added, nor mock-infected wells. Neither haemadsorption, nor neutralization were observed in any of the wells.
Table 3. Detection of HA in the cell culture supernatant of the various cell cultures tested, with and without added trypsin, according to the virus strain used to infect these cells and hours after infection.

<table>
<thead>
<tr>
<th>Virus strains</th>
<th>Wey/86</th>
<th>Wey/87</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>trypsin</td>
<td>trypsin</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Cells hours p.i.</strong></td>
<td><strong>reciprocal log$_{10}$ dilutions</strong></td>
<td><strong>2</strong></td>
</tr>
<tr>
<td>Vero</td>
<td>06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td>PK1</td>
<td>06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td>MDBK</td>
<td>06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td>MDCK</td>
<td>06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>-</td>
</tr>
</tbody>
</table>

* hours post-infection when the cell culture monolayer was examined.
** reciprocal log$_{10}$ exponents representing the virus dilutions used to infect the cells in the microplate wells.
*** reciprocal HA titres found in undiluted cell culture supernatant.
c = control well with only cell culture medium (negative control)
NT = not tested
- = no HA detected in undiluted cell culture supernatant.
HA tests on the PK1 cell supernatant was carried out 6, 12, 24, 48 and 72 hours after infection, and the results shown in Table 3. At 24 hours after infection, the cell monolayer had already started detaching from the surface of the plate. Unfortunately plaque formation could not be analysed, since the cell monolayer detached in all wells, including those in the negative controls. Haemadsorption was observed only in those wells where HA was detected, although the reaction was not very clear, due to partial detachment of the cells. Consequently, haemadsorption inhibition was only carried out in the duplicate wells giving a positive result. No haemadsorption was observed in those wells treated with specific antisera. Supernatant with HA was infective when inoculated into embryonated eggs.

Although the MDBK cells formed a confluent monolayer, which was maintained during the period of virus infection, virus production as determined by HA was only detected 72 hours after incubation in the wells with added trypsin, in low titres (1/4) for Wey/86 and slightly higher titres (1/8 to 1/16) for Wey/87. Haemadsorption and haemadsorption-neutralization tests produced erratic results which could not be analysed. Erythrocytes adhered to infected cells, but also to numerous non-infected dead cells, despite several washes of the monolayer.

Results of the H present in the supernatant of infected MDCK cells are expressed in Table 3. A steady increase in both the number of wells showing HA and in titre could be
observed on consecutive days. Haemadsorption was positive only in those wells where high HA was observed, and this was neutralised when pre-treating the monolayers with specific antisera. No CPE was observed until the 7th day post-infection. Those wells with agar overlay with trypsin showed discrete plaque formation, with plaques of different sizes for Wey/87 (Fig. 8).

2. Comparative quantitation

Results of HA titration of both viruses in the different cell cultures with and without added trypsin are shown in Figure 9. The range of mean titres for each virus in the different tissues were less than 1 log\textsubscript{10}. The mean titre for each of these viruses was significantly lower in MDBK than in any of the other cells. The HA titre in the cell cultures with added trypsin rose until 96 hours post-infection.

Cytopathic effect was not systematically studied in detail. However, cultures showing haemadsorption also had advanced CPE with few cells remaining attached to the plate. This phenomenon was noted with PK1 cells but not with MDCK or MDBK cells.

No strain was able to form plaques in cell cultures in the absence of trypsin in the culture medium (Fig. 8). Plaques formed in the presence of trypsin were not always
Fig. 8. Plaque formation of Wey/86 and Wey/87 in MDCK cell culture with and without added trypsin.

Plaque formation was only observed in virus dilutions up to $10^{-4}$ when trypsin was added to the medium. Plaques of different size were detected in the plates infected with Wey/87.
Fig. 9. Growth curves of porcine influenza viruses in cell cultures with and without added trypsin.

At regular intervals after inoculation, HA was determined in the cell culture medium. The growth curves shown demonstrate that production of virus was reduced in all cells compared with the HA titre obtained when inoculating the same viruses in embryonated eggs; furthermore, growth only occurred after the addition of trypsin to the cultures.
uniform in size and shape, and difficult to measure (Fig. 8), suggesting genetic heterogeneity of the virus strains used.

All cell culture media of infected wells that presented HA and/or FA were infective for embryonated eggs. Mock infected cell media, however, did not show any HA, nor produced H after 72 hours egg inoculation.

3. Immunoperoxidase test in microplate culture of MDCK cells

As the results of the preceding experiments indicated that MDCK cells developed CPE later than the other cell cultures tested, these cells were used for the IPX technique test. The results of the test are shown in Table 4. Every day, starting 3 days after cell infection, one of the five replicate plates was fixed and stored. Cell monolayers incubated for more than 5 days, whether infected or not, were already detaching from their well surfaces, so that IPX results from days 6 and 7 could not be considered reliable. Best readings were obtained from plates read on the 5th day post-infection, with the specific antiserum at a 1/10 dilution and conjugate at 1/100; or a 1/20 dilution of the specific antiserum and 1/300 conjugate dilution. Virus antigen could be detected up to the highest dilution of inoculum (1/1024).
Table 4: Detection of porcine influenza antigen using the immunoperoxidase technique.

<table>
<thead>
<tr>
<th>Incubation period</th>
<th>Antiserum dilution</th>
<th>1/100</th>
<th>1/200</th>
<th>1/300</th>
<th>1/400</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days</td>
<td>1/10</td>
<td>3.4*</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>1/20</td>
<td>-</td>
<td>**</td>
<td>3.1</td>
<td>2.8</td>
</tr>
<tr>
<td>4 days</td>
<td>1/10</td>
<td>3.4</td>
<td>3.7</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>1/20</td>
<td>-</td>
<td>3.1</td>
<td>1.9</td>
<td>3.4</td>
</tr>
<tr>
<td>5 days</td>
<td>1/10</td>
<td>4.0</td>
<td>3.7</td>
<td>3.7</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>1/20</td>
<td>-</td>
<td>3.4</td>
<td>4.0</td>
<td>3.1</td>
</tr>
<tr>
<td>6 days</td>
<td>1/10</td>
<td>3.4</td>
<td>2.5</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>1/20</td>
<td>-</td>
<td>3.1</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>7 days</td>
<td>1/10</td>
<td>1.3</td>
<td>1.3</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>1/20</td>
<td>-</td>
<td>1.3</td>
<td>1.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Results are expressed in reciprocal log_{10} exponents. The mean of the last wells showing positive results was used in the calculations.

** Negative results, so considered when no red colour could be observed microscopically in the cell monolayer.
Discussion

Epithelioid cells have proved to be the most sensitive in vitro system for propagation of the largest array of influenza A strains (Dowdle and Schild, 1975). Of these, primary monkey, human, and calf kidney cells have been most widely used. Epithelioid cell cultures derived from fully developed organs provided more uniform sensitivity to influenza viruses than cultures derived from organs in the embryonic state.

The results of these experiments indicated that MDCK cells, with added trypsin, were the most suitable for the cultivation of the Weybridge strains of porcine influenza virus, being the best single alternative to embryonated eggs. These cells were best for the FA and IPX techniques, as the cell monolayers stayed attached to the plate surface for up to 7 days. PK1 cells were very susceptible to the virus, but did not permit their use in tests where an intact cell monolayer was required, due to the early development of CPE.

Since virus was detected in the supernatant, from 24 hours post-infection onwards, it was concluded that infectious virus was being actively produced in the cells. Using the FA technique, virus dilutions up to $10^{-2}$ were still found to be positive for virus antigen in some cultures, showing that virus recovery from infected tissues is unlikely to be achieved at dilutions higher than $10^{-3}$
when using cell cultures for primary isolation.

The inability of the virus to form plaques in cell cultures in the absence of added trypsin confirms previous studies (Klenk and others, 1975) that only a few strains of influenza A viruses form plaques in cell cultures without added trypsin and showed that trypsin significantly enhances the infectivity of the strains tested.

The aim of this experiment was also to find the lowest possible influenza virus titre detectable in cell culture by one of the techniques used (CPE, haemadsorption, HA, FA, IPX) in the shortest period after incubation. This is of importance when analysing tissue suspensions or secretions for the presence of virus, specially when only very small amounts of virus are present.

Negroni and Tyrrell (1959) described the morphological changes in influenza A infected tissue cultures as cell rounding, vacuolization and granulation, following reduction in mitosis and the appearance of new virus detected by haemadsorption at the cell surface. However, no characteristic CPE was observed in our studies that could be considered typical, confirming the review by Pereira (1961) that cytopathic effects were not often used as evidence of influenza virus infection in cell cultures, as their effects may be extremely variable.

The haemadsorption technique (Vogel and Shelokov, 1957) may be a sensitive method for the detection of H at the surface of the infected cells, the absorption of
erythrocytes onto the infected cell monolayer indicating the presence of H on the cell surface but in these studies however, results were confusing and the haemadsorption difficult to read, especially when cell degeneration had started.

When comparing the amounts of H detected in cell culture fluid and those produced in the allantoic fluid of embryonated eggs, it becomes evident that cell cultures are not efficient producers of H, when no special techniques are employed. On the other hand, as cell culture wells on microplates may substitute for 96 embryonated eggs, they are very useful for virus screening in animal tissues and secretions. They are easier to handle, cheaper and quicker to examine for virus infection, provided that some basic equipment is available.

Fluorescent antibody techniques have been employed extensively to detect virus-specific antigens in infected cells, and proven to be useful in the study of respiratory viral diseases, by several authors (Coons and others, 1951; Hers, 1962; Tateno and others, 1962; Pensaert and others, 1986). Liu (1956) considered immunofluorescence techniques quicker than retrospective serological studies using the standard HI test, in the diagnosis of influenza virus from nasal samples, although less sensitive. Later, among others, Sugiura and others (1962) successfully followed the development of influenza virus antigens in tissue culture cells using the fluorescent antibody technique. These
studies confirm the usefulness of the direct fluorescent antibody technique for diagnosis of influenza in pigs, as also reported by Pensaert and others (1986). It permits a successful differential diagnosis from other acute respiratory tract diseases, such as Aujeszky's disease. The test is still efficient when part of the monolayer is destroyed. Nevertheless, the fluorescent antibody technique for porcine influenza antigen detection as described here was very laborious and time consuming.

Since the HA is not dependent on H cleavage (Klenk and others, 1975), presence of HA in the cell culture fluids does not necessarily indicate productive infection (Henle and others, 1955) and the production of infectious virus must be shown by other means, such as plaque formation in cell culture or pathogenicity for experimental hosts. Also, in abortive infections, parts of the cell membrane containing HA or NA may be liberated when CPE occurs (Lohmeyer and others, 1979). For these reasons, cell culture supernatant presenting HA were inoculated into embryonated eggs, to confirm the presence of mature and infectious virus.

Host-dependent differences in infectivity of influenza virus strains grown in different host systems exist, and the presence of cellular proteases activating the virus permits consequent spread of infection (Rott, 1979) (see Section 3.1 in Chapter I). Some influenza virus strains are produced with a cleaved H, while others are not, and only virus particles with a cleaved H are infectious (Rott and
Scholtissek, 1982). Whether an influenza virus strain possesses cleaved H or not may be tested by cultivating the virus in cell culture, with and without the addition of trypsin. Plaque formation indicates the presence of infectious particles, while no plaque formation in various cell cultures by Wey/86(H1N1) and Wey/87(H3N2) in the absence of trypsin suggested that these strains possessed uncleaved H. Incubation of the infected cell culture in the presence of trypsin was found to enhance both the productive growth and plaque formation by the influenza virus strains.

Since the presence of serum in cell cultures may cause non-specific neutralization of the virus and neutralization of the trypsin (Dowdle and Schild, 1975), no serum was incorporated into the culture media after infection of cell monolayers. Failure to adequately remove serum constituents before virus inoculation may lead to poor isolation rates or lower infectivity assays in susceptible cell cultures. On the other hand, the lack of serum in the culture media may be, in part, responsible for the detachment of cells from the plates.

Although the immunoperoxidase test as designed here took almost as long as the fluorescent antibody technique, it was clear that the results of the IPX test were more reliable. No non-specific staining was observed, the cell monolayers were easily viewed under the microscope, avoiding false positive results, and reading was much less tedious.
CHAPTER IV

RECOVERY OF INFLUENZA VIRUS FROM FRESH AND FROZEN TISSUES FROM INFECTED PIGS

Introduction

The influenza virus type H1N1 (A/swine/Weybridge/117316/86) has recently been introduced into the U.K., but how this occurred is not known (Roberts and others, 1987). According to Tofts (1987) the most recent outbreaks caused by the H1N1 subtype in Britain may have their origin in imported live pigs from Europe.

Animal influenza is widespread in Europe, U.S.A. and other countries (Nardelli and others, 1978; Tan and others, 1979; Ottis and Bachmann, 1980; Vandeputte and others, 1980; Muller and others, 1981; Masurel and others, 1983). It is now accepted that an influenza virus from one species may infect another species, and in such a way may cause several outbreaks, some epizootic or even panzootic (Fontaine and others, 1983).

The possibility of transmission of influenza viruses between different species has been questioned since the occurrence of the first recorded outbreaks of influenza (Koen, 1928; Shope, 1931), and subsequently, evidence of such transmission has been described by several authors (Tumova and Pereira, 1968; Kundin, 1970; Harkness and
A number of workers reported the possibility of influenza virus transmission from man to pig (Styk and others, 1971a; Alonso and others, 1976; Satsuta and others, 1981; Miwa and others, 1987) and vice-versa (Smith and others, 1976; Thompson and others, 1976; De Jong and others, 1986). In addition, evidence has also been presented that influenza A viruses may be transmitted through avian species to pigs (Laver and Webster, 1973; Hinshaw and others, 1978b; Pensaert and others, 1981). Experimental infections of various species with human and porcine or avian influenza viruses have proven that transmission is possible and live virus has been isolated from various organs and tissues of several types of experimentally infected animals (Blaskovic and others, 1969; Fontaine and others, 1983). Also, some experimental work on transmission of human influenza viruses to pigs has been done, with the detection of the virus and/or specific antibodies in the recipient (Nayak and others, 1965b; Styk and others, 1971a; Pospisil and others, 1973; Maes and others, 1984; Vannier and others, 1985).

Antibodies in man against porcine influenza virus have been frequently reported (Kluska and others, 1961; Woods and others, 1981; Masurel, 1976; Schnurrenberger and others, 1970). These antibodies were detected by HI tests, and it was considered that almost all positive individuals
had had a moderate to maximal exposure to the virus by means of aerosol infection.

Nothing is known about the survival of porcine influenza virus in pig carcasses, even though this is an important point to be considered with respect to export and import of meat. Literature search failed to reveal any report describing studies carried out on porcine influenza virus isolation from meat, and no data is available showing how long influenza virus can survive in the tissues of fattening pigs following slaughter.

The experiments described below were designed to evaluate the survival of porcine influenza virus in contaminated pork, and the consequences this may have on subsequent import/export.

Experiment 1 - In vitro contamination of meat

Cubes of fresh pig meat were treated with various dose levels of porcine influenza virus (Wey/86) and stored refrigerated (4°C) or frozen (-20°C). Meat samples were tested for the presence of live virus by inoculation of embryonated eggs, after 0, 1, 2, 3, 5, 8 and 15 days storage at each temperature.

Experiment 2 - Experimental infection of pigs

Nine pigs were inoculated intratracheally with the same British isolate of porcine influenza virus (Wey/86) and monitored clinically and virologically. One pig was killed
each day and its tissues examined for the presence of influenza virus. In addition, one pig was not inoculated, but was kept as a sentinel in contact with the group, and was killed 10 days after the start of the experiment. This pig served as a control of body temperature and weight gain, as well as to detect the virus transmissibility.

**Experiment 3 - Persistence of virus in infected tissue**

The survival of the virus was studied in tissues from the pig (from experiment 2) killed at 5 days post-inoculation. Duplicate blocks of tissues were stored for varying periods at 4°C or -20°C.

**Materials and methods**

**Virus** - Wey/86(H1N1) has been used throughout this study, after 5 to 7 egg passages (see Section 2 in Chapter II).

**Meat in vitro test** - cubes of 10 x 10 x 20 mm hind leg meat of a freshly slaughtered, healthy pig, 6 weeks old, were cut and put into separate screw cap bottles. Log₁₀ dilutions of virus were made in PBS from stock with an initial HA titre of 1:32 per 25 μl. These were inoculated into and around the meat cubes. Half of the meat was stored at 4°C, the other half at -20°C. On days 0, 1, 2, 3, 5, 8, and 15, the various meat pieces, one from each dilution, were thawed at room temperature for 30 minutes, cut into small pieces with
scissors, and ground up in a mortar, with the aid of a pestle, sterile sand and 10 ml tryptose phosphate broth with antibiotics (TPB+A). After centrifugation for 10 minutes at 700 g, the supernatants were collected and 0.2 ml of the dilutions inoculated into 9-day-old SPF fowls' eggs by either amniotic or allantoic sac routes. These eggs were incubated for 96 hours at 33°C and checked daily for embryonic death. Eggs were harvested after this period and the presence of virus in the amniotic and allantoic fluid detected by the standard micro HA test. If not stated otherwise, all procedures were carried out in a safety cabinet at room temperature. All negative fluids were stored at -70°C and a second passage made in the allantoic cavity of 9-day-old fowls’ embryonated eggs.

The virus dilutions used for meat inoculation were tested by HA test, and the titre of 1:32 confirmed for the 10° dilution. Dilutions greater than this did not show any HA.

Infection of pigs - Nine Large White, seronegative, 12-week-old pigs were sedated IM with 0.75mg/kg of Suicalm (4% azaperone; Janssen, Belgium) then inoculated intratracheally with 10⁵ EID₅₀ of the virus in 0.2 ml. The trachea was perforated just under the larynx through the skin, using a 1 ml syringe and a 19G x 1.5" needle, and the virus suspension injected towards the lung. One pig was not inoculated but left as a control among the infected ones.
**Pig accommodation** - all ten pigs were kept in a single isolation block, in two separate boxes of approximately 14 square metres each, with 5 pigs in each. The air in these boxes was kept at a negative pressure, and exhausted (10 litres/hour) through a high efficiency particulate (HEPA) sterilizing filter. With the exception of two cows no other animals were kept in the building, and these were kept in separate boxes, and stayed in the building throughout the whole experiment. Personnel had access to the animals only after changing into impermeable coats and wearing a face mask. Exiting the building required a shower and a change of outer clothing and boots. No material other than the slaughtered pig was taken out of the building, and all materials were sterilized prior to entry. The pigs were fed granulated food and had free access to fresh water. Bedding was cleaned when necessary and kept in plastic bags in the building up to the end of the experiment. At this time it was incinerated.

**Disease development** - during the ten consecutive days post-infection, weight, rectal temperature, clinical signs of illness, and antibody level were monitored for each animal. Nasal swabs were collected daily and stored in 50% of glycerol in PBS at -70°C until tested for the presence of virus.
Slaughter - each day, one of the experimental animals treated was killed using 20 ml of Expiral (Pentobarbitone sodium BP, 200mg/ml; Ceva, U.K.) intravenously. The animal was then bled out in the isolation building and taken to the post-mortem room in a sealed plastic bag.

Infection screening test - from each slaughtered animal, a piece of all tissues collected was screened for the presence of live virus, using cell cultures (all tissues) and 9-day-old embryonated eggs (lung, mediastinal lymph nodes, blood and nasal swab). The animal presenting the highest overall infection was selected for further studies. The infection rate was determined by HA tests on egg fluids and cell culture supernatant, as well as FA techniques on cell monolayers (see Chapter III).

Sampling - lungs (apical lobe), trachea, mediastinal lymph nodes (Nayak and others, 1965), kidney, spleen, liver, heart and brain were removed from the carcass, as well as parts of the intercostal muscle, ham, small intestine, rectum and oviducts (when female). Whole blood was also collected. Small samples were cut (10 x 10 x 10 mm) and stored in plastic vials.

Storage - all samples were kept at -70°C during the 10 days of sampling. The samples of the animal presenting the highest infection rate in the screening test were split into
2 parts, half being kept at 4°C, half at -20°C, thereafter.

**Testing of samples** - two samples of each tissue, one stored at 4°C, and one at -20°C, were tested daily for 15 days for the presence of live virus. This involved HA tests and inoculation into cell systems: 9-day-old embryonated fowls’ eggs, MDCK (CCL 34, Flow) and PK1 cell line (porcine kidney, CVL). The amniotic cavity was used for primary inoculation, followed by 2 passages in the allantoic cavity. Only lung, mediastinal lymph nodes and blood were tested in eggs; all samples were tested in the above mentioned cell types, grown in microplates, as described below.

The samples, after maceration, were diluted 1 in 10 in PBS, centrifuged at approximately 3000 g and the supernatant used for inoculation procedures. One egg was used for each sample, using 0.1 ml of the 1:10 dilution. Serial 2-fold dilutions of the initial 1:10 dilution were used to infect the cell cultures, up to 1:640. All microplates had positive and negative controls. The cell cultures were infected at the time of seeding. The presence of the virus was detected by HA testing on egg fluids and cell culture supernatants at 96 hours post-infection, and also by a FA technique applied to the cell monolayers, after 7 days incubation. Results were recorded as the highest HA titre (first, second or third egg passages) and the highest sample dilution showing specific fluorescence in the cell cultures.
Cell cultures - the PK 1 and MDCK cell cultures, grown in 96 well, flat-bottomed microplates (Nunc, Denmark) were selected according to previous studies (see Chapter III). Approximately $2.5 \times 10^4$ cells/ml were used for seeding, each well receiving 200 μl of cell suspension. Incubation took place at 33°C, with the microplates sealed with adhesive tape. Medium for MDCK cells was Eagle's Minimum Essential Medium (MEM) plus 5% fetal calf serum and antibiotics, and for PK 1 cells, medium 199 (Flow) plus 5% fetal calf serum, 3% bicarbonate, 5% lactalbumin hydrolysate, 1% L-glutamine and antibiotics. To both media was added 10 μg/ml trypsin, 2% of HEPES and 1% sodium bicarbonate (in a 7% solution).

TPB+A = Tryptose phosphate broth and antibiotics (100 UI penicillin, 100 μg streptomycin, 20 IU mycostatin/ml).

PBS pH 7.4 - prepared as described in Section 1 in Chapter II and added antibiotics, as above.

Virus detection - HA technique was performed according to standard micro-methods (WHO) (see Section 2 in Chapter II).

Serological examination - specific antibodies were demonstrated by the micro HI test in 96 well, U-bottomed microplates (Nunc, Denmark). The titres were expressed as the reciprocal of the highest serum dilution causing complete inhibition of 4 HAU of virus (see Section 2 in Chapter II).
Before testing, the sera were inactivated at 56°C for 30 minutes and treated with kaolin and 10% CRBC's to remove non-specific inhibitors (see Section 2 in Chapter II).

The FA technique used was the method already established at the CVL, Weybridge, adapted for microplates. (See Chapter II and III). In brief, cell monolayers grown in microplates were fixed in each cell with 75% acetone for five minutes, after removal of the cell culture medium. The plates were left on the bench to dry. Then, the plates were washed three times with TRIS (0.01M tris-HCl pH 8.7 containing 0.14M NaCl). The conjugate, prepared according to the method of Nairn (1976), was applied to all wells, using 50 μl/well, and left for 30 minutes at 37°C. The plates were washed again three times with TRIS, and dried by tapping them on paper towel. The plates were read, inverted, under a U.V. microscope, using a 50 W high pressure mercury lamp (WOTAN).

Results

1. In vitro experiment

The thawed meat pieces, triturated and suspended 1 in 10 (v/v) were inoculated into embryonated eggs, which allantoic and amniotic fluids were tested for HA.
The results of the *in vitro* experiment are summarized in Table 5 and demonstrate that even after 15 days of storage, the influenza virus present in the meat was infectious and able to replicate in embryonated eggs. Detailed results can be found in Appendix I.

It can be observed that during the first three days, virus survival was not greatly affected, although there was a higher virus recovery rate in the meat kept at -20°C. This becomes obvious at day 5. At 4°C, the virus was no longer isolated from the highest dilutions, and seemed not to survive much longer than 10 days. By day 8, only the lowest dilutions permitted virus recovery, and at day 15, an end-point seems to have been reached, with isolations made only from the 10⁻¹ dilution. From the meat kept at -20°C, isolations were still possible from the various virus dilutions on day 15.

Although the amniotic route is known to be more sensitive to the virus and permit higher recovery rate, in this experiment very few samples were positive by that route, possible due to technical inexperience.

A second passage of the fluids negative on first passage gave a positive result with some samples (Appendix I).

The large range of virus dilutions used to infect meat and inoculate the eggs enabled the determination of an end-point, even on day 0.
Table 5: Highest dilutions of Wey/86 infected meat suspension, kept at 4°C and -20°C which gave a positive HA result after egg inoculation.

### 4°C

<table>
<thead>
<tr>
<th>meat dose level (log10)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&gt;3*</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>2.</td>
<td>2</td>
<td>&gt;3</td>
<td>-</td>
</tr>
<tr>
<td>-1</td>
<td>2</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>2</td>
</tr>
<tr>
<td>-2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>&gt;3</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>-3</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>&gt;3</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>-4</td>
<td>1</td>
<td>&gt;3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### -20°C

<table>
<thead>
<tr>
<th>meat dose level (log10)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>-</td>
</tr>
<tr>
<td>-1</td>
<td>0</td>
<td>&gt;3</td>
<td>2</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>1</td>
<td>&gt;3</td>
</tr>
<tr>
<td>-2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>2</td>
</tr>
<tr>
<td>-3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>&gt;3</td>
<td>2</td>
<td>&gt;3</td>
<td>1</td>
</tr>
<tr>
<td>-4</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>&gt;3</td>
<td>&gt;3</td>
<td>&gt;3</td>
</tr>
</tbody>
</table>

* HA titre found in eggs inoculated with meat extract dilution, expressed in reciprocal log\(_{10}\) exponents.
0 = HA titre between 1/5 and 1/10
- = no HA titre detected
2. Evolution of experimental disease *in vivo*

No clinical signs of illness were observed among the pigs infected with Wey/86 during the ten days of the experiment, except that the pigs in box 01 were slightly depressed and reluctant to move between days 2 and 4 post-infection. Figures 10 and 11 show the temperature range and weight gain, respectively, during the experimental period. No statistically significant temperature rise could be observed on any day. However, there was a significant impairment in weight gain of infected pigs when compared with the control. Influenza A virus specific antibodies were only detected, at low levels, in 2 of the pigs, starting on day 8 post-infection. No other clinical evidence of infection was observed. The presence of virus in nasal swabs, collected daily, was very inconsistent when using the HA technique (Table 6). All gave HA titres less than 1/10, except that of pig 222, from which the virus was reisolated on day 05, with a HA titre greater than 1/256.

Paired serum samples collected from the two cows in the building were negative for the Weybridge strain of influenza virus by HI test.

No macroscopic lesions were observed in any tissue of the experimentally inoculated pigs.

The contact control 205 was the most lively pig of the group. No seroconversion was demonstrated, nor virus isolated from any of its tissues or nasal swabs (Table 6).
Fig. 10. Temperature range observed in pigs experimentally infected with Wey/86 (H1N1), during a 10 day observation period. Since the normal temperature of pigs is approximately 38.5°C, the temperature of the infected and control pigs during the experiment can be considered as being within the expected range of healthy pigs.
Days post-infection: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Temperature (°C): 36, 37, 38, 39, 40, 41

- Mean infected pigs
- Control
- Range infected pigs
Fig. 11. Weight gain during a 10 day period following experimental infection of pigs with Wey/86 influenza virus strain subtype H1N1. Infected pigs did not gain weight significantly, when comparing with the weight gain of the control pig over the same period.
Weight (Kg)

- mean infected pigs
- control
- range infected pigs

Days post-infection

Weight (Kg)

0 1 2 3 4 5 6 7 8 9 10
3. Infection screening test

The results of the screening test for the presence of live virus in tissues of infected pigs after slaughter are presented in Table 6. Virus detection was carried out in embryonated eggs and cell cultures, using HA and FA tests.

Influenza virus was most frequently detected in the nasal swabs, as well as in the lungs and mediastinal lymph nodes. Virus was isolated from the trachea, faeces and ovaries of five pigs. Other tissues presented erratic positive results, with less than 50 per cent of the animals exhibiting virus isolation from the same tissue.

Pig 222 was chosen for further investigation, because it yielded influenza virus in respiratory tissues, and also because, when considering the post-infection time and classical porcine influenza disease evolution, it was considered to have been slaughtered at the peak of the disease expression.

Pieces of tissue of pig 222, kept at 4°C and -20°C, were checked for the presence of live virus during 15 consecutive days. Results are expressed in Table 7. Live virus was detected on at least one day in all tissues examined, but the frequency of isolation gradually diminished with time. It was expected to find virus in the same tissues before and after storage, for at least the first test post-storage. This did not occur, perhaps due to the distribution of the virus in the tissues.
Table 6. Summary of results showing the presence of influenza virus in pig tissues infected with Wey/86 virus, at the time of slaughter, as detected in various cell systems, by HA and FA tests.

<table>
<thead>
<tr>
<th>Pig no</th>
<th>MH</th>
<th>MI</th>
<th>H</th>
<th>S</th>
<th>Li</th>
<th>K</th>
<th>Int</th>
<th>T</th>
<th>F</th>
<th>RO</th>
<th>ML</th>
<th>L</th>
<th>Bl</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>209</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>217</td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>208</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>222</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>206</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>219</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>207</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>216</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>205</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Total % positive: 30 40 30 20 40 30 40 50 50 50 20 80 80 60 90

+ = detection of virus by one of the techniques
- = no virus detected by the techniques used
* pigs arranged in order of slaughter.

** Tissue abbreviations used:

- MH = meat (Ham)
- MI = meat (intercostal muscle)
- H = heart
- S = spleen
- Li = liver
- K = kidney
- Int = large intestine
- T = trachea
- F = faeces
- RO = reproductive organs
- Br = brain
- ML = mediastinal lymph nodes
- L = lung
- Bl = blood
- NS = nasal swab
- NT = not tested
Table 7. Persistence of virus in the tissues of a Wey/86 influenza virus infected pig (no 222) on storage. The pig was slaughtered 5 days post-infection and samples of tissues were kept at 4°C and -20°C during a 14 day period.

<table>
<thead>
<tr>
<th>day</th>
<th>IM</th>
<th>K</th>
<th>RO</th>
<th>T</th>
<th>Ha</th>
<th>H</th>
<th>Li</th>
<th>Br</th>
<th>Int</th>
<th>F</th>
<th>NS</th>
<th>L</th>
<th>ML</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Summary</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>day</th>
<th>IM</th>
<th>K</th>
<th>RO</th>
<th>T</th>
<th>Ha</th>
<th>H</th>
<th>Li</th>
<th>Br</th>
<th>Int</th>
<th>F</th>
<th>NS</th>
<th>L</th>
<th>ML</th>
<th>Bl</th>
</tr>
</thead>
<tbody>
<tr>
<td>-20°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Summary</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ = detection of virus by HA in allantoic fluid of inoculated embryonated eggs.
- = no detection of virus by HA after egg inoculation.
* abbreviations of tissues tested:
MI = meat (intercostal muscle) F = faeces
H = heart RO = reproductive organs
S = spleen Br = brain
Li = liver ML = mediastinal lymph
K = kidney nodes
Int = large intestine L = lung
T = trachea B1 = blood
NS = nasal swab
NT = not tested
Discussion

The experiments described in this chapter were designed to determine if pigs infected with porcine influenza virus, slaughtered after infection, can transmit the virus to other pigs or personnel handling the diseased animal, through surviving virus in their tissues. It was considered possible that virus could be spread by a number of routes during the process of slaughtering: heavily infected lungs or blood could infect the meat through contact, or virus could already be present in the meat or other parts of economical value.

Recovery of porcine influenza virus from experimentally infected pigs has been achieved by several authors (Blaskovic and others, 1970; Fontaine and others, 1983). Tissues used for isolation have been taken from the affected respiratory tract and accessory lymph nodes, and also from blood samples and organs associated with filtration of blood, like the spleen. Nayak and others (1965b) studied the progression of the infection in experimentally infected pigs, and Vannier and others (1985) established the shedding period of this virus. In the above studies, no tissues other than the respiratory tract appeared to be involved.

The in vitro studies reported in this Chapter showed that, depending upon the condition of storage, porcine influenza can be transmitted through meat, provided that it
is processed within 15 days after slaughter and storage is at low temperatures (in this case -20°C). The population at risk may be meat processor employees in charge of handling crude meat soon after slaughter, or herds which are fed with pig wastage.

The results obtained in the in vivo experiments showed that pigs infected with influenza virus may harbour the virus in their tissues without clinical signs of illness. This confirmed the studies of Blaskovic and others (1970), Styk and others (1971a), Vandeputte and others (1981) and Vannier and others (1985). Although in the present studies no spread to other in contact pigs was observed, the possibility of spread exists, and contacts acquiring the infection, may pass unnoticed.

It is clear that after experimental infection, the porcine influenza virus strain used in these studies replicated poorly in pigs, with moderate or no pathogenicity. Similar observations have been made by several authors (Pospisil and others, 1973; Vandeputte and others, 1981; Maes and others, 1984; Vannier and others, 1985). A number of factors, including the age of the pigs, environmental temperature, virus strain used for infection, dose, route of inoculation, concommitant infections and stress, have all been shown to have influence on the expression of the influenza disease (see Section 3 in Chapter I).
Finally, it must be remembered that several egg passages were used to isolate the virus, and the original strain isolated from the pig may have altered its host and tissue specificity. The poor recovery rate of virus from the pigs when using cell culture lines may be in part due to poor host adaptation or no ability to grow in them.
INTERSPECIES TRANSMISSION AND PATHOGENICITY OF PORCINE INFLUENZA VIRUSES IN TURKEYS AND PIGS.

Introduction

Despite frequent speculation, the origins of the many outbreaks of influenza in birds and mammals have never been determined (Easterday and Tumova, 1972a; Alexander, 1982a; Murphy and Webster, 1985), although the ability of influenza virus isolates from one species to infect other species has been demonstrated (Shortridge, 1988).

With the exception of an outbreak of influenza in terns in South Africa in 1961 (Easterday and Tumova, 1972a), no significant disease problems caused by influenza viruses are known to occur among free-flying birds (Stallknecht and Shane, 1988). Migratory waterfowl have been suspected of spreading the virus and to be involved in producing new strains of virus by genetic reassortment (Wells, 1963; Easterday and Tumova, 1972a; Easterday and Beard, 1984). There have been no reports of disease outbreaks in humans that could be specifically attributed to influenza viruses of avian origin (Webster and others, 1981b) but there is evidence that suggests that an avian-porcine-human chain of events does occur (see Section 6 in Chapter I).
It has been shown that the Hong Kong variant of human influenza virus can be transmitted to pigs (Lief, 1970; Beare and others, 1971; Styk and others, 1971b; Kundin and Easterday, 1972; Pospisil and others, 1973; Ottis and others, 1982), cattle (Lopes, 1985; Lopes and Woods, 1987), dogs (Todd and Cohen, 1968; horses (Blaskovic and others, 1969); chickens and also wild birds (Sakstelskaja, 1975; Hinshaw and others, 1981c). So, many H3N2 subtype strains usually associated with human infections readily infect other animals. After the 1918-1919 pandemic, suggestions of natural infection of pigs with viruses that also infected humans have been made on a number of occasions (Shope, 1936; 1938). Other findings also suggested that pigs in 1976 were infected with influenza H3N2 strains similar to those first detected in man in 1968 and 1975 (Romvary and Vizy, 1971; McFerran and others, 1972; Shortridge and others, 1977; Alonzo and others, 1976; Tan and others, 1979; Aymard and others, 1980; Tumova and others, 1980a; Arikawa and others, 1981; Nerome and others, 1981; Ottis and others, 1982; Madec and others, 1984; Tumova and others, 1985). There was evidence that viruses of this kind were still present in some porcine populations in Europe in 1977, confirming the ability of this earlier H3N2 strain to persist in nature. Also, H1N1 viruses appeared in pigs all over Europe, after the appearance of the same virus in the human population (Aymard and others, 1980; Hannoun
In 1978, Chapman and others (1978) discussed the significance of the presence of antibodies against human influenza A viruses in pigs. They examined serum samples collected from pigs in Great Britain at various times between July 1971 and July 1977, and provided evidence of continuing infection with human H3N2 influenza viruses. Studies from Shortridge and others (1977) found that Hong Kong-like viruses isolated from pigs could be readily transmitted experimentally from pig to pig. However, the pathogenicity of the viruses studied for the subsidiary hosts was very low, and there were no reports of panzootics in pigs at that time. Some authors consider that after direct transmission of infection to another species, almost all viral particles produced are incomplete, leading to a sub-clinical infection, which is only manifested as clinical disease by interaction with other factors such as stress (Sakstelskaja, 1975). Similar opinions were expressed later by workers who found specific antibodies in the serum both of diseased pigs (Tumova and others, 1980b; Yasuhara and others, 1983) and pigs without typical clinical signs of the disease (Sinnecker and others, 1983b).

However, the findings of Shortridge and others (1987) for example, suggest that interspecies transmission from birds to pigs may occur in nature. The precursor H of the virus infecting pigs in China in the 1980s was an influenza virus of duck origin. If the influenza strains present in
pigs had their origin in migrating, wild or domestic birds, interspecies transmission occurs readily between these species.

Studies concerning antigenic analyses of influenza H1N1 viruses from different species led Aymard and others (1985) and others before them (Mohan and others, 1981; Hinshaw and others, 1984; Andral and others, 1985) to conclude that transfer of influenza viruses is possible between pigs and turkeys. They suggested that since serological surveys in Brittany, France, indicated the absence of influenza in turkeys before 1980, pigs possibly transferred the virus to these birds on that occasion. Mohan and others (1981) reported on an outbreak of influenza among turkeys on an Ohio farm where antibodies to porcine influenza were identified in the sera of the diseased turkeys. Also, some of the farm workers presented HA antibody titre against the same porcine virus strain.

There are few reports of experimental infection of avian species with mammalian influenza isolates (Alexander, 1982a). When attempted, virus was not always recovered, nor signs of disease observed (Easterday and Tumova, 1972a). Comparisons of H1N1 isolates from pigs, humans, ducks and turkeys with polyclonal and monoclonal antibodies, RNA-RNA competitive hybridization, and replication studies have been carried out by Hinshaw and others (1984). Their results suggest that both turkeys and pigs are involved in the
maintenance of influenza viruses and their transmission to humans. In their report, they describe influenza A subtype H1N1 isolates from turkeys that are almost indistinguishable from viruses typically associated with pigs. The three monoclonal antibodies used, which recognized different antigenic determinants on the H of H1 strains, showed that these determinants were shared by the recent turkey, human and porcine viruses but not by the duck viruses.

Natural influenza infection among turkeys, due to a porcine influenza virus subtype H1N1 has been reported by Andral and others (1985). The infection occurred after the spread of the virus in a pig herd located near the turkey breeding farm.

Also, since some porcine viruses infect humans (Hinshaw and others, 1978a) it is possible that infected farm personnel can transmit the viruses to turkeys, but only if these viruses infect and replicate in turkeys and also possess a tissue tropism similar to that of mammalian, rather than avian, viruses of the same subtype. In this manner, domestic turkeys, even when kept inaccessible to wild birds, can be infected by direct or indirect aerosol transmission, contaminated water, or brought in by humans by other means.

In earlier experiments, porcine influenza A viruses were recovered from experimentally infected pigs, which had developed signs of disease. Using the same porcine influenza isolates, infection of turkeys and interspecies
transmission of influenza between turkeys and pigs have been attempted, under experimental conditions. The objective was to find out whether the English porcine isolates could have originated in birds, and whether or not a transmission cycle could be established between pigs and turkeys.
A - EXPERIMENTAL INFECTION OF TURKEYS WITH SOME INFLUENZA VIRUS SUBTYPES OF PORCINE AND AVIAN ORIGIN.

The pathogenicity of two porcine influenza A viruses (H1N1 and H3N2) was assessed for young turkeys (4 weeks old) by inoculating them by various routes, and comparing the pathogenicity of turkey isolates of the same two subtypes (H1N1 and H3N2), by analysing clinical signs, replication and excretion period of the viruses.

Material and methods

Birds - Broad-breasted white turkeys were purchased as 1-day-old poultts (British United Turkeys, High Wycombe, Bucks), and reared at the Central Veterinary Laboratory (CVL) under SPF conditions.

Experimental design - In experiment I, two groups of 12 turkeys were housed in separate rooms of 50 m$^3$ each in the isolation unit at CVL. A constant temperature of 26°C was kept, negative air pressure was maintained with 15 air changes/hour and exhaust and input filtered through HEPA filters. Ten of each group were inoculated with an influenza A virus subtype, one group with a subtype of avian origin and the other group with a subtype of porcine origin. In each group, two turkeys were left uninoculated as contacts.
Experiment II had a similar arrangement except that no contacts were present.

Throughout both experiments a minimum of five non-infected controls were kept separately.

All birds were examined daily and temperature, signs of disease and any deaths recorded. Dead birds were examined for internal lesions.

Virus strains — Subtypes Wey/86 and A/turkey/England/250/79(H1N1) (turk/79) were used in experiments IA and IB, respectively, and Wey/87 and A/turkey/England/69(H3N2) (turk/69) were used in experiment IIA and IIB. The influenza strains were obtained from stock (see Section 2 in Chapter II).

Inoculum — All turkeys of experiment I, except the contacts, were inoculated with infective allantoic fluid; 0.2ml intratracheally and 0.1ml intranasally. Each group of experiment II turkeys was inoculated with infective allantoic fluid; 0.1ml intratracheally, 0.1ml intranasally and 0.05ml supra-conjunctivally. The EID$_{50}$ for all inocula ranged between $10^4$ and $10^5$.

Virus titrations — see Section 2 in Chapter II.

Virus isolation — Cloacal and tracheal swabs were collected at regular intervals (days 1, 2, 3, 4, 7, 8, 10, ...
14, 21, 24, 28 for cloacal swabs, days 7, 14, 21 for tracheal swabs in experiment I, and days 3, 5, 7, 11, 17, 23, 30 for cloacal swabs, days 7, 17, 23, 30 for tracheal swabs in experiment II), and placed in 1 ml phosphate buffered saline pH 7.4 (PBS) containing 10,000 units penicilllin (Glaxo), 10 mg streptomycin (Evans), 250 μg gentamicin (Pfizer) and 5000 units mycostatin (Squibb) per ml. The samples were clarified by low speed centrifugation and inoculated into 9-day-old embryonated fowls' eggs, into the allantoic fluid, using 0.2ml of the supernatant. Eggs were incubated at 33°C and checked daily. Allantoic fluid was collected from the dead embryos or at 3 days after inoculation, and tested for HA. Negative samples were passaged twice more.

**H titrations** were done in 96 well microplates (Nunc, Denmark), with U shaped wells. The technique used was based on the recommendations of the WHO Expert Committee on Influenza recommendations (see Section 2 in Chapter II).

**CFT diluent** (Oxoid) was used throughout these tests as a diluent (see Section 1 in Chapter II).

**CRBCs** at 1 % v/v were used for HA reactions (see Section 2 in Chapter II).

Serology - HI tests were performed on sera collected on days
0, 7, 14, and 28 from turkeys of experiment I and on days 0, 7, 11, 17, 23 and 30 from turkeys of experiment II. This test was performed in U-shaped microtitre plates (see Section 2 in Chapter II).

The sera were inactivated at 56°C for 30 minutes but otherwise untreated against non-specific inhibitors.

PBS pH 7.4 - see Section 1 in Chapter II.

Results

1. Clinical signs of disease

Of the four viruses tested only H1N1 viruses produced any signs of illness or deaths in the 4-week-old turkeys. These turkeys were all sick by the second day after infection. Clinical signs consisted of general depression, ruffled feathers, coughing and sneezing during experiment I. In some birds greenish diarrhoea and reluctance to move were also observed. No respiratory signs of infection were observed in the birds of experiment II.

No weight losses due to influenza illness were found (Table 8). In fact, infected birds gained more weight than their respective controls. Individually, it was noted that those birds with the severest clinical signs of infection showed less weight gain. A difference in weight was observed between the turkey groups inoculated with porcine influenza
Table 8: Weight of turkeys (in Kg) infected with influenza virus strains. Only mean values were recorded from the animals infected with virus subtypes H1N1.

<table>
<thead>
<tr>
<th>Turkeys inoculated</th>
<th>Animals infected with H1N1</th>
<th>Animals infected with H3N2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before infection (day 0)</td>
<td>weight gain</td>
</tr>
<tr>
<td></td>
<td>NT 1.65</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>NT 1.90</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>NT 2.00</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>NT 2.30</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>NT 1.80</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>NT 2.10</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>NT 2.35</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>NT 2.15</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>NT 2.10</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>NT 2.10</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>NT 2.10</td>
<td>0.72*</td>
</tr>
<tr>
<td></td>
<td>NT 1.30</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>NT 1.40</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>NT 1.60</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>NT 1.90</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>NT 1.45</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>NT 2.20</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>NT 1.85</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>NT 1.55</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>NT 1.40</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>NT 1.15</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>NT</td>
<td>0.59*</td>
</tr>
<tr>
<td></td>
<td>contr</td>
<td>0.62*</td>
</tr>
</tbody>
</table>

NT = not tested
* = mean values
contr = non-infected controls
( ) = standard error of the mean
strains and avian influenza strains, the former being heavier at the end of the experiment.

The temperature of turkeys was only monitored in experiment I. Since the normal body temperature of turkeys range between 40 and 43°C (Biester and Schwarte, 1959) no large variations occurred either in the turkey group infected with the H1N1 porcine subtype or in the group infected with the H1N1 turkey subtype (Fig. 12). Minor variations may be due to stress during handling.

2. Virus recovery

Virus recovery was attempted from dead and live birds.

In turkey 328, that had died 24 hours after infection, influenza virus was detected in the trachea, intestinal contents and kidneys. No internal lesions were observed. No deaths occurred among the turkeys receiving H3N2 viruses.

Haemagglutinating agents were isolated from cloacal swabs of all but three infected and contact turkeys. Virus recovery from tracheal or cloacal swabs of infected or in contact birds is shown in Tables 9 and 10. It was noted that successful isolations were more frequent from cloacal swabs. Virus was never recovered from the trachea without also being recovered from the cloaca, although the converse was frequently true. The highest proportion of birds excreting virus was found between days 5 and 7, and the last virus isolation was made at 17 days after infection.
Fig. 12. Temperature of turkeys following infection with porcine and avian strains, subtype H1N1.

Obs.: Only the turkeys in Experiment I had their rectal temperature controlled daily.

..... = decision boundary between which temperature were considered normal.

---- = reference value of mean normal temperature of birds.
Table 9. Isolation of H1N1 virus from cloacal and tracheal swabs collected from turkeys during experiment I, following egg passage and detection by HA.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>7</th>
<th>8</th>
<th>10</th>
<th>14</th>
<th>17</th>
<th>21</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>t</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>t</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>326</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>327</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>328</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>329</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>331</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>332</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>333</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>334</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>335</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pool</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>678</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>679</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>337</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>338</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>339</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>340</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>341</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>342</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>343</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>344</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>345</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>pool</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>676</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>677</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Key:
0 = no detectable HA (less than 2 HAU)
NT = not tested
+ = detectable HA (equal or greater than 2 HAU)
c = cloacal swab
t = tracheal swab
Table 10. Isolation of H3N2 virus from cloacal and tracheal swabs collected from turkeys during experiment II, following egg passage and detection by HA.

<table>
<thead>
<tr>
<th></th>
<th>days post-inoculation</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>11</td>
<td>14</td>
<td>17</td>
<td>23</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>t</td>
<td>c</td>
<td>c</td>
<td>c</td>
<td>t</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>501</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>502</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>503</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>504</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>505</td>
<td>NT</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>506</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>507</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>508</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>509</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>510</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>511</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>512</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>513</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>514</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>515</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>516</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>517</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>518</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>519</td>
<td>NT</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>520</td>
<td>NT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Key:
0 = no detectable HA (less than 2 HAU)
NT = not tested
+ = detected HA (equal or greater than 2 HAU)
c = cloacal swab
t = tracheal swab
with avian H1N1 virus and at 14 days after infection with porcine H3N2 virus.

With only a few exceptions, all turkeys became infected with H1N1 and H3N2 avian and porcine virus strains. The turkey group infected with porcine H3N2 strain included 3 birds from which no virus was isolated, although specific antibodies were detected in all (Appendix II). However, a second passage in the chick embryo was sometimes necessary to detect the virus by the HA test.

Virus isolation and immune response studies indicated that in contact turkeys in the H1N1 virus groups had been infected and recovered from infection.

3. Immune response

The mean immune response in birds surviving to 28 days after infection was measured by the HI test (Fig.13). All sera collected were tested against the turkey and porcine strains of that subtype. The contacts to H1N1 infected animals reached the highest titres, followed by the antibody titre of the infected animals. No antibodies against the avian H1N1 strain were detected in the birds infected with the porcine H1N1 strain. Among the birds infected with the avian H1N1 strain, however, antibodies against the porcine H1N1 strain were detected, which may be due to cross-reactivity. Again, contact birds showed a higher immunological response than the other birds.
Fig. 13. Results of HI tests on sera from turkeys of experiment I and II - presence of influenza virus specific antibodies.

13.1. Antibody response of turkeys infected with porcine strain subtype H1N1, against H1N1 porcine and avian strains.
13.2. Antibody response of turkeys infected with avian strain subtype H1N1, against H1N1 porcine and avian strains.
13.3. Antibody response of turkeys infected with porcine strain subtype H3N2, against H3N2 porcine and avian strains.
13.4. Antibody response of turkeys infected with avian strain subtype H3N2, against H3N2 porcine and avian strains.
13.1 Exp.1a

13.2 Exp.1b

13.3 Exp.1la

13.4 Exp.1lb

Ab titre = \( \log_2 \left( \frac{\text{reciprocal HI titre}}{10} \right) \)
No contact birds were present among the turkeys infected with the H3N2 strains, and no comparisons could be made on transmissibility. Antibodies against the porcine H3N2 strain were present in the turkeys infected with the homologous virus, and only a minor response against the avian strain. Birds exposed to the avian H3N2 strain responded to both avian and porcine strains. The antibody titre against the latter at the start of the experiment is possibly due to the heating of the sera before testing, since this has been shown to increase non-specific HA in turkey sera (D.J. Alexander, personal communication).

It was considered that a characteristic antibody response was only obtained with the birds infected with the H1N1 strains, and the peak of antibody titre varied between 17 and 30 days post-infection. A more lasting immunological response was obtained with the H1N1 avian strain. No correlation could be found between prolonged virus isolation and the observed immunological response.

An individual analysis shows that only one bird failed to show a positive HI titre to H1N1 virus infection in its serum at 28 days, despite the isolation of the same virus from this bird and from both of the contact birds (Appendix II).

Using a positive serological response as evidence of the establishment of an infection, it can be seen that of the four strains examined the avian H1N1 virus was most infectious for turkeys. The highest HI titres recorded in
this study were from the turkeys that survived infection and contact with the H1N1 viruses.

B - INTERSPECIES TRANSMISSION OF PORCINE INFLUENZA VIRUSES IN TURKEYS AND PIGS

The capacity of porcine influenza viruses to infect turkeys and transmit the virus back to pigs was investigated.

Material and methods

**Birds** - Broad-breasted white turkeys were purchased as 1-day-old poults (British United Turkeys, High Wycombe, Bucks), and reared at CVL under SPF conditions.

**Pigs** - four-week-old cross-breed Large White piglets were purchased from a farm where no porcine influenza has been reported in the six months preceding the experiments.

**Virus strains** - Subtypes Wey/86(H1N1) and Wey/87(H3N2) were used in both experiments. Both strains were isolated from disease outbreaks in pigs in England, in 1986 and 1987 respectively. Stock cultures were stored at -70°C and
passaged not more than four times in embryonated fowls' eggs (see Section 2 in Chapter II).

**Inoculum** - The first 10 turkeys introduced into each room were inoculated with crude infective allantoic fluid, 0.2ml intratracheally, and 0.1ml intranasally, containing $10^4$ to $10^5$ EID$_{50}$.

**Experimental design** - The same procedure was set up twice (experiments 1 and 2) for each of the two virus strains, H1N1 (group A) and H3N2 (group B). In each experiment, two groups of 10 turkeys were housed in separate rooms in the isolation unit at CVL. Each room, with a volume of 50 cubic metres, was kept at a constant temperature of 26°C. A negative air pressure was maintained, with input and exhaust of 15 air changes/hour, filtered through HEPA filters. On day 0, all ten of each group were inoculated with an influenza A virus subtype, one group with H1N1 and the other group with H3N2, both of porcine origin. They were kept in cages measuring two square metres, with free access to food and water.

After 3 to 4 days, six piglets were introduced into each room, and put into similar cages as the turkeys. Four of the pigs were in close contact with the infected turkeys. The other two, although kept in the same room, had no direct contact, since a plastic curtain separated their cages and avoided accidental transmission by means other than air.
The contact pigs and infected turkeys were changed daily into each other's cages, which had not been cleaned, thus allowing contact with faeces.

On day 14 the first group of turkeys was removed, their cages cleaned, and replaced by five influenza-virus-free turkeys. These turkeys were changed daily to the cages of the pigs, permitting close contact, for a further period of 15 days in order to detect any pig to turkey transmission of the viruses.

All birds and pigs were examined daily and temperature, signs of disease or deaths were recorded. Dead animals were post-mortemmed and any lesions noted. Blood samples were collected on day 0 and 14 and/or 21 days after exposure, and tested for specific antibodies to porcine influenza viruses.

Both experiments had a similar arrangement, except that in experiment 2 the temperature responses in the infected turkeys were not recorded.

**Virus titrations** - see Section 2 in Chapter II.

**Virus isolation** - Cloacal and tracheal swabs were collected at regular intervals and placed in 1 ml phosphate buffered saline pH 7.4 (PBS) containing 10,000 units penicillin (Glaxo), 10 mg streptomycin (Evans), 250 μg gentamicin (Pfizer) and 5000 units mycostatin (Squibb) per ml.

Samples were clarified by low speed centrifugation and 0.2 ml of the supernatant was inoculated into 9-day-old embryonated fowls' eggs, by the allantoic route. Eggs were
incubated at 33°C and checked daily for viability. Allantoic fluid was collected from the dead embryos or 3-days post-inoculation and tested for HA. Negative samples were passaged twice more, by inoculating the harvested allantoic fluid from the first passage undiluted into fresh 9-day-old embryonated eggs.

**Haemagglutinin titrations** - carried out in 96 well microplates (Nunc, Denmark), with U shaped wells (see Section 2 in Chapter II).

**CFT diluent (Oxoid)** - used as diluent throughout these tests. One tablet was dissolved in 100 ml distilled water (see Section 1 in Chapter II).

**CRBCs at 1% v/v suspension** were used for HA reactions (see Section 2 in Chapter II).

The identity of the isolates was confirmed by HI tests, using specific antisera produced in chickens (see Section 2 in Chapter II).

**Serology** - HI tests were done on sera collected on days 0, 14 and/or 21 from turkeys and/or pigs. This test was performed in U-shaped microtitre plates, and carried out as recommended by Palmer and others (1975) (See Section 2 in Chapter II).

All sera were inactivated at 56°C for 30 minutes.
Turkey sera received no further treatment, but porcine sera were treated with 25% kaolin w/v and 10% v/v fowl red blood cells, and thus tested at an initial dilution of 1 in 10 (see Section 2 in Chapter II).

PBS - see Section 1 in Chapter II.

Results

1. Clinical signs

During the first week, a slight rise in temperature was observed among all contact pigs in experiment 1 (Fig. 14). The control pigs of experiment 1A (group infected with H1N1 strain) maintained a very stable temperature, but this was not the case with the control pigs in experiment 1B (group infected with H3N2 strain). Although a rise in temperature was observed on the 6th day post-infection in this group, neither virus nor antibody rise was detected suggesting that no infection had taken place (Table 11 and Fig. 15). No significant temperature rise was observed in the pigs in experiment 2.

Clinical signs observed among the infected turkeys and contact pigs included cough, nasal discharge varying from mild to purulent, and ocular discharge. The infected birds had ruffled feathers in the first 3 days and sometimes
Fig. 14. Temperature monitoring of pigs during the first 6 days of experiment 1.

.... = decision boundary under which temperatures were considered normal.

---- = reference value considered normal according to mean of healthy pigs.
Table 11. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 1A (using virus strain H1N1).

<table>
<thead>
<tr>
<th>Days **</th>
<th>0</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>I R I R I R I R I R I R I R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Turkeys infected with virus (10)***

<table>
<thead>
<tr>
<th>Days</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>In contact pigs (4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control pigs (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

NT = not tested
I = intestinal tract
R = respiratory tract

* Number of positive samples detected in experiment 1A (using virus strain H1N1).

** Number of days post-exposure that the virus was detected.

*** Number of animals from which samples were taken.
serous discharge from the nasal clefts could be observed on the third and fourth days. None of the control turkeys kept in close contact with the pigs showed any clinical sign of illness.

During experiment 1A, one contact pig and one contact turkey died unexpectedly, with no clinical signs of illness and no macroscopical lesions. Also, one contact bird died after fighting in experiment 2A. Necropsy findings revealed only small patches of consolidation in the apical and cardiac lobes of both lungs of the pig, but no virus was isolated. No deaths occurred among the animals exposed to H3N2 viruses.

2. Virus recovery

The results of virus isolation from intestinal and respiratory tracts swabs of infected or in contact animals are shown in Tables 11, 12, 13 and 14. Very few isolations were made, and these occurred principally in experiment 2. A number of isolations were made from the infected turkeys in experiment 2A, and the pigs in experiment 2B. Generally, the isolations were not constant, a positive animal sometimes giving a negative result in a subsequent sampling. Although the two experiments were carried out using similar protocols, no common characteristics could be drawn between them.
Table 12. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 1B (using virus strain H3N2).

<table>
<thead>
<tr>
<th>Days **</th>
<th>0</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>I R I R I R I R I R I R I R I R I R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Turkeys infected with virus (10)*** 0*NT 0 0 0 NT 0 0 0 0 0 0 0 0 0 0 0 0 0

<table>
<thead>
<tr>
<th>Days</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>In contact pigs (4)</td>
<td>0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days</th>
<th>0 to 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control pigs (2)</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days</th>
<th>0 to 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>In contact turkeys (5)</td>
<td>0 0</td>
</tr>
</tbody>
</table>

NT = not tested
I = intestinal tract
R = respiratory tract

* Number of positive samples detected.

** Number of days post-exposure that virus was detected.

*** Number of animals from which samples were taken.
Table 13. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 2A (using virus strain H1N1).

<table>
<thead>
<tr>
<th>Days **</th>
<th>1</th>
<th>4</th>
<th>7</th>
<th>11</th>
<th>14</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Turkeys infected with virus (10)*** | NT | 1* | 1 | 0 | 6 | 2 | 8 | 0 | 8 | 0 | NT | NT |

<table>
<thead>
<tr>
<th>Days</th>
<th>0 to 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>In contact turkeys (5)</td>
<td>0</td>
</tr>
<tr>
<td>In contact pigs (4)</td>
<td>0</td>
</tr>
<tr>
<td>Control pigs (2)</td>
<td>0</td>
</tr>
</tbody>
</table>

NT = not tested
I = intestinal tract
R = respiratory tract

* Number of positive samples detected.
** Number of days post-exposure that virus was detected.
*** Number of animals from which samples were taken.
Table 14. Isolation of virus from swabs collected from the intestinal and respiratory tracts of turkeys and pigs in experiment 2B (using virus strain H3N2).

<table>
<thead>
<tr>
<th>Days **</th>
<th>1</th>
<th>4</th>
<th>7</th>
<th>11</th>
<th>14</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>I</td>
</tr>
<tr>
<td>Turkeys infected with virus (10)***</td>
<td>0*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>In contact pigs (4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Control pigs (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Days</td>
<td>0 to 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In contact turkeys (5)</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NT = not tested  
I = intestinal tract  
R = respiratory tract

* Number of positive samples detected.  
** Number of days post-exposure that virus was detected.  
*** Number of animals from which samples were taken.
Virus was never recovered from the trachea or cloaca of the turkeys in experiment 1, although viruses were present sporadically in the pigs, both those in direct contact and the controls exposed only by aerosol.

Among the turkeys, most isolations were obtained from the intestinal tract samples, especially in experiment 2A (using H1N1 virus). In contrast, in experiment 2B, no virus could be isolated from the infected turkeys; although its presence became apparent with its isolation from the pigs in the same room. The isolation of virus in the contact pigs was almost always some days earlier than in the controls. Confirmation of the identity of the isolated viruses by HI showed that these were of the same subtype as inoculated on day 0 in the turkeys.

3. Immune response

The results of serological tests are shown in Appendix III and Figures 15, 16, 17 and 18. The animals in the two experiments presented different immunological results, which were unexpected, when compared to preliminary experiments with only turkeys.

Where turkeys were infected with the H1N1 porcine strain (Figures 15 and 16), specific antibodies were detected in the infected turkeys, the contact pigs and the control pigs, increasing up to the 28th day post-contact. The control turkeys, however, only developed specific
Fig. 15. Mean antibody titre detected in animals exposed to the porcine H1N1 strain (experiment 1A).

\[ \text{Ab titre} = \log_2 \left( \frac{\text{reciprocal HI titre}}{10} \right) \]
Fig. 16. Mean antibody titre detected in animals exposed to the porcine H1N1 strain (experiment 2A).
Fig. 17. Mean antibody titre detected in animals exposed to the porcine H3N2 strain (experiment 1B).

\[ \text{Ab titre} = \log_{10} \left( \frac{\text{reciprocal H}1 \text{titre}}{10} \right) \]
Fig. 18. Mean antibody titre detected in animals exposed to the porcine H3N2 strain (experiment 2B).

The graph shows the antibody titre over days post-infection for different groups:
- Turkeys: (anti H1N1 Ab)
- Turkeys: (anti H3N2 Ab)
- Pig control: (anti H1N1 Ab)
- Pig control: (anti H3N2 Ab)
- Pig contact: (anti H1N1 Ab)
- Pig contact: (anti H3N2 Ab)
- Turkey control: (anti H1N1 Ab)
- Turkey control: (anti H3N2 Ab)

The antibody titre is calculated as:

\[ \text{Ab titre} = \log_2 \left( \frac{\text{reciprocal HI titre}}{10} \right) \]
antibodies in the second experiment. In the second experiment, antibodies against the H3N2 porcine strain in the H1N1 infected turkeys were found at the same level as those for the H1N1 strain.

The serological response of the animals infected with the H3N2 porcine strain are shown in Appendix III and Figures 17 and 18. The infected turkeys developed a transitory specific immune response with a peak between 7 and 15 days post-exposure. Contact pigs, control pigs and control turkeys developed only a small rise in HI antibodies in the second experiment, whereas during the first experiment only the control pigs demonstrated the presence of specific antibodies. The same sera, when tested for H1N1 antibodies, gave a variety of results. In experiment 1, the contact and control pigs had an increase in H1N1 antibody 8 days post-introduction into the isolation unit, although no antibodies against the same strain were detected in the infected or control turkeys. In the second experiment, both contact and control pigs already had a significant antibody titre against the H1N1 strain, which decreased steadily until day 10, when a small rise was noticed. No H1N1 antibodies were present in the turkeys, apart from a very slight antibody rise in the infected turkeys.
Discussion

The main focus of influenza research has been the definition of those characteristics which permit the virus to cause disease. This is related to the capacity to infect the largest possible number of tissues and hosts, producing infectious virions capable of spreading the infection.

The spread of influenza virus subtypes among humans, birds and mammals such as pigs has been studied by several authors. In the present studies, replication of the Weybridge porcine influenza virus strains H1N1 and H3N2 in turkeys, the spread of both viruses to pigs, and from the pigs to non-infected birds were followed, in order to throw more light on the transmissibility and pathogenicity of these isolates.

In a study similar to the one described here, Homme and Easterday (1970) analysed the effects of influenza A/Turkey/Wisconsin/1966 virus in ring-necked pheasants, mallard ducks, Canada geese, and domestic geese, by virus isolation and HI tests. They concluded that an inapparent carrier state can exist, and that turkeys may remain infected for a considerable period, shedding virus intermittently during this time. Virus persistence was also shown by the presence of HI antibody.

The ability of the viruses to establish infection or spread to in contact birds tested in these studies varied considerably. Only the H1N1 viruses showed any virulence
for turkeys, causing signs of disease. The other viruses did not produce any sign of disease, although virus isolation and the presence of specific antibodies indicated that infection with H3N2 viruses was established in the same turkeys. It is possible that if the experiment was carried on for a longer period, more isolations would have been obtained.

The presence of virus in the cloacal swabs of turkeys indicated that at least one of the birds was excreting virus during the period of day 3 to 17. The isolation of viruses from the nostrils and also rectum of the pigs may indicate that replication occurred in the respiratory as well as intestinal tracts, although contamination of the pig's anus with infected material on the bottom of the cage is more likely to have occurred.

The suggestion that "natural infection" produces a better immune response, possible due to virus adaptation to the host, was produced by contacts to H1N1 infected turkeys, by developing higher HA antibody titres than the infected ones.

The influenza antibodies present in some pigs at the beginning of the experiment presumably were of maternal origin. They may delay the active antibody response, when compared to pigs without these antibodies. It is also possible that the production of non-specific antibodies against influenza virus is stimulated by the presence of a
different influenza virus strain, if the subject had had a first antibody stimulation by this virus. This was described as "antigenic sin" by Schulman (1975) and later reviewed by Ada and Jones (1986).

Concluding, seroconversions indicated that infection was established in the experimentally infected turkeys and the contact pigs, but no pig to turkey transmission occurred, with the exception of experiment 2A, using H1N1 influenza virus, where the contact turkeys produced a transitory serological response to the virus. The low level of antibodies present in the pigs of experiment 2 on day 0 faded away in one month, and probably were of maternal origin. On comparing the results of experiment 1 and 2, it was concluded that no interference in active antibody production could be observed due to the presence of these maternal antibodies.

Economically, one could consider that when complications occur, influenza infection in birds with subtypes tested here can lead to significant expenses. The economic losses claimed due to death, weight loss, medication and technical assistance were not observed in these experiments. No therapy was applied to the infected birds and their final weight was similar to or higher than that of controls. Only one death occurred. Clearly, these results only apply to influenza virus subtypes used in these experiments and under the conditions specified.
CHAPTER VI

SEROLOGICAL SURVEY OF BRAZILIAN PIG HERDS FOR ANTIBODIES AGAINST INFLUENZA A VIRUS.

Introduction

There have been extensive serological surveys searching for H3N2 and H1N1 specific antibodies in pigs. Antibodies against A/Hong Kong/68 (H3N2) were reported by Styk and others (1971b) in pigs in Slovakia, Harkness and others (1972) in pigs in Great Britain, Shortridge and others (1977) in pigs in Hong Kong, Chapman and others (1978) in pigs in Great Britain, Shortridge and Webster (1979) in pigs in Southeast Asia, Tumova and others (1980a) in pigs in Czechoslovakia, and Masurel and others (1983) in pigs in the Netherlands. Miwa and others (1987) found antibodies in pigs against A/Hong Kong/85 (H3N2) in Japan. Ottis and others (1982) reported the isolation and characterization of an influenza virus from Italian pigs which was similar to the human H3N2 isolate A/England/42/72 and the detection of antibodies against human H3N2 influenza strains A/Hong Kong/1/68 and A/Victoria/3/75 in pig herds from different Italian provinces. High antibody titres against A/Port Chalmers/73 (H3N2) in pigs have been reported by Alonso and others (1976) in Italy, Shortridge and Webster (1979) in
southeast Asia, Tan and others (1979) in Malaysia, Yamane and others (1979a) in Japan, Tillon and others (1980) in France, and Tumova and others (1980b) in Czechoslovakia. Specific antibodies against A/Victoria/75 (H3N2) has been found in pigs by Tan and others (1979) in Malaysia, Yamane and others (1979a) in Japan, Tillon and others (1980) in France, Tumova and others (1980a) in Czechoslovakia, Masurel and others (1983) in the Netherlands, Madec and others (1984) in France, and Vagt and others (1984) in West Germany. Several authors have shown that the virus with the H3 antigen has adapted itself to pig reservoirs (Kundin, 1970; Sugimura and others, 1975; Milev and others, 1981; Sidoli and Guarda, 1982; Madec and others, 1984; Tumova and others, 1985).

The appearance of H1N1 viruses (or antibodies against H1N1 viruses) in pigs gave further evidence of human to pig spread of the virus (Aymard and others, 1980; Hannoun and Gourreau, 1980; Nerome and others, 1982; Roberts and others, 1987). However, there have been only a few reports on the occurrence of human H1N1 strains of influenza virus among pig populations, in contrast to those of human H3N2 strains (Nerome and others, 1982).

Antibodies against human H1N1 subtypes, like the A/New Jersey/75,76,77 have been detected in pigs, in various countries: Shortridge and Webster (1979) in Southeast Asia, Tan and others (1979) in Malaysia, Yamane and others (1979a) in Japan, Tillon and others (1980) in France, Vandeputte and
others (1980) in Belgium, Satsuta and others (1981) in Japan, Masurel and others (1983) in the Netherlands, Abusugra and others (1987) in Sweden, Miwa and others (1987b) and Goto and others (1988) in Japan. The H1N1 was reported to be antigenically different from that commonly present in the U.S.A., suggesting it came from a different origin.

In most of the cases, a significant antibody response was present against various virus subtypes, of human and/or porcine origin, suggesting that mixed infections occur frequently and that another source, other than human, was also infecting pigs.

Webster and others (1982) and Shortridge (1988) emphasized the risks of interspecies transmission and pandemic outbreaks of influenza virus (see also Section 6 in Chapter I). They studied, in depth, the relationship between the several subtypes that have been isolated to date, and the variations occurring in the amino acid sequence of the H and N proteins. This has been recently updated and reviewed by McCauley (1987).

In an attempt to investigate further the role of pigs in influenza epidemiology, the present study was undertaken to evaluate the prevalence of antibodies against porcine and human influenza viruses in Brazilian fattening pigs. At the same time, it was intended to obtain information about interspecies transmission of influenza viruses and its
latent infection in pigs.

All virus strains were chosen according to their prevalence in man or pig, and for little cross-reactivity (Webster and others, 1982; P. Chakraverty, personal communication).

The serum of many species contains inhibitory substances that may interfere with the specificity of the HI and other tests (see Section 4.2 in Chapter I). To avoid the interference of these inhibitory substances, tests that are not influenced by them may be used (e.g. single radial haemolysis), or they must be inactivated. Different laboratories use different methods for the inactivation of these inhibitors (Kaplan and Payne, 1959).

McCrea (1946) showed that heat treatment (62°C for 15 to 20 minutes) destroyed the non-specific inhibition in rabbit serum, by denaturation of the globulin responsible for this effect. Burnet and Stone (1947) demonstrated the activity of Vibrio cholerae filtrates in removing "virus receptors" from red blood cells, acting in a similar way to viruses of the influenza group attaching to the surface of red blood cells. Red blood cells treated with receptor destroying enzyme (RDE) by Burnet and Stone (1947) lose their virus receptors, and also agglutinate. Clarke and Casals (1958) recommended the adsorption of sera with kaolin, although, in their opinion, this treatment is entirely empirical, and is based on selective adsorption
under standardized conditions. They recommended a comparison of results with the various methods available using normal and immune sera from the species to be tested. Ananthanarayan and Paniker (1960) tested the usefulness of heating at 56°C, treatment with RDE, potassium periodate, or trypsin to inactivate non-specific inhibitors in animal and human sera. They also could not find a single technique that was suitable for all animal species sera tested, due to the qualitative and quantitative differences of the inhibitors. Cohen and others (1963) described inhibitors present in horse serum and their removal by heating, neuraminidase or potassium periodate treatment. Coleman and Dowdle (1969) tested sera from monkey, goat, chicken, human, rabbit, ferret, guinea-pig and horse, by treating them all with heat, trypsin, periodate, RDE and kaolin, to determine the most effective way of removing non-specific inhibitors. They concluded that the presence of influenza H inhibitors in animal sera was unpredictable and their reactivity complex. In their opinion, empirical findings should be taken into account in the interpretation of HI results.

The method of choice for routine pre-treatment of pig sera at CVL has been heating and kaolin adsorption, as well as treatment with 10 per cent CRBCs before testing (G. Wibberley, personal communication).

All sera tested in this series of experiments appeared to contain non-specific inhibitors of H, as well as agglutinins for the type of erythrocyte (fowl) used in the
test, all of which must be removed. Preliminary experiments were set up to choose the most efficient technique for their removal.
A - NON-SPECIFIC HAEMAGGLUTINATION INHIBITORS

To avoid the interference of non-specific inhibitors when carrying out HI tests on pig sera, a preliminary experiment was set up to evaluate the effectiveness of kaolin and RDE in removing these non-specific inhibitors, and also whether or not specific antibody levels were reduced by the process.

Material and methods

Selection of pig sera - individual sera from 9 pigs reared at the CVL (Minimal disease herd) were obtained and used in this experiment, together with two specific antisera against porcine influenza (H1N1 and H3N2) from CVL stock produced in pigs.

CRBCs absorption - since many sera contain naturally occurring agglutinins for fowl erythrocytes, all sera were routinely absorbed with such cells prior to their use in the test. The presence of non-specific H in normal sera was tested by adding 25 μl of 1 per cent CRBCs to 25 μl of each of the serial dilutions of sera in U shaped 96 well microplates (Nunc, Denmark). Readings were taken after 60 minutes at room temperature. All sera showing HA were first
treated with CRBCs in equal volumes, by adding 100 μl of 10% washed fowl erythrocytes per 100 μl of serum. Absorption took place for 20 minutes with occasional shaking, after which the tubes were centrifugated for 10 minutes at 400 g at 4°C. The supernatant was used in the HI test. All other sera were diluted 1 in 2 by adding CFT diluent in equal volumes.

**Kaolin production** - see Section 1 in Chapter II.

**RDE production** - Five ml of an overnight nutrient broth culture of *Vibrio cholerae* (RDE strain - VZ; National Collection of type cultures, Colindale, London) was used to produce 300 ml of culture in brain heart infusion medium by incubating the culture at 37°C for 18 hours in a screw capped bottle, aerated by a magnetic stirrer. This culture was centrifuged at 1750 g for 30 minutes and the supernatant used as the enzyme source (House, 1967) after sterilization through a 0.4 μ pore filter.

**RDE titration** (as recommended by WHO (Palmer and others, 1975)) - Serial two-fold dilutions of RDE from 1/2 to 1/4096 in CFT diluent were set up in duplicate in a 96 well microplate with U shaped wells. Volumes of 25 μl were used for each reagent. One volume of 1 % CRBCs was added to all RDE dilutions and also to two CRBC control wells. After an incubation period of 60 minutes at 37°C, during which the
CRBCs were resuspended frequently, one volume of 4 HAU of the reference virus strain was added to all wells except to the CRBC control wells, where one volume of CFT diluent was substituted. The plate was incubated at room temperature for 30 minutes or until the CRBC control wells formed a compact, negative pattern. The plate was then read, the end-point dilution of the RDE being considered the last well of the dilution series in which HA was absent.

The number of RDE units in 1 ml of undiluted stock was calculated by multiplying the end-point dilution factor by forty. One hundred RDE units/ml is required to be used as working solution, according to the recommendations of Palmer and others (1975).

Assessment of RDE activity - The ability of various dilutions of RDE to remove non-specific inhibitors from normal sera (Burnet and Stone, 1947) was tested using the micro HI technique.

Treatment of sera

Kaolin method - Four volumes of kaolin suspension were mixed with one volume of the serum sample and incubated at 37°C for 60 minutes. The suspension was centrifuged at low speed (400 g for 15 minutes) and the supernatant used in the test (effective starting dilution of 1/10, allowing for the
previous treatment with 10% CRBCs plus kaolin treatment).

**RDE method** - One volume of 10 per cent CRBC treated serum, mixed with four volumes of serial dilutions of freshly prepared RDE in CFT diluent (1/2 up to 1/8) was incubated at 37°C overnight, and then heated at 56°C for one hour to destroy residual enzyme. A final dilution of one in ten was thus obtained.

Serum controls in CFT diluent were included and held under the same conditions as the test samples, as well as a control diluent sample treated in the same way as the sera.

**HI test** - see Section 2 in Chapter II.

**Virus strains** - Wey/86 (H1N1); Wey/87(H3N2); A/duck/Hong Kong/196/77 (H1N2) (dck/77). All strains were maintained by serial egg passage using the allantoic route of inoculation, and stored at -70°C for use as virus stocks (see Section 2 in Chapter II).

Batches of virus were prepared as required, by inoculation of 9-day-old embryonated fowls'eggs by the allantoic route with 0.2 ml of the stock virus diluted $10^{-3}$ in PBS pH 7.4. After 72 hours incubation at 33°C the eggs were chilled and the allantoic fluids harvested, pooled, and stored at 4°C until required.

**Antisera** - the specific antisera used were produced as described in Section 2 in Chapter II.
Results

All test samples showed non-specific HA when reacted with CRBCs (Table 15). This was removed by treating the samples with 10 per cent CRBCs in equal volumes, before testing.

Using the RDE titration method as recommended by the WHO (Palmer and others, 1975), and described in Material and Methods, different optimal RDE solutions were obtained when using different virus strains (Table 16). The end-point dilutions of the RDE when using 4 HAU of Wey/86 (H1N1), Dck/77 (H1N2) and Wey/87 (H3N2) were 1/4, 1/1 and 1/8 respectively. By definition one unit of RDE is the quantity of RDE needed to inhibit agglutination of one volume of a 1 per cent CRBC suspension in the presence of one volume of 4 HAU of virus. This gave a variation of RDE units per ml between 40 and 320. This means that according to the virus strain, various amounts of RDE had to be used as working solution (100 units/ml), and that a new RDE titration would have to be set up each time a new virus was tested. Also, the RDE produced in this study sometimes did not reach 100 units/ml, and would have to be concentrated first (Table 16). This type of test did not take into account the origin of the test sera. In an attempt to overcome this problem, the RDE activity in the present study was assayed on pig sera directly. RDE dilutions of 1/1, 1/2, 1/4 and 1/8 were tested on the same serum samples, together with the
Table 15. Presence of non-specific HA in test and specific antisera.

<table>
<thead>
<tr>
<th>sera</th>
<th>not treated</th>
<th>after 10% CRBCs treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>H1N1 antisera</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H3N2 antisera</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = haemagglutinating activity  
- = no haemagglutinating activity

Table 16. RDE titrations using the method as recommended by the WHO.

<table>
<thead>
<tr>
<th>virus</th>
<th>end point dilutions</th>
<th>RDE units/ml*</th>
<th>dilution factor to obtain working stock (100 RDE units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wey/86</td>
<td>1/4</td>
<td>160</td>
<td>160/100 = 1.6</td>
</tr>
<tr>
<td>dck/77</td>
<td>1/1</td>
<td>40</td>
<td>40/100 = 0.4</td>
</tr>
<tr>
<td>Wey/87</td>
<td>1/8</td>
<td>320</td>
<td>320/100 = 3.2</td>
</tr>
</tbody>
</table>

* one RDE unit = quantity needed to inhibit HA of one volume of a 1% CRBC suspension in the presence of one volume of 4HAU of virus.
untreated and kaolin treated aliquots.

Non-specific inhibitors were present in all sera against all the virus strains tested. The results are shown in Table 17. This HI reaction was considered as non-specific because the sera originated from a minimal disease herd reared at CVL, in which no influenza had been detected in the six months prior to collection of the sera. Nine sera from normal pigs and 2 specific antisera were tested by HI against 4 HAU of unheated allantoic fluid antigens. A wide variation in inhibitory activity was found, according to the virus strain. Variation in effectiveness of the treatment was also observed from serum to serum.

All negative sera used showed non-specific HI when tested untreated in the HI test. This was indistinguishable from specific HI as observed with the specific antisera. The efficiency of kaolin treatment varied with the virus strain and serum sample tested, generally being better than RDE in the lowest dilution, with the exception of serum 2. When RDE was used neat to remove non-specific inhibitors for viruses Wey/86 and dck/77, both specific positive antisera were equally efficient at HI. RDE did not remove non-specific inhibitors for Wey/87 virus from specific antisera against H1N1 virus strain.

Kaolin also removed immunoglobulin fractions containing specific antibodies from the specific antisera (Table 18), and for this reason, it is not the method of choice when other methods of removing inhibitors are effective. The
Table 17. HI activity present in normal pig sera against influenza virus strains Wey/86(H1N1), dck/77(H1N2), and Wey/87(H3N2), when using kaolin or RDE to remove non-specific inhibitors.

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>treatment</th>
<th>sera 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>antisera</th>
<th>H1N1</th>
<th>H3N2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wey/86</td>
<td>none</td>
<td>16*</td>
<td>8</td>
<td>16=16</td>
<td>16</td>
<td>8</td>
<td>8=16</td>
<td>4</td>
<td>1280</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>kaolin</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>=16</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H1N1</td>
<td>RDE 1**</td>
<td>1</td>
<td>4</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>=16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>=16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td>4</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>=16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>1</td>
<td>8</td>
<td>16</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>=16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Wey/87</td>
<td>none</td>
<td>≥16</td>
<td>8</td>
<td>4 =16</td>
<td>=16</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>640</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>kaolin</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>=16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3N2</td>
<td>RDE 1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>=16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>=16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>1</td>
<td>-</td>
<td>=16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>8</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>=16</td>
<td>8</td>
</tr>
<tr>
<td>dck/77</td>
<td>none</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>32</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kaolin</td>
<td>2</td>
<td>4</td>
<td>-</td>
<td>4</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>16</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>H1N2</td>
<td>RDE 1</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>16</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>-</td>
<td>2</td>
<td>1</td>
<td>16</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>-</td>
<td>2</td>
<td>2</td>
<td>16</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>-</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>16</td>
<td>2</td>
</tr>
</tbody>
</table>

* reciprocal HI titre of the sera tested

** reciprocal log₂ dilutions of RDE

- = no HI observed
Table 18. Reciprocal of HI titre of specific pig antisera before and after treatment with RDE or Kaolin.

<table>
<thead>
<tr>
<th>Antisera</th>
<th>not treated</th>
<th>treated with RDE</th>
<th>treated with Kaolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1N1</td>
<td>1280</td>
<td>1280</td>
<td>320</td>
</tr>
<tr>
<td>H3N2</td>
<td>640</td>
<td>640</td>
<td>320</td>
</tr>
</tbody>
</table>
treatment with RDE did not influence the titre of homologous antibodies against the virus.

Discussion

As the inhibitory activity of sera of various animal species on biological assays is caused by different inhibitory substances, complete elimination of these inhibitors demands the application of a variety of procedures to treat the sera, according to the virus strain and the origin of the serum.

The observation that none of the treatments described above completely eliminated the inhibitors for all three virus strains tested is in agreement with studies by Antoniadis and Woytek (1970). They suggested that it is not only the inhibitor that should be regarded as the variable factor in the virus-inhibitor reaction, but also the virion, which, by changes in its receptors, becomes sensitive to the alpha, beta or gamma inhibitor, respectively, that is contained in the sera of different animal species or other biological material (See Section 4.2 in Chapter I).

Coleman and Dowdle (1969) found that kaolin treatment of sera effectively removed inhibitors in all species except horses. Unfortunately, this treatment also removed specific HI inhibition, lowering the HA titre by 10 fold. In
the present studies, a 2 to 4 fold decrease in HI titres could be observed in sera treated with kaolin.

In view of these results, it was decided to treat all pig sera in these studies with kaolin and CRBCs prior to HI tests, as this procedure was as effective as, less variable and easier to perform than RDE. However, in analyzing the results it was necessary to remember that there is a reduction in HI titre produced by the kaolin treatment.
B - SEROLOGICAL SURVEY FOR INFLUENZA VIRUS ANTIBODIES IN BRAZILIAN PIG HERDS.

During the period of April-September 1987, a total of 1031 pig serum samples were collected at abattoirs, from various areas of the South, South-East and East of Brazil. Sera were tested for the presence of antibodies against several representative strains of porcine and human influenza viruses.

Material and Methods

Sera - blood samples were obtained at abattoirs and The National Research Centre for birds and pigs, EMBRAPA, Concórdia, SC, Brazil. They were collected in the period of May to July 1987 from the abattoirs, and in August at the Research Institute, using sera collected in 1987 for earlier immunological surveys, that were kept at -20°C.

Sera were separated from the abattoir blood samples on the same day of collection, and stored at 4°C until treatment.

All sera were inactivated at 56°C for 30 minutes.

To avoid nonspecific reactions, all serum samples were treated with kaolin and CRBCs (see Section 2 in Chapter II). The dilution of 1/10 was the starting dilution used at
the HI test performed to check their influenza antibody titre.

**Influenza virus strains** -
- A/swine/Weybridge/117316/86(H1N1) (Wey/86),
- A/swine/Weybridge/163266/87(H3N2) (Wey/87),
- A/duck/Hong Kong/196/77(H1N2) (Dck/77),
- A/FM/1/47(H1N1) (FM/47),
- A/Singapore/1/57(H2N2) (Sing/57),
- A/Hong Kong/1/68 (H3N2) (HK/68),
- A/England/42/72(H3N2) (Eng/72),
- A/Port Chalmers/1/73(H3N2) (PC/73),
- A/Victoria/3/75(H3N2) (Vic/75),
- A/Brazil/11/78-PR/8/34 (H1N1)(Braz/78),
- A/Philippines/2/82(H3N2)(Phil/82)
- A/Leningrad/360/86(H3N2) (Lenin/86).

**Specific antisera** - prepared in ferrets and provided by Dr. Chakraverty (Central Public Health Laboratory, Colindale, London) and otherwise prepared in SPF chickens at the CVL (see Section 2 in Chapter II), were used as positive controls throughout the experiments.

**Serum antibody to reference strains mentioned above** - examined by the HI test using the microtitre method (see Section 2 in Chapter II).
**Virus multiplication** - in embryonated eggs as described in Section 2 in Chapter II.

**Virus titrations** - done before and after the HI tests, using the standard HA microtechnique (see Section 2 in Chapter II).

**Cross reactivity of viruses under test** - a chequer-board using all virus strains and respective antisera was set up initially to detect relationships between the strains used in this survey.

**Results**

No clinical evidence of an outbreak of influenza-like disease in pigs was observed in the areas surveyed during the period of blood collection. Antibody levels equal to or higher than 1/40 were considered as evidence of past contact or infection, as no vaccination against influenza has ever been practised on the farms surveyed.

The chequer-board set up to determine the antigenic relationship of the H of these viruses and the respective antisera is shown in Table 19. Only PC/73 reacted to various antisera in higher titres than to its own specific antisera. Also, several antisera neutralized the HA of heterologous viruses to a similar, or higher titre then the homologous virus [eg. dck/77(H1N2), FM/47(H1N1),
Table 19. Cross-reaction between porcine and human influenza A viruses in HA tests

<table>
<thead>
<tr>
<th>viruses</th>
<th>Wey/86</th>
<th>Wey/87</th>
<th>Dck/77</th>
<th>FM/47</th>
<th>Sing/57</th>
<th>HK/68</th>
<th>Eng/72</th>
<th>PC/73</th>
<th>Vic/75</th>
<th>Braz/78</th>
<th>Phil/82</th>
<th>Lenin/86</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wey/86(H1N1)</td>
<td>320*</td>
<td>80</td>
<td>10</td>
<td>80</td>
<td>160</td>
<td>20</td>
<td>10</td>
<td>40</td>
<td>40</td>
<td>10</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>Wey/87(H3N2)</td>
<td>10</td>
<td>1280</td>
<td>10</td>
<td>20</td>
<td>80</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>20</td>
<td>10</td>
<td>160</td>
</tr>
<tr>
<td>Dck/77(H1N2)</td>
<td>80</td>
<td>40</td>
<td>10</td>
<td>20</td>
<td>160</td>
<td>40</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>FM/47(H1N1)</td>
<td>10</td>
<td>320</td>
<td>10</td>
<td>640</td>
<td>320</td>
<td>640</td>
<td>80</td>
<td>640</td>
<td>320</td>
<td>160</td>
<td>10</td>
<td>320</td>
</tr>
<tr>
<td>Sing/57(H2N2)</td>
<td>10</td>
<td>320</td>
<td>10</td>
<td>40</td>
<td>640</td>
<td>320</td>
<td>160</td>
<td>320</td>
<td>320</td>
<td>160</td>
<td>10</td>
<td>160</td>
</tr>
<tr>
<td>HK/68(H3N2)</td>
<td>10</td>
<td>320</td>
<td>10</td>
<td>80</td>
<td>320</td>
<td>2560</td>
<td>160</td>
<td>640</td>
<td>640</td>
<td>160</td>
<td>20</td>
<td>320</td>
</tr>
<tr>
<td>Eng/72(H3N2)</td>
<td>10</td>
<td>320</td>
<td>10</td>
<td>80</td>
<td>320</td>
<td>1280</td>
<td>640</td>
<td>1280</td>
<td>640</td>
<td>160</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>PC/73(H3N2)</td>
<td>10</td>
<td>160</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>80</td>
<td>80</td>
<td>320</td>
<td>160</td>
<td>20</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>Vic/75(H3N2)</td>
<td>10</td>
<td>320</td>
<td>10</td>
<td>80</td>
<td>160</td>
<td>320</td>
<td>160</td>
<td>640</td>
<td>1280</td>
<td>160</td>
<td>10</td>
<td>160</td>
</tr>
<tr>
<td>Braz/78(H1N1)</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Phil/82(H3N2)</td>
<td>10</td>
<td>320</td>
<td>10</td>
<td>80</td>
<td>320</td>
<td>320</td>
<td>160</td>
<td>1280</td>
<td>640</td>
<td>320</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>Lenin/86(H3N2)</td>
<td>10</td>
<td>320</td>
<td>10</td>
<td>80</td>
<td>160</td>
<td>160</td>
<td>80</td>
<td>320</td>
<td>320</td>
<td>80</td>
<td>80</td>
<td>640</td>
</tr>
</tbody>
</table>

* The titre corresponds to the reciprocal of the HI titre found using 4 HAU of each virus strain.
Eng/72(H3N2), Phil/82(H3N2)].

The sera, after treatment, were tested against as many virus strains as possible depending on the amount of sera available. The results of the HI test of 1031 sera collected at random from 23 pig herds in Brazil during the period March - October 1987, in prevalence and in percentages, are summarized in Tables 20 and 21, respectively. The occurrence of specific antibodies against a certain virus strain varied from herd to herd. All herds had antibody titres to the H3N2 strains in more than 50 per cent of the sera examined. Fig. 19 presents the same results, ordered according to the geographical areas of origin. The approximate localization of each farm is shown on Fig 20.

The serological response against the viruses tested was not distinct. Some sera gave high antibody responses to several virus strains. An overall picture of the antibody levels found was produced by tabulating the highest serum antibody responses against the viruses and plotting these in Table 22. The highest antibody levels were found against H3N2 strains, principally Lenin/86. High antibody titres against PC/73 were also present, as well as to Sing/57, Vic/75 and Wey/87.

An analysis of the geographical incidence of specific antibodies (Fig. 21) shows that pig herds in South-East, Brazil, with only a few exceptions, possessed higher antibody levels against human H3N2 influenza viruses than
Table 20. Prevalence of positive antibody responses (*) against influenza virus in pig sera of various farms in Brazil.

<table>
<thead>
<tr>
<th>farms</th>
<th>Wey/86</th>
<th>Dck/77</th>
<th>Wey/87</th>
<th>F1/47</th>
<th>Sin/57</th>
<th>HK/68</th>
<th>Vic/75</th>
<th>Phi/86</th>
<th>Lenin/86</th>
<th>Eng/72</th>
<th>PC/73</th>
<th>Brazil/78</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>9/54**</td>
<td>0/54</td>
<td>3/54</td>
<td>8/46</td>
<td>12/46</td>
<td>17/46</td>
<td>2/25</td>
<td>NT</td>
<td>10/25</td>
<td>0/5</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>8</td>
<td>0/18</td>
<td>5/18</td>
<td>9/18</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
<td>4/4</td>
<td>0/4</td>
<td>1/4</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>0/36</td>
<td>12/36</td>
<td>12/36</td>
<td>2/21</td>
<td>5/21</td>
<td>1/21</td>
<td>6/12</td>
<td>1/12</td>
<td>5/12</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>12</td>
<td>0/40</td>
<td>0/40</td>
<td>17/40</td>
<td>4/51</td>
<td>38/51</td>
<td>0/51</td>
<td>1/6</td>
<td>0/14</td>
<td>6/14</td>
<td>0/7</td>
<td>2/7</td>
<td>0/7</td>
</tr>
<tr>
<td>18</td>
<td>18/62</td>
<td>13/62</td>
<td>28/62</td>
<td>9/37</td>
<td>20/37</td>
<td>17/37</td>
<td>2/18</td>
<td>0/18</td>
<td>5/18</td>
<td>0/3</td>
<td>1/3</td>
<td>0/3</td>
</tr>
<tr>
<td>17</td>
<td>0/38</td>
<td>0/38</td>
<td>9/38</td>
<td>2/37</td>
<td>1/37</td>
<td>5/37</td>
<td>2/32</td>
<td>13/32</td>
<td>21/32</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>18</td>
<td>4/43</td>
<td>0/43</td>
<td>16/43</td>
<td>7/28</td>
<td>14/28</td>
<td>2/28</td>
<td>0/12</td>
<td>1/12</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>1/24</td>
<td>0/24</td>
<td>10/24</td>
<td>1/10</td>
<td>9/10</td>
<td>1/10</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>0/33</td>
<td>0/33</td>
<td>13/33</td>
<td>0/22</td>
<td>21/22</td>
<td>0/22</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>1/45</td>
<td>5/45</td>
<td>25/45</td>
<td>9/37</td>
<td>13/31</td>
<td>6/31</td>
<td>1/7</td>
<td>0/7</td>
<td>3/7</td>
<td>1/4</td>
<td>1/4</td>
<td>3/4</td>
</tr>
<tr>
<td>10</td>
<td>0/23</td>
<td>6/23</td>
<td>5/23</td>
<td>0/21</td>
<td>3/21</td>
<td>0/21</td>
<td>4/5</td>
<td>1/5</td>
<td>13/15</td>
<td>0/3</td>
<td>3/3</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0/13</td>
<td>0/13</td>
<td>12/13</td>
<td>1/8</td>
<td>1/8</td>
<td>0/8</td>
<td>1/2</td>
<td>0/2</td>
<td>1/2</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>16</td>
<td>0/14</td>
<td>4/14</td>
<td>7/14</td>
<td>2/14</td>
<td>4/14</td>
<td>1/14</td>
<td>3/4</td>
<td>1/4</td>
<td>1/4</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>17</td>
<td>1/8</td>
<td>0/8</td>
<td>2/8</td>
<td>0/4</td>
<td>2/4</td>
<td>0/4</td>
<td>NT</td>
<td>0/4</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>19</td>
<td>0/11</td>
<td>2/11</td>
<td>4/11</td>
<td>0/9</td>
<td>6/9</td>
<td>0/9</td>
<td>1/3</td>
<td>0/3</td>
<td>1/3</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>1</td>
<td>0/13</td>
<td>2/13</td>
<td>5/13</td>
<td>0/10</td>
<td>5/10</td>
<td>0/10</td>
<td>1/3</td>
<td>1/3</td>
<td>2/3</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>0/11</td>
<td>0/11</td>
<td>22/51</td>
<td>6/38</td>
<td>6/38</td>
<td>7/38</td>
<td>11/17</td>
<td>1/17</td>
<td>7/17</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>0/24</td>
<td>0/24</td>
<td>1/24</td>
<td>0/23</td>
<td>21/23</td>
<td>0/23</td>
<td>4/12</td>
<td>0/12</td>
<td>3/12</td>
<td>0/6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
<tr>
<td>6</td>
<td>0/28</td>
<td>0/28</td>
<td>9/28</td>
<td>5/46</td>
<td>1/28</td>
<td>2/28</td>
<td>3/17</td>
<td>5/17</td>
<td>15/25</td>
<td>NT</td>
<td>1/5</td>
<td>1/5</td>
</tr>
<tr>
<td>13</td>
<td>0/103</td>
<td>7/103</td>
<td>25/10</td>
<td>4/87</td>
<td>3/87</td>
<td>4/87</td>
<td>11/71</td>
<td>13/71</td>
<td>65/71</td>
<td>0/44</td>
<td>10/44</td>
<td>1/44</td>
</tr>
<tr>
<td>21</td>
<td>0/63</td>
<td>0/63</td>
<td>1/63</td>
<td>11/63</td>
<td>2/63</td>
<td>2/63</td>
<td>14/63</td>
<td>0/63</td>
<td>19/63</td>
<td>0/63</td>
<td>43/63</td>
<td>0/63</td>
</tr>
<tr>
<td>22</td>
<td>0/50</td>
<td>0/50</td>
<td>0/50</td>
<td>8/49</td>
<td>0/49</td>
<td>3/49</td>
<td>16/49</td>
<td>0/49</td>
<td>19/49</td>
<td>0/49</td>
<td>26/49</td>
<td>1/49</td>
</tr>
</tbody>
</table>

NT = not tested
* a positive antibody response was considered HI titres equal or greater than 1/40
** relation between positive responses and total of sera tested
Table 21. Percentage of positive antibody responses (*) against influenza virus in pig sera of various farms in Brazil.

<table>
<thead>
<tr>
<th>Farms</th>
<th>Wey/86</th>
<th>Duck/77</th>
<th>May/87</th>
<th>FM/47</th>
<th>Sing/57</th>
<th>HK/68</th>
<th>Vic/75</th>
<th>Phil/84</th>
<th>Lenin/86</th>
<th>Eng/72</th>
<th>PC/73</th>
<th>Braz/78</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>17**</td>
<td>0</td>
<td>6</td>
<td>17</td>
<td>26</td>
<td>37</td>
<td>8</td>
<td>NT</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>28</td>
<td>50</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>25</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>34</td>
<td>34</td>
<td>10</td>
<td>24</td>
<td>5</td>
<td>50</td>
<td>8</td>
<td>42</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td>8</td>
<td>75</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>43</td>
<td>43</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>29</td>
<td>21</td>
<td>45</td>
<td>24</td>
<td>54</td>
<td>46</td>
<td>11</td>
<td>0</td>
<td>28</td>
<td>0</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td>3</td>
<td>11</td>
<td>14</td>
<td>35</td>
<td>4</td>
<td>25</td>
<td>9</td>
<td>53</td>
<td>0</td>
<td>31</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>5</td>
<td>3</td>
<td>14</td>
<td>6</td>
<td>41</td>
<td>65</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Mean</td>
<td>8</td>
<td>12</td>
<td>30</td>
<td>11</td>
<td>33</td>
<td>15</td>
<td>31</td>
<td>10</td>
<td>42</td>
<td>11</td>
<td>33</td>
<td>3</td>
</tr>
</tbody>
</table>

** East

<table>
<thead>
<tr>
<th>Farms</th>
<th>Wey/86</th>
<th>Duck/77</th>
<th>May/87</th>
<th>FM/47</th>
<th>Sing/57</th>
<th>HK/68</th>
<th>Vic/75</th>
<th>Phil/84</th>
<th>Lenin/86</th>
<th>Eng/72</th>
<th>PC/73</th>
<th>Braz/78</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>9</td>
<td>0</td>
<td>37</td>
<td>25</td>
<td>50</td>
<td>7</td>
<td>0</td>
<td>8</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>0</td>
<td>42</td>
<td>10</td>
<td>90</td>
<td>10</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>95</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>11</td>
<td>56</td>
<td>24</td>
<td>42</td>
<td>19</td>
<td>14</td>
<td>0</td>
<td>43</td>
<td>25</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>26</td>
<td>22</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>27</td>
<td>7</td>
<td>87</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>0</td>
<td>92</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>29</td>
<td>50</td>
<td>14</td>
<td>29</td>
<td>7</td>
<td>75</td>
<td>25</td>
<td>25</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>19</td>
<td>13</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>NT</td>
<td>0</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Mean</td>
<td>4</td>
<td>8</td>
<td>45</td>
<td>11</td>
<td>48</td>
<td>5</td>
<td>33</td>
<td>7</td>
<td>51</td>
<td>12</td>
<td>63</td>
<td>38</td>
</tr>
</tbody>
</table>

** South-East

<table>
<thead>
<tr>
<th>Farms</th>
<th>Wey/86</th>
<th>Duck/77</th>
<th>May/87</th>
<th>FM/47</th>
<th>Sing/57</th>
<th>HK/68</th>
<th>Vic/75</th>
<th>Phil/84</th>
<th>Lenin/86</th>
<th>Eng/72</th>
<th>PC/73</th>
<th>Braz/78</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>18</td>
<td>36</td>
<td>0</td>
<td>67</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>33</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>15</td>
<td>38</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>33</td>
<td>33</td>
<td>67</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>43</td>
<td>16</td>
<td>16</td>
<td>18</td>
<td>65</td>
<td>6</td>
<td>41</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>91</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>32</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>18</td>
<td>29</td>
<td>60</td>
<td>NT</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>7</td>
<td>24</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>15</td>
<td>18</td>
<td>92</td>
<td>0</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>17</td>
<td>3</td>
<td>3</td>
<td>22</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>0</td>
<td>0</td>
<td>16</td>
<td>0</td>
<td>6</td>
<td>33</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>53</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0</td>
<td>5</td>
<td>22</td>
<td>8</td>
<td>29</td>
<td>5</td>
<td>31</td>
<td>11</td>
<td>48</td>
<td>0</td>
<td>36</td>
<td>5</td>
</tr>
</tbody>
</table>

NT = not tested
* a positive antibody response was considered HI titres equal or greater than 1/40
** % of sera positive for a specific virus strain
Fig. 19. Geographical distribution of antibody titres against the different influenza virus strains (*) found in pig herds in Brazil, expressed in percentages.

* only HI titres equal or greater than 1/40 were considered positive and included.
Fig. 20. Geographical location of the farms where the sera used in the studies were collected.

a = Santo Antonio do Monte - 8
b = Ponte Nova - 3, 10, 15, 20
c = Rio Casca - 12
d = Teixeiras - 17
e = São José do Rio Preto - 18
f = Magé - 9, 11, 14, 16, 19
g = Bragança Paulista - 5
h = Guarulhos - 7
i = Ponta Grossa - 2, 6, 21
j = Laranjeiras do Sul - 13
k = Paulo de Frontin - 1
l = São Miguel do Oeste - 4
m = Concórdia - 22, 23
n = Guarapuava - 21
Table 22. General percentage (*) of antibody levels detected in pig sera against the various virus strains tested.

<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>General Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eng/72 (H3N2)</td>
<td>2%</td>
</tr>
<tr>
<td>Wey/86 (H1N1)</td>
<td>2%</td>
</tr>
<tr>
<td>HK/68 (H3N2)</td>
<td>3%</td>
</tr>
<tr>
<td>Dck/77 (H1N2)</td>
<td>3%</td>
</tr>
<tr>
<td>FM/47 (H1N1)</td>
<td>4%</td>
</tr>
<tr>
<td>Phil/82 (H3N2)</td>
<td>4%</td>
</tr>
<tr>
<td>Braz/78 (H1N1)</td>
<td>6%</td>
</tr>
<tr>
<td>Vic/75 (H3N2)</td>
<td>13%</td>
</tr>
<tr>
<td>Wey/87 (H3N2)</td>
<td>13%</td>
</tr>
<tr>
<td>Sing/57 (H2N2)</td>
<td>15%</td>
</tr>
<tr>
<td>PC/73 (H3N2)</td>
<td>17%</td>
</tr>
<tr>
<td>Lenin/86 (H3N2)</td>
<td>19%</td>
</tr>
</tbody>
</table>

* Percentage of sera that presented the highest HI antibody titre against each of the virus strains tested.
Fig. 21. Analysis of highest antibody levels detected in pig sera according to geographical area of origin, against the various viruses tested.
those from the East or South of the country.

Although the highest antibody titre present in pigs from different farms varied considerably regionally, no significant variation was detected in the geographical distribution of the virus strains themselves. Nonetheless, a markedly low percentage of positive sera against H1N1 strains and the H1N2 strain was observed.

Discussion

The potential dangers of porcine influenza spreading from pigs to humans was first suggested by Shope (1936) and later studies on the subject intensified world-wide after the Fort Dix incident, New Jersey, U.S.A. in 1976. If human influenza viruses which were present in the human population several decades ago are shown to still be circulating in pig populations of today, this may represent a risk for new pandemics to break out in the future.

To determine whether or not influenza infection has established itself as a continuous disease in pigs, serological screening of pig serum specimens from different parts of the world to detect influenza antibody should be undertaken.

The role and importance of animals in the epidemiology of influenza was highlighted by the WHO, by surveying a
large number of equine and porcine sera for specific antibodies to influenza viruses (Kaplan and Payne, 1959). Sera from all over the world were included, and tested against A/Singapore/1/57(H2N2) and A/swine/1976/31(H1N1). The authors concluded that the H2 strain could cause natural infection in pigs and also spread back to humans, for example, by close contact with live animals or pork products from abbatoir pigs. The isolation of influenza viruses from man that are antigenically related to influenza viruses isolated from pigs has led to increased surveillance of pig populations throughout the world for the detection of human influenza viruses.

Influenza virus infection among Brazilian pig herds has not been reported before. However, with the increase in pig meat production at an industrial level in the last 10 years, and the knowledge that influenza viruses can be passed between different species suggested that a serological study of porcine sera from Brazil would be useful. The study involved the use of influenza reference strains isolated from human influenza outbreaks, and some recent porcine isolates from both subtypes isolated from pigs.

Hinshaw and others (1984) showed that H1N1 viruses circulating currently in pigs included at least 2 antigenically distinct groups, and that the presence of one or/and the other is associated with the geographical location of the animals. Consequently this study
incorporated some non-porcine H1N1 and H1N2 influenza virus types. Low levels of antibodies to human H1N1 virus (FM/47), and even lower antibody titres to H1N1 porcine virus circulating currently in Europe (Wey/86) were detected, in addition to low titres against the avian H1N2 strain (dck/77).

The presence of Lenin/86 in the Brazilian human population detected in the year preceding the collection of the porcine sera (PHLS Communicable Disease Surveillance Centre, unpublished data, 1988), and the detection of significant antibody levels against this same virus strain in the herds studied in this thesis may indicate interspecies transmission of this strain from humans to pigs.

The large number of sera with high antibody levels against Sing/57 (H2N2) may be due to cross-reaction with the neuraminidase. Also, the close relationship between the H3N2 viruses PC/73, Vic/75 and Wey/87 found by Wibberley and others (1988) agreed with the serological findings in these studies (Table 19) and is probably responsible for the high antibody levels found against these viruses.

The higher antibody levels against human H3N2 influenza viruses detected on farms in the South East region of Brazil may be explained by the higher incidence of small family-run farms, where man to pig transmission may occur more readily, in contrast to the more industrial farming in the
East and South, where contact between humans and the herd is minimal.

The observed low percentage of positive sera against H1N1 and H1N2 strains may suggest that Brazilian pig herds came into contact with principally H3N2 strains of human origin. The remaining sera had either low HI titres of 1/20 or less, or were negative to influenza H1N1 and H1N2 antigens.

The results of this study indicate that pigs in Brazil, like those in several other countries around the world, have antibodies against various human influenza strains, and most probably harbour the virus. This makes them potential reservoirs for future human pandemics. Miwa and others (1986;1987) isolated human H1N1 and H3N2 influenza virus from abattoir pigs in Japan and also found serological evidence of pig infection with the isolated strain. Their findings confirm suggestions made that pigs and humans were infected simultaneously during the epidemic of human influenza in that area, in 1985, and that interspecies transmission occurs readily.
CHAPTER VII

PORCINE REASSORTANT INFLUENZA VIRUSES IN ENGLISH PIG HERDS

Introduction

Much evidence has accumulated by several workers to suggest that influenza viruses isolated from birds may replicate in mammals, and also that viruses isolated from mammals could possibly replicate in birds, with a variable range of clinical disease (see Section 6 of Chapter I). Coexistence of two distinct influenza A viruses in pigs has been reported by several authors in various countries (Yamane and others, 1979a; Roberts and others, 1987), as well as the occurrence of reassortment between them (Hirst and Gotlieb, 1953; Webster and Laver, 1975; Stuart-Harris and Schild, 1976, Yamane and others, 1978).

Serological evidence of H1N2 reassortant influenza virus infection among pigs was reported by Sugimura and others (1980) and Arikawa and others (1981). Their results indicated that genetic reassortment occurred between porcine strains of H1N1 and H3N2 subtypes in pigs in Japan. The isolation of a reassortant influenza virus (H1N2) from pigs in Japan confirmed these observations (Sugimura and others, 1980). Also Nerome and others (1983) isolated a reassortant influenza virus (H1N2) from pigs in Japan, which was
antigenically different from the Kanagawa isolate described by Sugimura and others (1980). The latter had growth characteristics similar to those of avian influenza viruses (such as the capacity to grow at high temperatures, e.g. 42°C).

Serological evidence of the simultaneous presence of influenza virus H1N1 and H3N2 in English pigs would imply the possibility of genetic reassortment between the two infecting viruses.

The above possibility was investigated by screening pig sera obtained from various pig farms in England for the simultaneous presence of antibodies against H1N1 and H3N2 porcine influenza virus strains. The pig herds from where these sera originated were then examined in more detail, and a subsequent search for the presence of influenza viruses carried out, in the hope of isolating a reassortant influenza virus.

Material and methods

Sera - blood samples were obtained from English pig herds in various areas of England (Fig.22), and their serum separated, inactivated and treated as described in Section 2 in Chapter II.

Serological screening tests - sera from all over England, received at the CVL for diagnostic routine purposes, were
screened for antibodies against certain influenza virus strains, including influenza A H1N1 and H3N2 strains. Pig herds from farms whose sera contained these antibodies were detected and used in this study. HI activity in these sera involved the use of the influenza A virus strains Wey/86(H1N1), Wey/87(H3N2) and Dck/77(H1N2). The sera under test were distributed at random on the test microplates, to avoid influence in reading. Selected sera were also tested for specific N antibodies. Identification of antibodies against the H and N glycoproteins was accomplished by HI and spot NI tests (see Section 2 in Chapter II), and also confirmed by the SRID test, using small special immunoplates (Hyland) which were filled with 3 ml of 1.5% agarose (A37 - Industrie Biotechnique Francaise) dissolved in phosphate-buffered saline (pH 7.2), 0.1% sodium azide (Fisons) and 0.1 ml of purified antigen. The wells (3 mm in diameter) were cut with a special template and the agarose plugs removed by vacuum suction. They were filled with the test sera and kept at room temperature in a moist chamber for 24 hours. The plates were then soaked in PBS (pH 7.2) for 3-4 hours, stained 15 minutes with aqueous 0.5% Kenacid blue (BDH Chemicals), again soaked in PBS (pH 7.2) for 24 hours and read under an oblique light and dark background. The presence of a halo indicated the presence of specific H and/or N antibodies in the serum under test. H antibodies produced a small dense halo, whereas N antibodies produced a
more diffuse halo, larger than that of H (Schild and others, 1972).

**Virus isolation** - eighty five nasal swabs were collected at slaughtertime at the abattoir, from the same 6-months-old slaughter pigs originated from farm 73, from which serum samples has been collected. Thirteen nasal swabs were collected directly from farm 73, together with blood samples from the same animals, either with or without respiratory signs. Swabs were collected, in 1 ml PBS (pH 7.4), from the upper respiratory tract of the animal under study. The swabs plus PBS were centrifuged at 1000 g for 10 minutes to deposit any debris, and 0.2 ml aliquots inoculated into the allantoic cavity of 9-day-old embryonated fowls’ eggs. The fluid was harvested 96 hours after inoculation and those showing HA with CRBCs were purified. When characterized as influenza A viruses, three allantoic passages of a $10^{-3}$ dilution of the allantoic harvest from the first passage were made to increase the virus yield. The allantoic fluid from the third passage constituted the virus stock used for further identification.

**Virus identification** - specific antisera, prepared as described previously (Section 2 in Chapter II), were used to identify the internal viral proteins and the H and N of the isolated influenza A viruses, using the IDD (see later in this section), HI and spot NI tests (see Section 2 in
Chapter II). A sample of each isolated virus was also sent to WHO World Influenza Centre in London for subtyping using monoclonal antibodies.

**IDD test** - after a positive HA screening test, showing the HA of the isolate, the sample was inoculated at ten-fold dilutions into embryonated eggs and the highest dilution giving a positive HA was used for typing using the IDD technique. This involved the concentration and purification of the virus (see Section 2 in Chapter II). The concentrated virus was treated with lauresol sarcosine (Sigma) to rupture the virus and expose the internal proteins, and then 10 μl was dispensed into 5 mm wells cut into agar gel plates. Positive antigen samples were included, as well as positive and negative reference antisera (purified H3 from CVL stock), which were located in alternate wells. If a common precipitin line developed within 48 hours between the treated samples and positive antisera, but not between the treated samples and negative antisera, it was considered as a line of identity and proof of the homology between sample and antisera.

**Agar gel plates** - agar gel was prepared as a 1% ion agar (Oxoid) containing 8% NaCl and 0.01% thimerosol (Sigma), in PBS (pH7.2) solution. The mixture was autoclaved, and when cooled to 75°C, poured into 10 cm diameter petri plates to give a uniform 2.5 mm thick coat of agar (Samadieh and Bankowski, 1971).
Lauresol sarcosine - a stock of 10% w/v was prepared in PBS pH7.2, final concentration of 1% being obtained by diluting the stock solution 1 in 10.

PBS (pH7.2) - see Section 1 in Chapter II.
PBS (pH7.4) - see Section 1 in Chapter II.

Results

1. Serological studies

The search for the presence of reassortant influenza A virus in English pigs involved looking for pig herds with serological evidence of mixed infection with H1N1 and H3N2 porcine strains. One hundred and four English pig herds, distributed as shown on Fig. 22, were analysed for a routine influenza virus antibody survey (Table 23) and summarized (Table 24). Antibodies against all influenza virus types, A, B and C, were detected. Pig farms in England are mainly concentrated in the East of the Country, and consequently the number of farms analysed from that area was larger than from any other area. No specific geographical distribution of influenza virus types could be observed. HI antibodies against influenza A was found on 76 farms, against influenza
Fig. 22. Location of pig farms from which sera were collected in the survey for reassortant influenza viruses.
Table 23. Screening of pig farms for the presence of HI antibodies against influenza virus subtypes (porcine H1N1 and H3N2, human USSR/77, type B, type C) in England.

<table>
<thead>
<tr>
<th>Farm no</th>
<th>Localization</th>
<th>Positive to influenza virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>North Preston</td>
<td>negative</td>
</tr>
<tr>
<td>02</td>
<td>East Wretham</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>03</td>
<td>Caldecote</td>
<td>H1N1</td>
</tr>
<tr>
<td>04</td>
<td>Ford</td>
<td>H1N1</td>
</tr>
<tr>
<td>05</td>
<td>Ford</td>
<td>H1N1, influenza C</td>
</tr>
<tr>
<td>06</td>
<td>Great Milton</td>
<td>H1N1</td>
</tr>
<tr>
<td>07</td>
<td>Little Milton</td>
<td>H1N1, USSR</td>
</tr>
<tr>
<td>08</td>
<td>Castlecary</td>
<td>H1N1, H3N2, influenza B</td>
</tr>
<tr>
<td>09</td>
<td>Beeford</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>10</td>
<td>Newport</td>
<td>USSR</td>
</tr>
<tr>
<td>11</td>
<td>Berwick</td>
<td>H1N1, influenza C</td>
</tr>
<tr>
<td>12</td>
<td>North Piddle</td>
<td>influenza C</td>
</tr>
<tr>
<td>13</td>
<td>Glinton</td>
<td>H1N1, influenza C</td>
</tr>
<tr>
<td>14</td>
<td>Shrewsbury</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>15</td>
<td>Bourton</td>
<td>influenza C</td>
</tr>
<tr>
<td>16</td>
<td>Dalkeith</td>
<td>negative</td>
</tr>
<tr>
<td>17</td>
<td>South Milford</td>
<td>influenza C</td>
</tr>
<tr>
<td>18</td>
<td>Garvestone</td>
<td>H1N1, influenza C</td>
</tr>
<tr>
<td>19</td>
<td>West Lavington</td>
<td>H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>20</td>
<td>South Bury</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>21</td>
<td>Beeford</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>22</td>
<td>Ganton</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>23</td>
<td>Great Moulton</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>24</td>
<td>Longworth</td>
<td>H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>25</td>
<td>Walberswick</td>
<td>H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>26</td>
<td>Hook Norton</td>
<td>H1N1, H3N2, USSR, influenza B &amp; C</td>
</tr>
<tr>
<td>27</td>
<td>Loughmore</td>
<td>influenza C</td>
</tr>
<tr>
<td>28</td>
<td>Pitkennedy</td>
<td>influenza C</td>
</tr>
<tr>
<td>29</td>
<td>Nottingham</td>
<td>influenza C</td>
</tr>
<tr>
<td>30</td>
<td>High Gilwern</td>
<td>influenza B &amp; C</td>
</tr>
<tr>
<td>31</td>
<td>Hunmanby</td>
<td>H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>32</td>
<td>Balcombe</td>
<td>negative</td>
</tr>
<tr>
<td>33</td>
<td>Rugeley</td>
<td>USSR, influenza C</td>
</tr>
<tr>
<td>34</td>
<td>Beeford</td>
<td>H1N1, H3N2, USSR</td>
</tr>
<tr>
<td>35</td>
<td>Beckermet</td>
<td>H3N2, USSR</td>
</tr>
<tr>
<td>36</td>
<td>Tillington</td>
<td>negative</td>
</tr>
<tr>
<td>37</td>
<td>Aspull</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>38</td>
<td>Helmingham</td>
<td>H1N1, H3N2</td>
</tr>
<tr>
<td>39</td>
<td>Brandon Bank</td>
<td>H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>40</td>
<td>Lower Brailes</td>
<td>H3N2</td>
</tr>
<tr>
<td>Farm</td>
<td>sera no</td>
<td>Localization</td>
</tr>
<tr>
<td>---------------</td>
<td>---------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Walsham</td>
<td>04</td>
<td>H1N1, H3N2</td>
</tr>
<tr>
<td>Eye</td>
<td>25</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>North Bury</td>
<td>21</td>
<td>H1N1, H3N2, influenza C</td>
</tr>
<tr>
<td>Peasenhall</td>
<td>25</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>North Bury</td>
<td>10</td>
<td>influenza B &amp; C</td>
</tr>
<tr>
<td>Thelnetham</td>
<td>08</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Brenteleigh</td>
<td>26</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Sedgeford</td>
<td>11</td>
<td>influenza C</td>
</tr>
<tr>
<td>Great Harwood</td>
<td>43</td>
<td>influenza C</td>
</tr>
<tr>
<td>Great Harwood</td>
<td>39</td>
<td>influenza C</td>
</tr>
<tr>
<td>Catforth</td>
<td>07</td>
<td>influenza C</td>
</tr>
<tr>
<td>Bury</td>
<td>12</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Walgrave</td>
<td>20</td>
<td>H1N1, H3N2, USSR</td>
</tr>
<tr>
<td>Abingdon</td>
<td>01</td>
<td>negative</td>
</tr>
<tr>
<td>North Sutton</td>
<td>24</td>
<td>H1N1, H3N2, USSR</td>
</tr>
<tr>
<td>Selby</td>
<td>42</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>East Ardsley</td>
<td>14</td>
<td>H1N1, H3N2, influenza C</td>
</tr>
<tr>
<td>Caxton</td>
<td>01</td>
<td>H3N2</td>
</tr>
<tr>
<td>Abingdon</td>
<td>43</td>
<td>H1N1, H3N2, USSR, influenza B &amp; C</td>
</tr>
<tr>
<td>Neyland</td>
<td>06</td>
<td>H3N2</td>
</tr>
<tr>
<td>Abingdon</td>
<td>06</td>
<td>negative</td>
</tr>
<tr>
<td>Northop</td>
<td>29</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>Red Hill</td>
<td>20</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>Cambridge</td>
<td>05</td>
<td>Negative</td>
</tr>
<tr>
<td>North Shrewsbury</td>
<td>12</td>
<td>H1N1, influenza C</td>
</tr>
<tr>
<td>North Shrewsbury</td>
<td>02</td>
<td>negative</td>
</tr>
<tr>
<td>Trowbridge</td>
<td>12</td>
<td>H1N1, USSR, influenza C</td>
</tr>
<tr>
<td>Barcheston</td>
<td>12</td>
<td>H1N1, USSR, influenza C</td>
</tr>
<tr>
<td>Cold Hanworth</td>
<td>06</td>
<td>influenza C</td>
</tr>
<tr>
<td>Houghton</td>
<td>46</td>
<td>H3N2, influenza C</td>
</tr>
<tr>
<td>Easton</td>
<td>14</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Wornborouh</td>
<td>18</td>
<td>H1N1, influenza C</td>
</tr>
<tr>
<td>Stradbrooke</td>
<td>38</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Yorcombe</td>
<td>02</td>
<td>H1N1, H3N2</td>
</tr>
<tr>
<td>Wye</td>
<td>20</td>
<td>H1N1, H3N2, USSR, influenza B &amp; C</td>
</tr>
<tr>
<td>Northhallerton</td>
<td>06</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Newby</td>
<td>22</td>
<td>H1N1, H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Stowmarket</td>
<td>06</td>
<td>USSR, influenza C</td>
</tr>
<tr>
<td>Banham</td>
<td>03</td>
<td>negative</td>
</tr>
<tr>
<td>Glatton</td>
<td>14</td>
<td>USSR</td>
</tr>
<tr>
<td>Bodenham</td>
<td>02</td>
<td>negative</td>
</tr>
<tr>
<td>Sparsholt</td>
<td>13</td>
<td>negative</td>
</tr>
<tr>
<td>Denham</td>
<td>01</td>
<td>negative</td>
</tr>
<tr>
<td>Costessey</td>
<td>01</td>
<td>H1N1, USSR</td>
</tr>
<tr>
<td>Barway Walk</td>
<td>46</td>
<td>H3N2, USSR, influenza C</td>
</tr>
<tr>
<td>Kingscross</td>
<td>01</td>
<td>H3N2, influenza C</td>
</tr>
</tbody>
</table>
Table 23. (cont.)

<table>
<thead>
<tr>
<th>Farm no</th>
<th>Farm</th>
<th>Localization</th>
<th>Positive to influenza virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>Monewden</td>
<td>H1N1, H3N2, USSR, influenza C</td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>Heptonstall</td>
<td>USSR, influenza B &amp; C</td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>Shrewsbury</td>
<td>negative</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>Matton</td>
<td>H1N1, H3N2, influenza B &amp; C</td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>North Allerton</td>
<td>H1N1, H3N2, USSR, influenza C</td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>Hunt End</td>
<td>H3N2, influenza C</td>
<td></td>
</tr>
<tr>
<td>93</td>
<td>Caldecote</td>
<td>H1N1, H3N2</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>Deal</td>
<td>H3N2, influenza B &amp; C</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>Saxtead</td>
<td>H1N1, H3N2, USSR, influenza C</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>Thornton</td>
<td>H1N1, H3N2, USSR</td>
<td></td>
</tr>
<tr>
<td>97</td>
<td>Norwich</td>
<td>H1N1, H3N2, USSR, influenza C</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>B. in Wharfdale</td>
<td>influenza C</td>
<td></td>
</tr>
<tr>
<td>99</td>
<td>Impington</td>
<td>H1N1, USSR, influenza C</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Hastoe</td>
<td>H3N2</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>Modder</td>
<td>H3N2</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>Weybridge</td>
<td>influenza C</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>Raydon</td>
<td>USSR</td>
<td></td>
</tr>
<tr>
<td>104</td>
<td>Drax</td>
<td>H3N2, USSR</td>
<td></td>
</tr>
</tbody>
</table>

H1N1 = Wey/86  
H3N2 = Wey/87  
USSR = A/USSR/0098/77  
influenza B = B/England/21/68  
influenza C = C/Glasgow/85

* positive was taken as a HI titre equal or greater than 1/10  

** number of serum samples from each farm used in this study.
Table 24. Detection of specific antibodies against various influenza virus types and subtypes in pig sera originated from different areas in England.

<table>
<thead>
<tr>
<th>Farms</th>
<th>H1N1</th>
<th>H3N2</th>
<th>USSR</th>
<th>B</th>
<th>C</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>North</td>
<td>8*</td>
<td>14</td>
<td>11</td>
<td>2</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Centre</td>
<td>3</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>South</td>
<td>13</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td>18</td>
<td>4</td>
</tr>
<tr>
<td>East</td>
<td>18</td>
<td>17</td>
<td>19</td>
<td>0</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>West</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>56</td>
<td>43</td>
<td>9</td>
<td>67</td>
<td>13</td>
</tr>
</tbody>
</table>

* number of farms on which specific antibodies were detected

H1N1 = Wey/86
H3N2 = Wey/87
USSR = A/USSR/0098/77
B = B/England/21/68
C = C/Glasgow/85
negative = no antibodies against any of the viruses tested were detected (HI titre ≤ 1/10).
C on 67 farms, and against influenza B only on 9 farms, from a total of 104 farms studied. Farms on which pigs with antibodies against porcine H1N1 and H3N2 influenza viruses were present were separated. A number of pigs of 24 farms, selected in this way, were tested for serum antibodies against porcine Wey/86 (H1N1) and Wey/87 (H3N2), as well as an avian H1N2 influenza virus (Dck/77). Results of sera screening by HI, of the 24 selected farms over England, are summarized in table 25. Herds in which antibodies against the H1N2 strain were also found were selected for further studies.

No antibodies against any of the viruses tested were found in sera from farms 74 and 75. Sera from pigs on farms 8, 9, 46, 76, 77, 87 and 93 only contained antibodies against the H1N1 strain, whereas sera from pigs of farm 90 only had antibodies against strain H3N2. However, only a very small number of sera contained antibodies against the H1N2 strain, and when present, their HA titre was always lower (8-fold) than that of the other two strains present. These farms (42, 47, 56, 57, 71, and 73) were mainly situated in the East of England (Norfolk and Suffolk), and two of them in Yorkshire. Only animals from farms 56 and 73 presented H1N1, H3N2 and H1N2 antibodies simultaneously, in titres equal to or higher than 1/40 (Table 25). Accordingly, as the basis of the criteria described below, farm 73 was selected for further studies. It contained the largest number of animals with the highest HA antibody titre.
Table 25. Animals presenting antibody levels equal to or greater than 1/40 HI against influenza strains Wey/86, Wey/87 and Dck/77.*

<table>
<thead>
<tr>
<th>Farm**</th>
<th>Wey/86(H1N1)</th>
<th>Wey/87(H3N2)</th>
<th>Dck/77(H1N2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 (B)</td>
<td>9/19 ***</td>
<td>0/19</td>
<td>0/19</td>
</tr>
<tr>
<td>9 (B)</td>
<td>7/30</td>
<td>0/30</td>
<td>0/30</td>
</tr>
<tr>
<td>23</td>
<td>4/9</td>
<td>3/9</td>
<td>0/9</td>
</tr>
<tr>
<td>34</td>
<td>4/5</td>
<td>1/5</td>
<td>0/5</td>
</tr>
<tr>
<td>37</td>
<td>9/15</td>
<td>9/15</td>
<td>0/15</td>
</tr>
<tr>
<td>38</td>
<td>4/8</td>
<td>3/8</td>
<td>0/8</td>
</tr>
<tr>
<td>42</td>
<td>16/25</td>
<td>15/25</td>
<td>1/25</td>
</tr>
<tr>
<td>46 (B)</td>
<td>4/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>47</td>
<td>16/26</td>
<td>0/26</td>
<td>4/26</td>
</tr>
<tr>
<td>55</td>
<td>16/24</td>
<td>6/21</td>
<td>0/24</td>
</tr>
<tr>
<td>56</td>
<td>34/42</td>
<td>25/37</td>
<td>2/42</td>
</tr>
<tr>
<td>57</td>
<td>4/13</td>
<td>4/13</td>
<td>1/13</td>
</tr>
<tr>
<td>71</td>
<td>10/14</td>
<td>1/14</td>
<td>1/14</td>
</tr>
<tr>
<td>73 (D)</td>
<td>28/38</td>
<td>9/36</td>
<td>8/38</td>
</tr>
<tr>
<td>74 (A)</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>75 (A)</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>76 (B)</td>
<td>4/6</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>77 (B)</td>
<td>9/22</td>
<td>0/22</td>
<td>0/22</td>
</tr>
<tr>
<td>87 (B)</td>
<td>8/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>90 (C)</td>
<td>0/30</td>
<td>13/30</td>
<td>0/30</td>
</tr>
<tr>
<td>91</td>
<td>7/32</td>
<td>12/32</td>
<td>0/32</td>
</tr>
<tr>
<td>93 (B)</td>
<td>7/20</td>
<td>0/20</td>
<td>0/20</td>
</tr>
<tr>
<td>96</td>
<td>9/12</td>
<td>6/12</td>
<td>0/12</td>
</tr>
<tr>
<td>97</td>
<td>2/20</td>
<td>2/20</td>
<td>0/20</td>
</tr>
</tbody>
</table>

* The tabulated data refer to pig sera originated from farms of various areas of England, chosen to take part in these studies, based on previous serological studies (Table 23).
** A - no specific antibodies found against any of the viruses tested
    B - specific antibodies only against strain Wey/86(H1N1)
    C - specific antibodies only against strain Wey/87(H3N2)
    D - farm selected based on the simultaneous presence in the same serum of specific HI antibodies against Wey/86(H1N1), Wey/87(H3N2) and Dck/77(H1N2) tested, in significant titres ($\geq 1/40$).
*** - number of positive sera/total number of serum samples
against the H1N2 strain in their serum, was located in an intensive pig farming area, was run on an industrial basis (being part of a large pig rearing organization which operated farms in other counties), and was open to cooperate with research work.

The serological and diagnostic results of, respectively, the sera and nasal swabs of the pigs from farm 73 are shown in Appendix IV and summarized in Table 26. SRID tests were initially used as a screening test. However, no clear reactions were observed to confirm the presence of a specific N, so the HI and spot NI tests were subsequently used, and the SRID test only for confirmation of the results.

Serological evidence for the possible presence of reassortant influenza virus was found. In the preliminary survey, the breeding stock from this farm was used for serum sampling. They presented significant antibody levels against Wey/86(H1N1) and Wey/87(H3N2) strain, simultaneously (Table 26). The detailed survey among slaughter pigs presented antibodies mainly against the H3N2 porcine strain (Appendix IV). Although the interval between the two samplings was less than six months (May - September), samples taken an additional three months later (November) from the breeding stock and weaning pigs, presented mainly antibodies against H1N1 virus.

Very few abattoir pigs reacted in the HI test against
Table 26. Results of the serological screening test carried out on sera collected from pigs originated from farm 73.

<table>
<thead>
<tr>
<th>No of sera presenting</th>
<th>abattoir pigs</th>
<th>farm pigs</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1N1 antibodies alone</td>
<td>0 (&lt;3.5%)**</td>
<td>0 (&lt;7.7%)</td>
<td>0 (&lt;5.1%)</td>
</tr>
<tr>
<td>H3N2 antibodies alone</td>
<td>59* (69%)</td>
<td>1 (8%)</td>
<td>60 (61%)</td>
</tr>
<tr>
<td>H1N2 antibodies alone</td>
<td>0 (&lt;3.5%)</td>
<td>0 (&lt;7.7%)</td>
<td>0 (&lt;5.1%)</td>
</tr>
<tr>
<td>H1N1 and H3N2 antib.</td>
<td>23 (27%)</td>
<td>10 (77%)</td>
<td>33 (34%)</td>
</tr>
<tr>
<td>Antibodies against H1N1, H3N2 and H1N2 simultaneously</td>
<td>3 (3.5%)</td>
<td>2 (15%)</td>
<td>5 (5%)</td>
</tr>
</tbody>
</table>

Number of sera tested 85 13 98

Obs.: An antibody response was considered positive when HA titres were equal or greater than 1/40.

* number of sera

** percentage of total tested
the H1N2 strain, although when antibodies were found, the titre was relatively high (>1/80) when compared with that against H1N1 virus in the same samples, always 2 to 8-fold less (Appendix IV).

This was the only indication to suggest the presence of a reassortant virus. The spot N test indicated only the presence of antibodies against the N2 subtype, which was concurrent with the other findings.

2. Isolation of influenza virus from nasal swabs

Influenza virus was only isolated from 2 nasal swabs, collected from live animals on the farm. They were from a 2-month-old piglet (isolate 1) with, and a 3-year-old sow (isolate 2) without clinical signs of respiratory disease. These isolates proved to be influenza A H3N2 subtypes, as tested by IDD test (Fig. 23) and HI and spot NI test (Table 27). They were analysed for their H and N subtype by HI and NI tests using chicken antisera to each subtype virus of influenza A viruses according to the procedure described by Webster and others (1973). HA was strongly inhibited by antisera to H3N2 strains, and also a H12 avian strain. NI test for the characterization of the neuraminidase of the isolates was carried out using the spot test. NA of the isolates was inhibited only by antiserum to virus strains with the N2 neuraminidase. In the IDD test, a common
Fig. 23. Photograph of the IDD test plate showing the identity of the two influenza viruses isolated from pigs from farm 73.

The location of the samples in wells are shown in diagram.

- 1 - isolate 1 (piglet)  
- 2 - isolate 2 (sow)  
- Pa - positive antigen (NP)  
- - = negative SPF serum

(3 - purified H3 (1979)  
4 - purified H3  
5 - purified H3 (1975)  
6 - purified H12  
7 - purified H3 (1968)

Obs.: The reaction observed between wells 6 and Pa is probably due to egg protein.
Table 27. Results of HI and NI tests carried out on influenza isolates obtained from nasal swabs of pigs reared on farm 73.

<table>
<thead>
<tr>
<th>Antisera against</th>
<th>piglet isolate</th>
<th>sow isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HI</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/duck/Alabama/35/76 (H1N1)</td>
<td>4</td>
<td>neg</td>
</tr>
<tr>
<td>A/turkey/England/250/79 (H1N1)</td>
<td>2</td>
<td>neg</td>
</tr>
<tr>
<td>A/duck/Hong Kong/196/77 (H1N2)</td>
<td>128</td>
<td>pos</td>
</tr>
<tr>
<td>A/Singapore/57 (H2N2)</td>
<td>32</td>
<td>pos</td>
</tr>
<tr>
<td>A/turkey/England/69 (H3N2)</td>
<td>64</td>
<td>NT</td>
</tr>
<tr>
<td>A/swine/Taiwan/1/70 (H3N2)</td>
<td>64</td>
<td>NT</td>
</tr>
<tr>
<td>A/avian/756/88 (H3N2)*</td>
<td>128</td>
<td>NT</td>
</tr>
<tr>
<td>A/duck/Ukraine/1/63 (H3N8)</td>
<td>128</td>
<td>neg</td>
</tr>
<tr>
<td>A/equine/Miami/1/63 (H3N8)</td>
<td>32</td>
<td>NT</td>
</tr>
<tr>
<td>A/duck/Czechoslovakia/56 (H4N6)</td>
<td>64</td>
<td>NT</td>
</tr>
<tr>
<td>A/tern/South Africa/61 (H5N3)</td>
<td>8</td>
<td>NT</td>
</tr>
<tr>
<td>A/turkey/Maes/3760/65 (H6)</td>
<td>64</td>
<td>NT</td>
</tr>
<tr>
<td>A/turkey/England/199/79 (H7N7)</td>
<td>16</td>
<td>NT</td>
</tr>
<tr>
<td>A/turkey/Ontario/6118/67 (H8N4)</td>
<td>8</td>
<td>neg</td>
</tr>
<tr>
<td>A/Hong Kong/91/76 (H9N2)</td>
<td>64</td>
<td>NT</td>
</tr>
<tr>
<td>A/Turkey/Scotland/70 (H9N7)</td>
<td>16</td>
<td>neg</td>
</tr>
<tr>
<td>A/chick/Germany/10/49 (H10N7)</td>
<td>8</td>
<td>NT</td>
</tr>
<tr>
<td>A/duck/England/56 (H11N6)</td>
<td>16</td>
<td>NT</td>
</tr>
<tr>
<td>A/duck/Alberta/60/76 (H12N5)</td>
<td>16</td>
<td>NT</td>
</tr>
<tr>
<td>A/gull/Maryland/704/77 (H13N6)</td>
<td>16</td>
<td>NT</td>
</tr>
<tr>
<td>A/duck/Memphis/546/74 (H11N9)</td>
<td>16</td>
<td>neg</td>
</tr>
</tbody>
</table>

HI = haemagglutination inhibition  
NI = Neuraminidase inhibition  
neg - no affinity detected with antibodies for this strain  
pos - homology shown with antibodies for this strain  
* pooled sample from quarantaine birds collected in England  
NT = not tested
### Haemagglutination Inhibition test

#### Post-infection ferret sera

<table>
<thead>
<tr>
<th></th>
<th>X31</th>
<th>A/Eng/42/72</th>
<th>A/PC/1/73</th>
<th>A/Vic/3/75</th>
<th>Sw/OMS3633/84</th>
<th>Sw/Wey/87</th>
<th>A/Hiss/1/85</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Hong Kong/1/68</td>
<td>1280</td>
<td>160</td>
<td>40</td>
<td>40</td>
<td>&lt;</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>A/England/42/72</td>
<td>160</td>
<td>2560</td>
<td>160</td>
<td>80</td>
<td>160</td>
<td>&lt;</td>
<td>40</td>
</tr>
<tr>
<td>A/P.Chalmers/1/73</td>
<td>40</td>
<td>320</td>
<td>640</td>
<td>160</td>
<td>320</td>
<td>&lt;</td>
<td>40</td>
</tr>
<tr>
<td>A/Victoria/3/75</td>
<td>40</td>
<td>320</td>
<td>80</td>
<td>1280</td>
<td>80</td>
<td>40</td>
<td>80</td>
</tr>
<tr>
<td>sw/OMS/3633/84</td>
<td>40</td>
<td>40</td>
<td>80</td>
<td>40</td>
<td>1280</td>
<td>40</td>
<td>&lt;</td>
</tr>
<tr>
<td>sw/Weybridge/87</td>
<td>40</td>
<td>40</td>
<td>80</td>
<td>80</td>
<td>640</td>
<td>&lt;</td>
<td></td>
</tr>
<tr>
<td>A/Mississippi/1/85</td>
<td>&lt;</td>
<td>40</td>
<td>&lt;</td>
<td>40</td>
<td>&lt;</td>
<td>&lt;</td>
<td>2560</td>
</tr>
<tr>
<td>sw/Suffolk/1/88*</td>
<td>40</td>
<td>160</td>
<td>320</td>
<td>40</td>
<td>320</td>
<td>40</td>
<td>&lt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sw/Suffolk/2/88**</td>
<td>40</td>
<td>160</td>
<td>320</td>
<td>40</td>
<td>320</td>
<td>40</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

* isolate 1  
** isolate 2

### Haemagglutination Inhibition test

**monoclonal antibodies prepared against Sw/OMS/3633/84 (Ascitic fluids)**

<table>
<thead>
<tr>
<th></th>
<th>HC25</th>
<th>HC26</th>
<th>HC30</th>
<th>HC39</th>
<th>HC64</th>
<th>HC77</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Hong Kong/1/68</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>A/England/42/72</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>6400</td>
</tr>
<tr>
<td>A/P.Chalmers/1/73</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>6400</td>
</tr>
<tr>
<td>A/Victoria/3/75</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>6400</td>
</tr>
<tr>
<td>sw/OMS/3633/84</td>
<td>6400</td>
<td>6400</td>
<td>6400</td>
<td>6400</td>
<td>6400</td>
<td>6400</td>
</tr>
<tr>
<td>sw/Suff/1/88*</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>3200</td>
</tr>
<tr>
<td>sw/Suff/2/88**</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>3200</td>
</tr>
</tbody>
</table>

* isolate 1  
** isolate 2

### Neuraminidase-Inhibition test

**monoclonal antibodies (ascitic fluids)**

<table>
<thead>
<tr>
<th></th>
<th>X31</th>
<th>A/PC/1/73</th>
<th>A/Tex/3633/84</th>
<th>MRC 11 3633/84</th>
<th>MRC 17 3633/84</th>
<th>MRC 22 3633/84</th>
<th>MRC 34 3633/84</th>
<th>MRC 42 3633/84</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Hong Kong/1/68</td>
<td>2560</td>
<td>160</td>
<td>&lt;</td>
<td>40</td>
<td>40</td>
<td>&lt;</td>
<td>2560</td>
<td>&lt;</td>
</tr>
<tr>
<td>A/P.Chalmers/1/73</td>
<td>80</td>
<td>1280</td>
<td>160</td>
<td>640</td>
<td>5120</td>
<td>&lt;</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>Sw/OMS/3633/84</td>
<td>80</td>
<td>320</td>
<td>80</td>
<td>1280</td>
<td>160</td>
<td>2560</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>sw/Suff/1/88*</td>
<td>80</td>
<td>640</td>
<td>160</td>
<td>640</td>
<td>5120</td>
<td>&lt;</td>
<td>2560</td>
<td>2560</td>
</tr>
<tr>
<td>sw/Suff/2/88**</td>
<td>80</td>
<td>640</td>
<td>160</td>
<td>640</td>
<td>5120</td>
<td>&lt;</td>
<td>2560</td>
<td>2560</td>
</tr>
</tbody>
</table>

* isolate 1  
** isolate 2

< = <100

< = <20
precipitin line developed in 24 hours between the treated samples and positive antisera, but not between the treated samples and negative antisera (Fig.23).

The isolates were sent to the WHO World Influenza Centre in London for confirmation of their identity using monoclonal antibodies (Table 28). The isolates, called A/swine/Suffolk/1/88 (isolate 1) and A/swine/Suffolk/2/88 (isolate 2), were considered very similar to A/PC/73, which disappeared from the human population in 1973 but continues to circulate in European pigs (see Section 6 of Chapter II).

Discussion

Taking into consideration that interspecies transmission may occur with influenza viruses, continuing surveillance of influenza virus infection among pig herds is of great epidemiological importance to detect new strains of the virus and to determine their pathogenicity for the various susceptible hosts.

The aim of this study was to determine whether or not a reassortant strain of influenza virus was present in English pigs, with the consequent potential for pandemic spread to man and/or birds. Although no such reassortant was isolated, the serological results suggest the possibility of such an infection, and further suggest that genetic
reassortment between infecting viruses H1N1 and H3N2 occurs among the English pig population. The lack of antibodies to the H1N1 virus in the abattoir samples, but present in breeding stock from the same farm in the same period suggests that infection with H1N1 subtype is not currently active on that farm, but may have been present in the herd in the past, and antibodies are passed passively by the sows to their offspring. Accordingly, it seems likely there is no stimulus for active production of antibodies against H1N1 virus, but only against H3N2 viruses. Thus the former disappears gradually from the blood stream of those pigs after weaning and when separated from the breeding herd. It must also be considered that the animals sent for slaughter were clinically healthy, whereas those tested on site included piglets with respiratory signs of illness. This may justify the isolation of virus from the breeding stock but not from the slaughter pigs.

The inability to isolate a H1N2 reassortant from the pig farms studied does not disprove the occurrence of such an influenza virus in England. This study only involved a limited number of pigs from few farms and modern technology was not used for the detection of reassortants. It is possible that influenza strains other than those examined, such as combinations of H3N2 strains of various origins, would be detected if monoclonal antibodies and RNA genome analysis were used.

Although the main objective of this study was to detect
reassortant influenza virus, other findings are worthy of comment. During the initial screening tests, antibodies against influenza virus B and C were also detected. This may indicate that these influenza viruses are present in English pig herds. Further studies on this subject would be of interest, as to date no influenza B has been isolated from pigs, and very few reports on the presence of influenza virus type C among pigs have been published.
CHAPTER VIII

GENERAL DISCUSSION

1. Goals

This thesis describes \textit{in vitro} and \textit{in vivo} experiments which were devised to obtain further understanding of influenza epidemiology. The main points were a) diagnostic procedures for influenza that could improve disease identification qualitatively, quantitatively and in the shortest period of time, b) the possibilities of interspecies transmission and c) some considerations concerning the primary origin and periodic reappearance of viruses with slightly changed structures.

The opportunities for diseases such as influenza to spread are increasing. Besides the role of migrating birds in disseminating the viruses, there is a growing trend for transit of human beings to all corners of the world, and an increasing international trade in live animals and products to remote areas.

After the Fort Dix, New Jersey, U.S.A., episode (Weekly Epidemiological Record, 1976), interest grew in animal influenza, especially concerning the origin of the virus and its possible anthropozoonotic properties. In this context, the present trend in influenza research is to determine the
relationships and affiliation of an isolate, in comparison with all others isolated previously. Strains described as "avian", and "mammalian" or "human" refer either to virus properties found most frequently among isolates from that species, or to the species from which isolation was made. The labels do not imply that their ultimate origin was the species mentioned (Hinshaw and others, 1981b), and the appearance of a virus normally associated with avian species in seals (Webster and others, 1981c) and horses (Gibson, 1987) supports this argument. Confusion about origin is even greater when some of the attributed properties are separated in reassortants (Webster and others, 1981b).

2. Diagnostic procedures

Rapid diagnosis of influenza infections has always been sought, and, periodically, new approaches have emerged for the cultivation and isolation of the virus present in clinical samples. A culture system in which all influenza viruses replicate to high titre, producing infectious virus particles and without undue adverse effects on the host cell would be ideal for diagnostic tests. The studies carried out in this work indicated, however, that the variability of the virus under study makes it very difficult to develop a single technology for all types and subtypes of the virus. It was considered that each virus passage may produce a genetic variation in the population, as well as selection in
type and/or subtype, depending upon the virus growth and tissue affinity characteristics. In addition, a number of authors have demonstrated that the inoculation of influenza A viruses into cell cultures may produce several effects. The virus may replicate yielding infectious virus efficiently (productive infection), or small quantities over a long time period (persistent infection). The virus may also undergo an incomplete growth cycle, presenting virus antigens only inside and at the cell surface (abortive infection) with no production of infectious virus particles; non-infectious virus (such as DI particles) may be formed (see Section 2.2.3. in Chapter I); or the virus may fail to infect if its H is not cleaved. In the opinion of the author, efficient infectious virus replication in a certain host is only realistically tested when comparing infectivity doses in this same host. However, not all factors involving the host could be controlled. Field conditions could not be reproduced exactly as they were during the outbreak and different results were obtained under experimental conditions. When only small quantities of virus were produced, they were not detected by the techniques available. Abortive infection could only be detected by FA or IPX techniques and confirmed by absence of HA in the medium. The production of DI particles was avoided by using high virus dilutions for inoculation, and distinguished from influenza virus particles with an uncleaved H by treating
the samples with H cleaving enzymes (e.g. trypsin). The presence of HA in the supernatant of the culture may have represented inoculum residue, the presence of DI particles, infectious virus, non-infectious virus due to an uncleaved H, or virus antigens adhering to ruptured cell membranes. FA and IPX techniques indicated virus antigen production in the cell, but did not distinguish productive from abortive infection. It was concluded that the nature of the virus could only be properly defined by using the above mentioned techniques, in association with plaque-formation analysis and egg inoculation. Animal inoculation may then be used to confirm virus pathogenicity for a certain host. Embryonated eggs may also exert selection pressures upon the virus, which are different from those exerted by the different cell cultures. Thus, it was very difficult to establish with certainty if the virus under study was similar to the one causing the outbreak from which it was originally isolated.

2.1. Cell culture susceptibility

It was very difficult to compare cell culture susceptibility for the viruses under study, and to judge the effect of each virus on each type of cell. It was found that besides the cell morphology (epithelial-like, fibroblast-like, or a mixture of both) that may affect influenza A virus affinity, each cell culture had its own growth and plating efficiency, affecting the ability to form confluent monolayers (ATCC, 1983). Cell size may also affect
virus replication since with larger cells there are fewer cells per area and a different multiplicity ratio per cell. Some cells are more sensitive to ambient factors, such as handling, pH of the medium, trypsin and temperature variations. Contamination is often encountered with cell culture, but is less often a problem with egg techniques. This limits the usefulness of the cell cultures, although they may still be appropriate for situations in which large numbers of incoming samples must be screened.

Cells originating from different animal species, from different breeds or even individuals of a given species may vary in susceptibility to virus infection. Variation in susceptibility was found in vivo and in vitro and may be determined by a single dominant genetic factor (Pereira, 1961). Cells from susceptible animals may become entirely resistant to infection on cultivation. Variations in susceptibility have frequently been described arising in the course of prolonged cultivation of cell lines. This change is often correlated with transformation of the cells (reviewed by Pereira, 1961). The present studies showed that, when searching for suitable cultures for a certain influenza virus strain, an empirical approach should be adopted, as determined by the individual conditions of each laboratory. The overall conclusion that the MDCK cell line was the most susceptible cell culture was in general agreement with other workers.
2.2. Cytopathogenicity

Klenk and others (1977) showed that some influenza viruses are able to spread to and infect vital tissues and organs, while others have restricted replication sites (Bosch and others, 1979) (See Section 3.1.2 in Chapter I). The present studies showed that the infectivity of the influenza strains tested depended upon the presence of trypsin in the tissues to cleave its H. Trypsin is known to be present in embryonated eggs naturally, and therefore responsible, at least in part, for this being considered the best culture system.

The use of CPE as a diagnostic tool in influenza virus infection in these studies was restricted to plaque formation observations in agar overlay cultures with and without the addition of trypsin-like enzymes. Plaque-forming capacity of an influenza virus is indicative of a cleaved H. The cytopathic property may therefore be exchangeable between strains after reassortment of the H gene (Simpson and Hirst, 1961). In this manner, the host range of the virus may be broadened and its infectivity increased for a larger number of cells. However, CPE may occur due to other factors not related with the viral infection, whilst it must also be considered that the development of CPE can be inhibited by several virus inhibitors of different natures, such as antibodies, and also by any factor acting on reversible metabolic alterations which render the cells incapable of supporting
virus replication (see Sections 2.4 and 3.1 in Chapter I). It was concluded that MDCK cells were the most suitable of the cell cultures tested for influenza virus isolations and diagnostic techniques tested, provided the restrictions mentioned above were taken into consideration, since they permitted virus growth without CPE during a longer period than all other cell cultures tested.

3. Influenza virus in infected pig tissues

Recovery of porcine influenza virus from experimentally infected pigs has been achieved by several authors (Blaskovic and others, 1969; Fontaine and others, 1983). Tissues used for isolation have been taken from the affected respiratory tract and accessory lymph nodes, and also from organs connected with blood circulation, such as the spleen. Nayak and others (1965b) studied the progression of the infection in experimentally infected pigs, and Vannier and others (1985) established the shedding period of this virus. In all studies, no tissues other than the respiratory tract appeared to be involved.

The in vitro studies reported in this thesis demonstrate that, depending upon the condition of storage, porcine influenza can remain viable within, and potentially be transmitted through, meat, if processed within 15 days after slaughter and stored at low temperatures (in this case -20°C). The human population most at risk to infection
may be meat processor employees in charge of handling crude meat soon after slaughter, or animals which are fed with uncooked pig products. Legislation in most countries imposes the treatment of pig meat products before being used for animal food purposes, thus making disease transmission impossible by this means. However, influenza may be spread through carcasses of dead animals in the wild.

The results obtained in the in vivo experiments showed that pigs infected with influenza virus may harbour the virus in their tissues in the absence of clinical signs of illness. This confirmed the studies of Blaskovic and others (1970), Styk and others (1971a), Vandeputte and others (1981) and Vannier and others (1985). The inference from the above is that infected lungs or blood could infect the meat through contact, or even be already present in the meat or other parts of economical value, and the virus spread by this route to susceptible animals. Similarly, the contacts acquiring the infection may pass unnoticed.

Clearly, the presence of virus in tissue collected in the present studies after slaughter does not necessarily indicate that viral replication was occurring in that tissue. Industrial slaughter conditions were reproduced as near as possible, so that samples of meat, intercostal muscle, intestine, heart, brain and reproductive organs were processed on the same site where the respiratory tract had been processed. Therefore it would appear likely that the samples became contaminated on that occasion. Nonetheless,
the experiment proves that the virus can remain viable, even as a contaminant, in tissues of dead animals. The inconsistent isolation of infectious virus may represent the situation in nature or be attributed to the small size of the samples analysed, and the possibility that samples were not always collected from infectious foci or contaminated parts of the tissues.

It was clear that after experimental infection, the porcine influenza virus strain used in these studies replicated poorly in pigs, with moderate or no pathogenicity. Similar observations have been made by several authors (Pospisil and others, 1973; Vandeputte and others, 1981; Maes and others, 1984; Vannier and others, 1985). A number of factors, both viral and host derived (including the age of the pigs, environmental temperature, virus strain used for infection, dose, route of inoculation, concomitant infections, stress), have all been shown to influence the expression of influenza disease (see Section 3 in Chapter I and later in this Chapter).

The presence of the virus in the faeces of the infected pigs may indicate a possible route of transmission to other animals, such as birds, especially as untreated slurry may be spread over fields as a fertiliser. The isolation of virus from the reproductive organ samples (ovaries) also has significance as it raises the question whether or not influenza virus can be transmitted ante-natally, and the
further possibility that the virus could be a causative agent of reproductive disorders in pigs (Mensik, 1959; Gourreau and others, 1985). However, from the design of these studies it cannot be excluded that the virus isolated from the ovary was a contaminant, rather than actively replicating in the ovaries of the infected gilt.

4. Interspecies transmission

Since influenza viruses similar to major human subtypes have been isolated from avian species (Webster and Pereira, 1968), studies were carried out to determine if porcine influenza A viruses could replicate in turkeys. The relatively high titres of specific antibodies, 14 days after infection, left no doubt that this was possible, and was in accordance with experiments by Webster and others (1977b) testing the replication of mammalian influenza viruses in ducks. Although the strains they tested did not replicate in the intestinal tract of the ducks, successful isolations were obtained from the upper respiratory tract. In contrast, the present studies demonstrated that the porcine strain tested was present in the intestinal tract of turkeys infected by it.

Outbreaks of porcine influenza in two turkey breeder flocks were described by Mohan and others (1981). A respiratory tract disease occurred in the pig herd on the
same farm, immediately before the turkey flocks showed clinical signs. Attempts to isolate virus from turkeys and pigs failed, but all sera collected were positive in HI tests for porcine influenza antibodies. Also, three of the six farm workers had HI antibodies against the porcine influenza A virus. Their investigations indicated pigs as being a reservoir for the virus. Persistence of avian influenza A viruses in turkeys following natural infection was not well defined at that time, but in the present studies it was shown that turkeys can be experimentally infected and may excrete porcine influenza virus for up to 17 days post-infection. In this manner, turkeys, or other birds, can be part of the interspecies transmission chain of influenza infection over large areas.

The extreme variation in pathogenicity between strains of influenza A viruses (Klenk and Rott, 1988) is an indication of the difficulties encountered in assessing virus virulence. The experiments on this subject carried out in the present work, which were designed to assess the transmissibility of the English porcine isolates, also indicated a similar variability in these viruses. The contrasting results of the primary experiments (successful experimental disease production in turkeys and pigs with English porcine influenza isolates) and the later experiments are a demonstration of this variability. Such variability in pathogenicity of influenza isolates has been reported (Klenk and Rott, 1988; Shortridge, 1988), and
several explanations are possible for the failure to produce experimental clinical disease in turkeys at this time and the lack of clinical disease in pigs in contact:

The host origin of the viruses used may affect its ability to cause disease. For example, strains that have a so called avian origin but have been isolated from pigs, may only infect humans with difficulty, even in close contact, but will spread easily to other pigs and birds. Also, the so-called avian viruses possess the ability to replicate in the intestinal as well as in the respiratory tract of avian hosts, while "human" strains only replicate in the respiratory tract of birds. The amino acid composition and cleavability of the H is one of the virus properties responsible for the above, as discussed earlier. One of the experiments (see Chapter III) demonstrated that the porcine influenza strains H1N1 and H3N2 used throughout these studies produce plaques in MDCK cells only after addition of trypsin to the medium. The amino acid composition at the cleavage site of the H of these viruses is probably cleaved only by trypsin, and not by other cellular proteases. Trypsin is normally present in the gut as well as the upper respiratory tract, permitting these mild virus strains to replicate at these sites (Rott, 1979). On the other hand, if the virus strains possessed a different amino acid sequence at the cleavage site, other cellular proteases present in a much broader range of cells would be able to
cleave the H, making these virus strains much more pathogenic (Klenk and Rott, 1988).

A possible explanation for the experimental results obtained in Chapter V may lie in the host specificity of the influenza virus NP, as the pathogenicity of an influenza isolate may vary considerably with the species infected. It has been speculated that the position of amino acids in the NP gene is responsible for specific avian or human affinity (Klenk and Rott, 1988). Scholtissek and others (1985) showed that ducks infected with human or porcine viruses did not excrete the virus from the trachea or cloaca. However, when the same ducks were infected with avian influenza viruses, these could be isolated for longer than 2 weeks after infection from both sites. Antibodies against the human and porcine strains, however, were detected at higher titres than those against the avian strain. Turkey influenza viruses (H1N1) tested by Webster and others (1978) only replicated in the respiratory tract of birds, thus demonstrating a tissue tropism similar to that of mammalian, rather than avian viruses of the same subtype. It was observed during some experiments carried out in this study that characteristic clinical signs were more frequent in animals infected with strain H1N1 than H3N2, probably indicating a different affinity of this H3N2 strain for the host cells infected.
The virulence of an influenza isolate can vary after passaging of the virus. Egg passages necessary to amplify the virus may attenuate it, changing its host range and/or tissue tropism (Green and others, 1957). Antigenic variants of influenza A virus strains also emerge on serial passage in ovo principally in the presence of immune sera against different but related strains (Archetti and Horsfall, 1950; Klenk and Rott, 1988). A point mutation may also alter the temperature range of optimal growth of an influenza virus (Murakami and others, 1988) resulting in the virus becoming unable to replicate in the lungs.

Genetic reassortment between avian and mammalian influenza viruses was successfully achieved in 1971 by Webster and others (1971, 1973), when new influenza viruses were isolated from turkeys and pigs after mixed infection with influenza viruses, these new viruses proved to be pathogenic for animal hosts (turkeys, chickens and pigs).

The large and small plaques produced in the MDCK cell cultures by the viruses used in the present studies (see Chapter III) may be indicative of a population of viruses consisting of at least two subpopulations of influenza viruses. Genetic reassortment between these different virus populations, with the production of less pathogenic reassortants for the hosts tested, may have occurred.

Fontaine and others (1983) showed that the severity of the disease is linked to associated environmental and
aetiological factors as well as to the virulence of the virus strains.

Roberts and others (1987) recovered the influenza A virus (H3N2) used in these experiments from an outbreak of influenza among pigs in East Anglia. The virus was similar, but not identical, to some other human and porcine H3N2 strains. The degree of severity of the disease was thought to be due to secondary infection and stress. Serological surveys for specific antibodies showed that similar virus had been present in the animals for some time (Chapman and others, 1978). Klenk and Rott (1988) consider that if a host is co-infected with trypsin-like enzyme producing microrganisms in tissues where influenza virus with uncleaved H is present, the cleavage of the influenza virus strain's H by the co-infecting microrganism would permit the influenza virus to spread to other cells, causing disease. It is possible that this phenomenon occurred in the first experiments described in Chapter V, when infecting only turkeys, and not in the second experiments, producing the observed variance in pathogenicity. The H of the porcine influenza viruses used in the latter experiments may not have been activated in the turkeys, after a first cycle, in the absence of trypsin. The viruses isolated from faecal material were possibly contaminants encountered during the isolation procedure. The isolation of infectious viruses was probably due to the amplification in the first cycle in
fowls' eggs.

5. Serological studies in Brazil

A serological analysis was also used as an approach to study the distribution of influenza virus in pig populations and possible interspecies transmission.

Frequent detection in the sera from Brazilian pig herds of HA antibodies against influenza virus strains present in humans a decade ago, together with recent human variants suggests that H3N2 influenza A virus strains may be harboured by pig herds in Brazil, and that animals were infected with virus with antigenic properties similar to those of the virus circulating at the corresponding time in the human population. The higher percentage of positive sera against PC/73 and Lenin/86 antigens in the majority of the herds under study may suggest the possibility that these viruses were introduced into pig herds by man during and after the epidemics, but further work would be required to substantiate this hypothesis. Similar findings concerning isolation or serological demonstration of influenza A/H3N2 virus variants in pigs have been demonstrated by a number of authors in Europe, North America and Asia (see Section 1 and 6 in Chapter I and Introduction of Chapter VI). In North America, continuing evidence of the incidence of classical porcine influenza A/H1N1 virus has been reported several times (see Section 6 in Chapter I).
6. Reassortment studies and their implications within influenza virus origin and interspecies transmission.

The possible occurrence of genetic reassortment among influenza A viruses was described early in this century, very soon after the first isolation of the virus (Burnet and Lind, 1951a and b). Since then, numerous workers have confirmed this observation.

Serological examinations of influenza A virus infection among English pigs carried out since 1968 revealed that although antibodies against H3N2 strains had been present for a number of years, H1N1 antibodies were not detected until 1986 (Chapman and others, 1978; Roberts and others, 1987). Outbreaks of classical porcine influenza in pigs were first reported in 1986 (Roberts and others, 1987) and accordingly the opportunity for reassortment between H1N1 and H3N2 influenza virus strains may have only been possible since this date.

The simultaneous presence of different influenza virus strains in English pigs brings us back to the question how these viruses were introduced, and what was their origin, birds or other mammals?

Pigs and birds may be the reservoirs of influenza viruses and also the hosts in which genetic reassortment occurs, but, to date, there have been very few reports which claimed that man has been infected naturally with avian influenza viruses without genetic reassortment in
another species (Kovalchuk-Ivanyuk and others, 1975; Ottis and Bachmann, 1980; Hinshaw and others, 1981; Oxford, 1987; Shortridge, 1988). Passage in pigs of avian strains appears essential before transmission to man (see Section 6 in Chapter I).

The findings that pig farms localised mainly in East England, especially Norfolk and Suffolk, contained animals with antibodies to several influenza virus subtypes may be relevant. In that same area, duck and turkey flocks with high prevalence of influenza virus antibodies have been detected (Alexander, 1988a), and some outbreaks including those with virus isolation (including extremely virulent virus strains) have been reported (Alexander and Spackman, 1981; Alexander and Allan, 1982). The presence of waterfowl migration routes over that area have been commented on, and related to the influenza outbreaks among the poultry flocks (Alexander, 1988b). Fresh introduction of influenza virus to ducks and turkeys could happen every time migration occurs. It is possible that pigs are infected by waterfowl, perhaps indirectly, mechanically or by infected poultry and small free-living birds. At least the primary introduction may be due to migrating birds. Migrating birds may even have picked up influenza virus strains from pig herds in Europe or other continents where they spend part of their life. One of the influenza strains isolated from turkey herds in England in 1979 (Alexander and Spackman, 1981)
showed antigenic similarity with strains of porcine origin, although no evidence of infection of pigs with that particular strain of virus was reported in this country at that time (Chapman and others, 1978). Isolations of influenza strains antigenically related to porcine influenza H1N1 viruses from feral ducks have been reported earlier in other countries (Hinshaw and others, 1978b; Ottis and Bachmann, 1980).

The use of monoclonal antibodies would permit the analysis of small antigenic changes that may have occurred in the H and/or N. RNA analysis may detect reassortment from other genes than those producing the external proteins. The presence of different properties in the reassortant than those of the parent virus will also show that reassortment took place. Gene segments coding for internal proteins of avian influenza viruses may reassort with gene segments coding for the H and N proteins of human or porcine influenza virus, the resultant virus being serologically identical, or very similar to the parent human or porcine virus, but with differing biological properties such as host range, temperature sensitivity and pathogenicity.
7. Prospects

This work is by no means conclusive. Each new experiment, although answering or confirming the questions which they proposed to cover, also brought forwards new fields to be explored. Several have been mentioned in the respective Chapters.

Studies on the real variability of influenza virus strains by passaging, using different culture systems, should be undertaken, to permit a correct choice of culture systems for influenza diagnosis. Molecular biology should be used to distinguish small, but important, variations affecting virus-host cell affinity.

The various growth patterns of influenza viruses in culture systems need further studies, to elucidate the conditions under which they occur and their implications.

The possibility of latent infection with influenza virus in eggs and cell cultures, or vertical transmission of the virus, should be defined to permit precise diagnosis of the disease.

To define the cleavability of the H of the virus strains tested, further studies including amino acid sequencing of the H gene will be useful, permitting the characterisation of the cleavage site and range of enzymes able to cleave the H, and consequently the range of host cells by which these particular virus strains may be activated.
Another point that needs further study is the heterogenic size of plaques formed by the virus population under study. Cloning of these plaques, and studies on the causes of the different size observed, by modern technology, may contribute to the definition of other pathogenicity factors than those already known.

Influenza spread by means of infected pig products is unlikely when legislation on the subject is strictly followed. But clandestine slaughter, often practised on family-run farms in less developed geographical areas may spread the virus to the whole community and their livestock, by the practise of sharing the slaughter labour and processing of the meat. Subproducts are often used to feed dogs and cats, and the carcasses left to birds of prey. The latter may be responsible for taking the virus to more distant places. This is only a theory, and influenza disease spread through carcasses in the wild requires further studies, perhaps by virological and serological surveys on wild mammals and prey birds which come in close contact with humans.

Although the present studies demonstrated that interspecies transmission of the virus strains tested was possible, no clear-cut picture was obtained with reference to conditions necessary for this transmission, and direct nucleotide sequencing of the various viral genes involved in host and cell affinity, principally H and NP, are required, to permit more substantial results. This would also produce
more evidence concerning the role of pigs as "mixing vessel" for the virus.

The serological studies on porcine influenza carried out in Brazil demonstrated that the virus has been circulating in the herds. Further studies are required to isolate the virus and study its involvement in disease, as well as its incidence and origin.

Studies on HI antibodies alone may not be sufficient when carrying out serological surveys, and speaking generally, it became evident that a more precise monitoring of different leukocyte subpopulations, such as T-cells, will be of value for the study of pathogenesis of diseases in pigs. Monoclonal antibody analysis of the virus strains would contribute in detecting alterations in neutralising sites, and other changes that would affect host immunity.

The serological evidence of influenza C in English pigs needs further studies, principally related to the incidence of such an influenza virus and the significance in disease production.

Urgency for amino acid sequencing of English pig influenza isolates was clear by the results obtained in the studies on reassortant viruses. Without a detailed picture of the amino acid sequences of the genes of the virus strains under study, all findings are mere speculation about their origin and very few conclusive answers can be given.

As no certainty is available about which genes are
involved in influenza virus pathogenicity, all genes should be mapped and differences between strains analysed.

There is one aspect so far ignored as a possible transmission and reassortment source of influenza viruses: fresh water fish.

The rapid increase in human population in the developing world demands a major expansion in food production. Combining aquaculture with agriculture, fish-farming projects are being supported in various countries because they are labour intensive and provide low-cost fish feeds and pond fertilisers. In Brazil, integrated farming and aquaculture systems involving fowl, livestock and fish are being actively encouraged. In such systems, both excess food from farm animals and their faecal material are either consumed directly by fish or act as pond fertilisers. Adoption of these recommendations will result in increased co-location of pigs, humans, ducks, and fish concentrated in artisanal aquaculture industries, in a number of areas throughout the world (Scholtissek and Naylor, 1988). Fish maintained in water in contact with other animals infected with influenza may be infected and transmit the virus. Although no influenza outbreaks have been described among fish, Klenk (1973) reported that at temperatures lower than 25°C, the glycoprotein H accumulates in the cell and no cleavage occurs. When the temperature rises to 37°C the amount of H decreases and H₁ and H₂ can be detected, showing
that an optimum temperature is necessary for H cleavage to occur and consequently permit infection. These findings suggest that the lower body temperature in fish may prevent them from being actively infected, but that they may act as reservoirs and transmit the virus to other species with optimum body temperature for virus replication and H cleavage. Further epidemiological studies on influenza in areas where association between several cultures is practised should be considered a priority.
It appears that influenza A virus subtypes follow the universal pattern of species evolution. They are produced by random variability while selective environmental pressures favour certain variants, and eliminate others less suited to the particular environmental niche. Their zoonotic character, together with their capacity for reassortment, are the key-points in understanding the epidemiology of influenza.

Growth of the influenza virus and/or its ability to infect cells depends on viral genetic characters correlated with virulence, may depend on factors such as specific pH and temperature, and alterations in these factors may inhibit cytopathogenicity of the virus. The embryonated egg is still the most stable culture system available, and no cell culture substitute was found that could replace them totally. Nonetheless, MDCK cells were found to be suitable for primary isolation, when large numbers of samples had to be tested, and also appropriate to be used with the IPX technique developed.

Efficient diagnostic procedures for influenza virus infections have not yet been developed for pathogenicity
determination. Only very sophisticated techniques analysing each of its genes may produce a true picture of the virus under study, and only if assumed that no variation took place in the period during which the analysis is carried out.

The immunoperoxidase technique tested in this study was validated as a diagnostic tool for influenza infection, as it gave a correct diagnosis in all the samples tested. After some improvements, it may be possible to read the results even by naked eye. Using the same technique, porcine influenza antigens, as well as their antibodies in sera may be quickly identified for large surveys, even more quickly when microplates are used. This technique, in comparison to HA and HI tests, merits further studies.

It is possible that the events causing influenza spread in the human and animal populations are linked in that the same factors control the development of epidemics of influenza infections in all species. Also, the virus may be transmitted from one reservoir species to the other. Some porcine influenza virus strains may be spread actively, through infected pig tissues or clinically healthy pigs carrying the virus, to other populations (human, other mammals, avian) with or without producing disease and/or seroconversion. Other strains may be spread passively, through infected material and aerosol. Birds, picking up the virus at one site may carry it in their intestinal
tracts to distant places. This may be possible as studies in recent years have suggested that while influenza A viruses do not pass to and from man to other animals with complete freedom, under some conditions such transmission does occur (Alexander, 1982a; Kaplan, 1982; Bachmann, 1983; Oxford, 1987; Shortridge, 1988).

The major findings of the serological studies in this work are two-fold. Firstly, the known range of porcine H1N1 and H3N2 influenza virus has been extended, to include a large area of Brazil. Antibodies against Wey/87 were detected in pigs of all areas studied. Secondly, the presence of antibodies in pigs of influenza viruses circulating in human populations in the 1970s indicates probable interspecies exchange of viruses and the persistence of influenza virus among the pig population.

It should be emphasised that these results refer only to a point prevalence study of HI antibodies. Further studies are required to investigate the temporal evolution of influenza virus infection of pigs in Brazil, and involving a number of biological assays including NA and neutralisation.

These studies confirm those by Roberts and others (1987) that influenza viruses of the H3N2 subtype continue to circulate in pigs in England many years after their antigenic counterparts have disappeared from humans. This
had been demonstrated earlier in Great Britain with the A/Hong Kong/68 (H3N2) virus (Harkness and others, 1972; Chapman and others, 1978). These viruses may be reassortants but will require further studies to substantiate this possibility. If it could be proved that influenza viruses present in pigs are of avian origin, adaptation to replicate in humans by reassortment in pigs would explain the periodical resurgence of some influenza strains in humans, after long periods of absence. From the results obtained so far, it appears that the H1N1 virus strain is more closely related to avian strains, since it was more able to infect turkeys than the H3N2 strain.

Further studies would include the determination of the relatedness of influenza virus strains isolated from pigs, turkeys and migratory birds in the area under study, and the seasonality of these outbreaks.

A continuous surveillance of influenza virus infection among pigs is recommended, principally to study if their pathogenicity for the various susceptible hosts remains unchanged.
REFERENCES


ADA GL & JONES PD (1986) The immune response to influenza infection. Current Topics in Microbiology and Immunology, 128: 1-54


303


ANDREWES CH, LAIDLAW PP & SMITH W (1935) Influenza: observations on the recovery of virus from man and on the antibody content of human sera. British Journal of Experimental Pathology, 16:566-582


ARCHETTI I & HORSFALL FL, Jr (1950) Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. Journal of Experimental Medicine, 92:441-462


BARRETT ADT & DIMMOCK NJ (1986) Defective interfering viruses and infections of animals. Current Topics in Microbiology and Immunology, 128:53-84

BARRY RD (1964) The effects of Actinomycin D and ultraviolet irradiation on the production of fowl plague virus. Virology, 24:563-569


BUTTERFIELD WK, CAMPBELL CH, WEBSTER RG & SHORTRIDGE KF (1978b) Identification of a swine influenza virus (HswlN1) isolated from a duck in Hong Kong. The Journal of Infectious Diseases, 138(5):686-689


CHAPMAN MS, LAMONT PH & HARKNESS JW (1978) Serological evidence of continuing infection of swine in Great Britain with an influenza A virus (H3N2). Journal of Hygiene (Cambridge), 80:415-422


COMPANS RW (1973) Influenza virus proteins. II. Association with components of the cytoplasm. Virology, 51:56-70


DAVENPORT FM (1977) Reflections on the epidemiology of myxovirus infections. Medical Microbiology and Immunology, 164:69-76


309


DRZENIEK R (1972) Viral and bacterial neuraminidases. Current Topics in Microbiological Immunology, 59:35-74


310


EASTERTDAY BC & TUMOVA B (1972b) Avian influenza viruses in avian species and the natural history of influenza. Advances in Veterinary Science and Comparative Medicine, 16:201-222


311


FRANK AL, COUCH RB, GRIFFIS CA & BAXTER ED (1979) Comparison of different tissue cultures for isolation and quantitation of influenza and parainfluenza. Journal of Clinical Microbiology, 10:32-36


FROMHAGEN LH, KNIGHT CA & FREEMAN NK (1959) The ribonucleic acid, lipid and polysaccharide constituent of influenza virus preparation. Virology, 8:176-197


GREEN IJ, LIEBERMAN M & MOGABGAB WJ (1957) The behavior of influenza virus in various tissue culture systems. Journal of Immunology, 78:233-239

GESSER I, TOVEY MG, MAURY C & RANDU MT (1976) Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. Journal of Experimental Medicine, 144:1316-1323


HANNOUN C & DEVAUX JM (1980) Circulation enzootique permanente de virus grippeaux dans la baie de la somme. Comparative Immunology, Microbiology and Infectious Diseases, 3:177-183

HANNOUN C & COURREAU JM (1980) Surveillance de la grippe chez les porcs sains. Comparative Immunology, Microbiology and Infectious Diseases, 3:133-136


313


HENLE W & LIU OC (1951) Studies of host-virus interactions in the chick embryo influenza virus system. VI. Evidence for multiplicity reactivation. Journal of Experimental Medicine, 94:305-322

HERS JFP (1962) Fluorescent antibody technique in respiratory viral diseases. Research in Respiratory Diseases, 88:316-332


HINSHAW VS, WEBSTER RG & RODRIGUEZ J (1981d) Influenza A viruses: Combinations of hemagglutinin and neuraminidase subtypes isolated from animals and other sources. Archives of Virology, 67:191-206


HINSHAW VS, WEBSTER RG & TURNER B (1979) Water-borne transmission of Influenza A viruses? Intervirology, 11:66-68

HIRST GK (1941) Agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. Science, 94:22-23

HIRST GK (1942) Adsorption of influenza haemagglutinin and virus by red blood cells. Journal of Experimental Medicine, 76:195-209
HIRST GK (1948) The nature of the virus receptors of red cells. II The effect of partial inactivation of influenza virus on the destruction of red cell receptors and the use of inactivated virus in the measurement of serum inhibitors. Journal of Experimental Medicine, 87:315-328


HOISTON JL & DOXIE WR (1973) Influenza A neuraminidase antibody assay with sensitized erythrocytes. Applied Microbiology, 25:97-102


316


KÄRBER G von (1931) Beitrag zur kolletiven Behandlung pharmakologischer Reiherversuche. Archiv für Experimentelle Pathologie und Pharmakologie, 162:480-483

KASEL JA, FULK RV & COUCH RB (1969) Antigenic relationship between the equine and the Hong Kong human variant of influenza type A2 virus. Journal of Immunology, 102:530-532

KATES M, ALLISON AC, TIRRELL DAJ & JAMES AT (1961) Lipids of influenza virus and their relation to those of the host cell. Biochimica et Biophysica Acta, 52:455-466


KOEN JS (1928) Swine "flu". American Journal of Veterinary Medicine, 23:457-459


KOEP JV, KEMPF JE & KROEGER AV (1968) Cytoplasmic inclusions observed by electron microscopy late in influenza virus infection of chicken embryo fibroblasts. Virology, 36:681-683

KOSKINEN P, VUORINEN T & MEURMAN O (1987) Influenza A and B virus IgG and IgM serology by enzyme immunoassays. Epidemiology and Infection, 99(1):55-64

KOVALCHUK-IVANYUK TB, ROGENLY EG & URIN AI (1975) Search for influenza foci associated with wild and domestic birds. Ekologie of viruses, 3:77-79

KRUG RM (1972) Cytoplasmic and nucleoplasmic viral RNPs in influenza virus infected MDCK cells. Virology, 50:103-113


321


MASUREL N, DE BOER GF, ANKER WJJ & HUFFELS ADNHJ (1983) Prevalence of influenza viruses A-H1N1 and A-H3N2 in swine in the Netherlands. Comparative Immunology, Microbiology and Infectious Diseases,6:141-149


323


MENSIK J (1959) [Intra-uterine infection and persistence of swine influenza virus in the organism of infected piglets and sows.] Sbornik Ceskoslovenske Academie Zemedelskych Veterinarni Medicina, 4:797-806

MENSIK J (1962) [Experimental infection of pregnant sows with influenza suis virus. I. Proof of virus in placental tissue and in organs of newborn piglets.] Vedecke Prace - Vyzkumnho Ustavu Veterinarnho Lekanstvi v Ume, 2:31-47


MILLER GL & STANLEY WM (1944) Quantitative aspects of the red blood cell agglutination test for influenza viruses. Journal of Experimental Medicine, 79:185


MogaGBaG WC SIMPSON GI & GREEN IJ (1956) Growth characteristics and
cytotoxic effects of influenza A and B in cultures of human
embryo tissue. Journal of Immunology, 76:314-327

Serologic and epidemiologic evidence of infection in turkeys with an
agent related to the swine influenza virus. Avian Diseases, 25:11-26

Isccon, a novel structure for antigenic presentation of membrane

MOSS BA & BROWNLEE G (1981) Sequence of DNA complementary to a small
RNA segment of influenza virus A/NT/60/68. Nucleic Acids
Research, 9:1941-1947

auftreten der Schweineinfluenza in Norddeutschland. Der Praktische
Tierarzt, 8:669-673

growth behavior of human, swine, equine and avian influenza viruses
at a high temperature. Archives of Virology, 100:231-244

MURPHY BR, BARON S, CHAEHQB EG, IHEENDCRF CP & CHANOCK RM (1973)
Temperature-sensitive mutants of influenza virus. IV. Induction of
interferon in the naso-pharynx by wild-type and a
temperature-sensitive recombinant virus. Journal of Infectious
Diseases, 128:488-493

MURPHY BR, KASEL JA & CHANOCK RM (1972) Association of serum
antineuraminidase antibody with resistance to influenza in man. New
England Journal of Medicine, 286:1329-1332

B and others(ed), Raven Press, New York.

MURPHY JS & BANG FB (1952) Observations with the electron microscope on
cells of chick allantoic membrane infected with influenza virus.
Journal of Experimental Medicine, 95:259

NAEVE CW, HINSHAW VS & WEBSTER RG (1983) Phenotypic variation in
influenza virus reassortants with identical gene constellations.
Virology, 128:331-340

NAIRN RC (1976) Fluorescent protein tracing. 4 ed. Livingstone,
Edinburgh. p.369

in-vitro the glycoproteins of influenza and sendai viruses. Sixth
International Congress of Virology, 1-7 September, Sendai, Japan.


327


POSPIŠIL Z, MENSÍK J, TUMOVA B, STUMPA A & ČERNÝ M (1973) Experimental infection of colostrum-deprived, specific-pathogen free piglets with A/Hong Kong (H3N2) influenza virus. Zentralblatt für Veterinarmedizin, B,20:139-152


Pritchard GC, DICK IGC, WIBBERLEY G & ROBERTS DH (1987) Porcine influenza outbreak in East Anglia due to influenza A virus (H3N2). Veterinary Record,121:548

RICHARDSON CD, SCHEID A & CHOPPIN PW (1980) Specific inhibition of paramyxoviruses and myxoviruses replication by oligopeptides with amino acid sequences similar to those at the N-termini of the F1 or HA2 viral polypeptides. Virology,105:205-222


ROMARY J & VIZY L (1971) [Infection of pig herds with swine influenza and human influenza A-2 (Hong Kong) viruses.] Magyar Allatorvosok Lapja, January:49-51

ROSENBERGER JK, KRAUSS WC & SLEMONS RD (1974) Isolation of Newcastle disease and type A influenza viruses from migratory waterfowl in the Atlantic flyway. Avian Diseases,18:610-613

329


330


SCHILD GC, NEWMAN RW, WEBSTER RG, MAYOR D & HINshaw VS (1980) Antigenic analysis of influenza A virus surface antigens: considerations for the nomenclature of influenza virus. Comparative Immunology, Microbiology and Infectious Diseases, 3:5-18


331


SHELTON A, VOGEL JE & CHI L (1958) Hemadsorption (adsorption hemagglutination) test for viral agents in tissue culture with special reference to influenza. Proceedings of Experimental Biology and Medicine, 97:802-809

SHOEPE RE (1931) Swine influenza. III. Filtration experiments and etiology. Journal of Experimental Medicine, 54:373-385


SHOEPE RE (1938) Serological evidence for the occurrence of infection with human influenza virus in swine. Journal of Experimental Medicine, 67:739-748


333

SHORIRIDGE KF & WEBSTER RG (1979) Geographical distribution of swine (Hsw1N1) and Hong Kong (H3N2) influenza virus variants in pigs in Southeast Asia. Intervirology, 11:9-15


SMITH W, ANDREWS CH & IAILAW PP (1933) A virus obtained from influenza patients. Lancet,2:66-68


STUART HARRIS CH (1937) Influenza virus infection of rats and guinea-pigs. British Journal of Experimental Pathology,18:485-492

STUART HARRIS CH & SCHILD GC (1976) Influenza, the Viruses and the Disease, Edward Arnold Ltd., London.


SUGIURA A (1972) Influenza viral RNA and ribonucleoprotein synthesized in abortive infection. Virology,47:517-520


335


TAYLOR AR, SHARP DG, BEARD D, BEARD JW, DINGLE JH & FELLER AE (1943) Isolation and characterization of influenza A virus (PR8 strain). Journal of Immunology, 47:261


336


TOMÁVÁ B, ŠVANDČÍKA E & STUMPA G (1968) Findings of antibodies to animal influenza viruses in human sera and their significance for the study of interval antigenic relationship. Journal of Hygiene, Epidemiology, Microbiology and Immunology, 12:284-295


337
untersuchung zur vertreibung des Schwei m inf 1  uenza
virus in Belgien. Vlaams Diergeneeskunde Tijdschrift,49:1-7

of influenza A and B infections by detection of isotypic antibody
response. Sixth International Congress of Virology, 1-7 September,
Sendai, Japan.

Van deisen RA, Hinshaw VS, Senne DA & Pellacani D (1983)
Micro-neuraminidase-inhibition assay for classification of influenza
A virus neuraminidases. Avian Diseases,27(3):745-750

between influenza viruses of different geographic regions. Archives
of Virology,76:63-67

Van Nieuwenhuyze JL (1983) Mecanismes immunologiques de defense contre le
virus influenza. In: La grippe: grippe humaine, grippes animales,
les vaccins. Collection Fondation Marcel Merieux. pp.17-20

antigenic determinants of influenza virus haemagglutinin. II.
Original antigenic sin: a bone-marrow derived lymphocyte memory
phenomenon modulated by thymus-derived lymphocytes. Journal of
Experimental Medicine,140:1571

immunity in host defence against influenza A in mice. Postgraduate
Medical Journal,52:332-337

Vogel I & Shlekov A (1957) Adsorption-hemagglutination test for
influenza virus in Monkey Kidney tissue culture. Science,126:358-359

Von Magnus P (1954) Incomplete forms of influenza virus. Advances in
Virus Research,2:59-79

Wallace GD (1977) Swine influenza and lungworms. Journal of Infectious
Diseases,135:490-492

Wallace GD (1979) Natural history of influenza in swine in Hawaii:
prevalence of infection with A/HK/68(H3N2) subtype virus and its
Research,40:1165-1168

Walsh JJ, Diehllein LF, Low PN, Burch GE & Mogabgab WJ (1961)
Bronchotracheal response in human influenza. Archives of
International Medicine,108:376-388

338


WEBSTER RG, HINSHAW VS & BEAN WJ (1977a) Antigenic shift in myxoviruses. Medical Microbiology and Immunology, 154:57-68


WELLS RJH (1963) An outbreak of fowl plague in turkeys. Veterinary Record, 75:783-786


YASUHARA H, HIRAHARA T & NAKAI M (1983) Further isolation of a recombinant virus (H1N2, formerly H5W1N2) from a pig in Japan in 1980. Microbiology and Immunology, 27:43-50


Appendix I: HA activity of allantoic and amniotic fluids harvested from chick embryos, inoculated with infected meat suspensions on days 0, 1, 2, 3, 5, 8 and 15.

### Meat kept at 4°C:

<table>
<thead>
<tr>
<th>Dilutions into eggs</th>
<th>Virus dilutions inoculated into meat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^0</td>
</tr>
<tr>
<td>10^0 allantoic amniotic</td>
<td>+</td>
</tr>
<tr>
<td>10^-1 allantoic amniotic</td>
<td>+</td>
</tr>
<tr>
<td>10^-2 allantoic amniotic</td>
<td>-</td>
</tr>
<tr>
<td>10^-3 allantoic amniotic</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virus dilutions inoculated into meat</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^0</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>+</td>
</tr>
</tbody>
</table>

### Meat kept at -20°C:

<table>
<thead>
<tr>
<th>Dilutions into eggs</th>
<th>Virus dilutions inoculated into meat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^0</td>
</tr>
<tr>
<td>10^0 allantoic amniotic</td>
<td>NT</td>
</tr>
<tr>
<td>10^-1 allantoic amniotic</td>
<td>-</td>
</tr>
<tr>
<td>10^-2 allantoic amniotic</td>
<td>+</td>
</tr>
<tr>
<td>10^-3 allantoic amniotic</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virus dilutions inoculated into meat</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^0</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>(+)</td>
</tr>
</tbody>
</table>

### Notes:
- NT = not tested (contaminated)
- + = positive results
- (+) = second passage results
- - = negative results
### Appendix I: (Cont.)

#### Meat kept at 4°C:

<table>
<thead>
<tr>
<th>Dilutions into eggs</th>
<th>Day 03</th>
<th>Day 05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus dilutions inoculated into meat</td>
<td>Virus dilutions inoculated into meat</td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>10⁻¹</td>
</tr>
<tr>
<td><strong>10⁰ allantoic amniotic</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>10⁻¹ allantoic amniotic</strong></td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td><strong>10⁻² allantoic amniotic</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>10⁻³ allantoic amniotic</strong></td>
<td>-</td>
<td>(+)</td>
</tr>
</tbody>
</table>

**NT** = not tested (contaminated)

#### Meat kept at -20°C:

<table>
<thead>
<tr>
<th>Dilutions into eggs</th>
<th>Day 03</th>
<th>Day 05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus dilutions inoculated into meat</td>
<td>Virus dilutions inoculated into meat</td>
</tr>
<tr>
<td></td>
<td>10⁰</td>
<td>10⁻¹</td>
</tr>
<tr>
<td><strong>10⁰ allantoic amniotic</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>10⁻¹ allantoic amniotic</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>10⁻² allantoic amniotic</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>10⁻³ allantoic amniotic</strong></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**NT** = not tested (contaminated)

+ = positive results

(+) = second passage results

- = negative results
### Appendix I: (Cont).

#### Meat kept at 4°C:

<table>
<thead>
<tr>
<th>Dilutions into eggs</th>
<th>Day 08</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>virus dilutions inoculated into meat</td>
<td>virus dilutions inoculated into meat</td>
</tr>
<tr>
<td></td>
<td>$10^0$</td>
<td>$10^{-1}$</td>
</tr>
<tr>
<td>$10^0$ allantoic amniotic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-1}$ allantoic amniotic</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-2}$ allantoic amniotic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-3}$ allantoic amniotic</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

#### Meat kept at -20°C:

<table>
<thead>
<tr>
<th>Dilutions into eggs</th>
<th>Day 08</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>virus dilutions inoculated into meat</td>
<td>virus dilutions inoculated into meat</td>
</tr>
<tr>
<td></td>
<td>$10^0$</td>
<td>$10^{-1}$</td>
</tr>
<tr>
<td>$10^0$ allantoic amniotic</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-1}$ allantoic amniotic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$10^{-2}$ allantoic amniotic</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>$10^{-3}$ allantoic amniotic</td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

**NT = not tested (contaminated)**

$+$ = positive results  
$+$ = second passage results  
$-$ = negative results
Appendix II:

Results of haemagglutination inhibition tests on sera from turkeys of experiment I and II – presence of influenza virus specific antibodies.

<table>
<thead>
<tr>
<th>virus tested</th>
<th>Experiment I</th>
<th></th>
<th></th>
<th></th>
<th>Experiment II</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>07</td>
<td>14</td>
<td>21</td>
<td>28</td>
<td>07</td>
<td>11</td>
<td>17</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Turkey</td>
<td>Swine</td>
<td>Turkey</td>
<td>Swine</td>
<td>Turkey</td>
<td>Swine</td>
<td>Turkey</td>
<td>Swine</td>
<td>Turkey</td>
</tr>
<tr>
<td>326</td>
<td></td>
<td>0*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>501</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>327</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>502</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>328</td>
<td></td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>503</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>329</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>504</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>330</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>505</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>331</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>506</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>332</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>507</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>333</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>508</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>334</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>509</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>335</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>510</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>678</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>511</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>679</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>512</td>
<td>0</td>
<td>160</td>
</tr>
</tbody>
</table>

* = HI titres lower than or equal to 1/10

** = reciprocal HI values

NT = not tested
## Appendix II: (Cont.)

<table>
<thead>
<tr>
<th>Day</th>
<th>Virus Tested</th>
<th>Experiment I</th>
<th>Experiment II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>07</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Turkey</td>
<td>Swine</td>
<td>Turkey</td>
</tr>
<tr>
<td>336</td>
<td>0*</td>
<td>0</td>
<td>640</td>
</tr>
<tr>
<td>337</td>
<td>0</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>338</td>
<td>0</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>339</td>
<td>0</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>340</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>341</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>342</td>
<td>0</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>343</td>
<td>0</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>344</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>345</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>676</td>
<td>0</td>
<td>0</td>
<td>160</td>
</tr>
<tr>
<td>677</td>
<td>0</td>
<td>0</td>
<td>160</td>
</tr>
</tbody>
</table>

* = HI titres lower than or equal to 1/10  
** = reciprocal HI values  
NT = not tested
Appendix III: Antibody level detection in sera of animals taking part in experiments 1 and 2 (HI response)

<table>
<thead>
<tr>
<th>Exp 1</th>
<th>H1N1**</th>
<th>H3N2</th>
<th>Exp 2</th>
<th>H1N1</th>
<th>H3N2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0</td>
<td>9</td>
<td>16</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>A turkeys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0*</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>40</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>40</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0</td>
<td>20</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>control pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>contract pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>control turkeys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>NT</td>
<td>0</td>
</tr>
</tbody>
</table>

* 0 = HI titres ≤10
all values represent the reciprocal HI titre
NT = not tested
** all sera were tested against the H1N1 and H3N2 porcine strain
Appendix III (Cont).

<table>
<thead>
<tr>
<th>Exp 1</th>
<th>(H1N1^{**})</th>
<th>(H3N2)</th>
<th>Exp 2</th>
<th>(H1N1)</th>
<th>(H3N2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0 9 16</td>
<td>0 9 16</td>
<td>0 11 14 18 25 32</td>
<td>0 11 14 18 25 32</td>
<td></td>
</tr>
<tr>
<td>B turkeys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0* 0 0</td>
<td>0 80 20</td>
<td>0 NT 0</td>
<td>0 NT 80</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 NT 0</td>
<td>0 NT 40</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0 0 0</td>
<td>0 80 20</td>
<td>0 NT 0</td>
<td>0 NT 40</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0 0 0</td>
<td>0 80 20</td>
<td>0 NT 0</td>
<td>0 NT 40</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0 0 0</td>
<td>0 80 0</td>
<td>0 NT 10</td>
<td>0 NT 40</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0 0 0</td>
<td>0 0 10</td>
<td>0 NT 0</td>
<td>0 NT 40</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0 0 0</td>
<td>0 0 10</td>
<td>0 NT 0</td>
<td>0 NT 40</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0 0 0</td>
<td>0 40 10</td>
<td>0 NT 0</td>
<td>0 NT 80</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>NT NT 0</td>
<td>NT 40 0</td>
<td>0 NT 0</td>
<td>0 NT 40</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 NT 0</td>
<td>0 NT 80</td>
<td></td>
</tr>
<tr>
<td>control pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 0 20</td>
<td>0 0 10</td>
<td>80 10 NT 10 0 0</td>
<td>0 20 NT 20 0 0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0 0 0</td>
<td>0 0 10</td>
<td>80 0 NT 20 0 0</td>
<td>0 0 NT 20 0 0</td>
<td></td>
</tr>
<tr>
<td>contact pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 0 0</td>
<td>10 10 10</td>
<td>40 0 NT 10 0 0</td>
<td>0 10 NT 10 0 0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0 0 10</td>
<td>10 10 10</td>
<td>80 20 NT 10 0 0</td>
<td>0 40 NT 10 0 0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0 0 20</td>
<td>20 20 20</td>
<td>40 10 NT 40 0 0</td>
<td>0 20 NT 40 0 0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0 0 20</td>
<td>20 20 10</td>
<td>40 0 NT 40 0 0</td>
<td>0 10 NT 80 0 0</td>
<td></td>
</tr>
<tr>
<td>contact turkeys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 NT NT NT 0 0</td>
<td>0 NT NT NT 40 0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 NT NT NT 0 0</td>
<td>0 NT NT NT 0 0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 NT NT NT 0 0</td>
<td>0 NT NT NT 40 0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 NT NT NT 0 0</td>
<td>0 NT NT NT 0 0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0 0 0</td>
<td>0 0 0</td>
<td>0 NT NT NT 0 0</td>
<td>0 NT NT NT 0 0</td>
<td></td>
</tr>
</tbody>
</table>

* 0 = HI titres \(\leq 10\)

all values represent the reciprocal HI titre

NT = not tested

** all sera were tested against the H1N1 and H3N2 porcine strain
Appendix IV: Serological and diagnostic results of sera and nasal swabs of pigs from farm 73 (HI, NI, SRID and egg inoculation).

<table>
<thead>
<tr>
<th>No</th>
<th>Reciprocal HI titres</th>
<th>NI test</th>
<th>SRID</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1N1</td>
<td>H1N2</td>
<td>H3N2</td>
<td>N1</td>
</tr>
<tr>
<td>Abbatoir Pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>0</td>
<td>2560</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>0</td>
<td>640</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0*</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>+/-</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>80</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>40</td>
<td>80</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>640</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>80</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>640</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>20</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>80</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>23</td>
<td>20</td>
<td>320</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
</tbody>
</table>

0 = negative results
+ = positive results
+/- = weak positive results
* = all HI titres ≤ 10 were recorded as negative
Appendix IV (Cont).

<table>
<thead>
<tr>
<th>No</th>
<th>H1N1</th>
<th>H1N2</th>
<th>H3N2</th>
<th>NI test</th>
<th>SRID</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>40</td>
<td>0</td>
<td>640</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>27</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>29</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>31</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td></td>
<td>H3</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>33</td>
<td>0</td>
<td>0</td>
<td>640</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>34</td>
<td>0</td>
<td>0</td>
<td>320</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>35</td>
<td>20</td>
<td>0</td>
<td>2560</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>36</td>
<td>0</td>
<td>0</td>
<td>640</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>37</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>38</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>39</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>41</td>
<td>0</td>
<td>0</td>
<td>320</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>42</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>43</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>44</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>45</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>46</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>47</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>0</td>
<td>640</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>49</td>
<td>40</td>
<td>0</td>
<td>640</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
<tr>
<td>50</td>
<td>160</td>
<td>0</td>
<td>320</td>
<td>0</td>
<td>+</td>
<td>H3</td>
</tr>
</tbody>
</table>

0 = negative results
+ = positive results
+/- = weak positive results
* = all HI titres ≤ 10 were recorded as negative
Appendix IV:(Cont).

<table>
<thead>
<tr>
<th>No</th>
<th>Reciprocal HI titres</th>
<th>NI test</th>
<th>SRID</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H1N1</td>
<td>H1N2</td>
<td>H3N2</td>
<td>N1</td>
</tr>
<tr>
<td>51</td>
<td>0</td>
<td>0</td>
<td>2560</td>
<td>0</td>
</tr>
<tr>
<td>52</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>53</td>
<td>80</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>54</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>55</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>56</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>57</td>
<td>0</td>
<td>320</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>58</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>59</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>80</td>
<td>0</td>
<td>160</td>
<td>0</td>
</tr>
<tr>
<td>61</td>
<td>20</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>62</td>
<td>0</td>
<td>0</td>
<td>160</td>
<td>0</td>
</tr>
<tr>
<td>63</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>64</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>65</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>66</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>67</td>
<td>0</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>68</td>
<td>0</td>
<td>0</td>
<td>640</td>
<td>0</td>
</tr>
<tr>
<td>69</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>70</td>
<td>0</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>71</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
<td>0</td>
<td>2560</td>
<td>0</td>
</tr>
<tr>
<td>73</td>
<td>20</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>74</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>75</td>
<td>0</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>76</td>
<td>0</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>77</td>
<td>0</td>
<td>0</td>
<td>320</td>
<td>0</td>
</tr>
<tr>
<td>78</td>
<td>40</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>79</td>
<td>0</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>160</td>
<td>0</td>
<td>1280</td>
<td>0</td>
</tr>
</tbody>
</table>

0 = negative results  
+ = positive results  
+/− = weak positive results  
* = all HI titres < 10 were recorded as negative
Appendix IV: (Cont).

<table>
<thead>
<tr>
<th>No</th>
<th>Reciprocal HI titres</th>
<th>NI test</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1N1</td>
<td>H1N2</td>
<td>H3N2</td>
</tr>
<tr>
<td>81</td>
<td>0*</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>82</td>
<td>0</td>
<td>0</td>
<td>1280</td>
</tr>
<tr>
<td>83</td>
<td>640</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>84</td>
<td>640</td>
<td>0</td>
<td>1280</td>
</tr>
<tr>
<td>85</td>
<td>0</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>86</td>
<td>20</td>
<td>0</td>
<td>640</td>
</tr>
<tr>
<td>87</td>
<td>640</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>88</td>
<td>320</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>89</td>
<td>640</td>
<td>0</td>
<td>320</td>
</tr>
<tr>
<td>90</td>
<td>320</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>91</td>
<td>640</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>92</td>
<td>640</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>93</td>
<td>320</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>94</td>
<td>640</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>95</td>
<td>320</td>
<td>0</td>
<td>1280</td>
</tr>
<tr>
<td>96</td>
<td>640</td>
<td>0</td>
<td>640</td>
</tr>
<tr>
<td>97</td>
<td>320</td>
<td>0</td>
<td>2560</td>
</tr>
<tr>
<td>98</td>
<td>320</td>
<td>0</td>
<td>2560</td>
</tr>
</tbody>
</table>

0 = negative results  
+ = positive results  
+/− = weak positive results  
* = all HI titres ≤ 10 were recorded as negative