PROCEEDINGS OF
THE JOINT FIRST ANNUAL MEETINGS OF THE NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES OF THE EUROPEAN COMMUNITIES

HELD IN BRUSSELS,
5-6TH OCTOBER 1993

Edited by Dennis J. Alexander

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JOINT ANNUAL MEETINGS OF EEC NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES 1993

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	Dr. Jim Moynagh

AGENDA

Tuesday 5th October 1993

09.30: Welcome and introduction.

09.45: SESSION I COUNTRY REPORTS

a: AVIAN INFLUENZA

1. Belgium/Luxembourg
2. Spain
3. Portugal
4. Denmark
5. The Netherlands
6. Greece
7. France
8. Ireland
9. Germany
10. Italy
11. United Kingdom
12. Sweden
13. Finland
14. Austria

b. NEWCASTLE DISEASE

1. United Kingdom
2. Greece
3. Denmark
4. Ireland
5. Spain
6. Italy
7. Sweden
8. Finland
9. Austria
10. France

12.45: LUNCH

14.00: SESSION I continued

11. Germany
12. The Netherlands
13. Belgium/Luxembourg
14. Portugal

15.00: EC RESEARCH FUNDING *J. Connell*

15.10: SESSION II EC REPORTS

15.10: Report from the Commission on Newcastle disease and avian influenza in the European Community. *Jim Moynagh*

15.30: SHORT BREAK

15.45: Report of the EC Reference Laboratory for avian influenza. *Dennis Alexander*

16.00: Report of the EC Reference Laboratory for Newcastle disease. *Dennis Alexander*

16.30: SESSION III - NATIONAL LABORATORY FACILITIES

Wednesday 6th October 1993

09.30: PRELIMINARY DISCUSSION
Proposals of topics to be discussed in SESSION V

09.50: SESSION IV - ORIGINAL CONTRIBUTIONS

09.50: Blocking ELISA to detect group-specific antibodies against influenza virus. *Guus Koch*

10.10: Questions raised by the highly pathogenic influenza outbreak in Norfolk in 1991. *Dennis Alexander*

10.30: Direct identification and characterization of PMV1 from suspicious organs by Nested PCR and automatic sequencing. *Veronique Jestin*

10.50: Differentiation of NDV strains of different virulence: recent developments *Guus Koch*

11.10: SHORT BREAK

11.30: Assessment of the molecular basis of virulence of the "pigeon variant PMV1 virus". *Dennis Alexander*

11.45: VIDEOFILM: "Two of a kind: avian influenza and Newcastle disease" - film produced by CSIRO Australian Animal Health Laboratory in the series Recognising Exotic Livestock Diseases.

12.05: VIDEOFILM: Newcastle disease in The Netherlands *Jan Cees van den Wijngaard*

12.30: LUNCH

13.45: SESSION V - CONCLUSIONS AND RECOMMENDATIONS

This session will be a general discussion of the current situation in Europe of the two diseases and the ability of the existing legislation and diagnostic facilities to bring about their control and eradication. Finally, recommendations will be made which could concern any aspect of control, diagnosis, disease reporting, the roles of the National and Reference Laboratories or the future format of the annual meeting of the Laboratories.

15.15: CLOSING REMARKS

15.30: CLOSE

THE JOINT FIRST ANNUAL MEETINGS OF THE NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES OF THE EUROPEAN COMMUNITIES

WELCOME AND INTRODUCTION

By Jorgen Westergaard for the Commission

Dr. Westergaard welcomed the participants to Brussels and to the Joint Meeting of the EC National Laboratories for Newcastle disease and Avian influenza. He particularly welcomed those participants representing EFTA countries.

He pointed out how opportune it was to hold the meeting just a few days after Directive 92/66/EEC had come into force [on 01.10.93] and drew the participants' attention to that and the other legislative documents covering the two diseases and trade both between member states and with third countries.

Dr. Westergaard further stressed how serious the current Newcastle disease situation in the Community was and the important role that the National Laboratories must play in ensuring that the Community's control measures are brought to bear in any outbreak as quickly and efficiently as possible.

He also mentioned the effect the two diseases can have on world trade and the importance of countries throughout the world eventually reaching a common agreement on definitions of the diseases.

In addition to reaching a globally accepted definition of disease it was important that the animal welfare aspects of tests to assess virulence and define disease were also addressed. The current *in vivo* tests involving inoculation of animals with virus isolates were unsatisfactory in this respect and it was important that alternative procedures were investigated and implemented if practicable and of equal sensitivity.

The necessity of virus isolation and *in vivo* tests before disease could be confirmed meant that there was considerable delay from suspecting disease to bringing into force the full control measures. Dr. Westergaard hoped that during the meeting there would be either formal or informal discussions on the feasibility of introducing rapid *in vitro* tests for the detection and assessment of virus based on new technology.

In conclusion Dr. Westergaard noted that this was the First Annual Meeting and hoped that, indeed, representatives of the National Laboratories would meet each year in the future.

SESSION I - COUNTRY REPORTS

a: AVIAN INFLUENZA

Representatives of the following countries made statements that no outbreaks of avian influenza had occurred in their countries during 1992 to 1993:

Belgium
Portugal
Greece
Austria

The following countries produced combined avian influenza and Newcastle disease reports which can be found in section b: of Session I:

Spain
Sweden
Finland

AVIAN INFLUENZA - CURRENT SITUATION IN DENMARK.

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Definition.

The definition of Avian Influenza (AI) in Denmark will follow the definition presented in the EC directive 92/40/EEC of introducing Community measures for the control of Avian Influenza.

Diagnostic methods.

There are no regional laboratories in Denmark.

The diagnostic methods for AI applied in Denmark will conform in details to the recommendations of the above mentioned EC directive. At present the National Laboratory for AI performs inoculation in embryonated SPF eggs, IVPI test on SPF chickens and HA/HI test employing H5 and H7 reference antisera and antigens.

Epidemiological situation.

AI has not been diagnosed in Denmark.

Most samples received for routine virology are inoculated by the allantoic route in SPF eggs. Allantoic fluid is harvested and tested for HA activity from all of them according to the procedure. No AI virus has been isolated up to the present time.

The compulsory national surveillance for avian diseases does not include routine laboratory test for AI and consequently poultry flocks are not routinely tested serologically for antibodies to AI.

REPORT ON THE CURRENT AVIAN INFLUENZA SITUATION IN THE NETHERLANDS

G. Koch[1] and J.C. van den Wijngaard[2]

[1]Avian Virology Department, Central Veterinary Institute, The Netherlands and [2]Poultry Health Department, Animal Health Service in the Southern Netherlands

Current definition of Avian Influenza (AI)

AI is a (subclinical infection or) clinical disease of birds caused by influenza viruses of the Orthomyxovirus group, designated as type A subtypes H5 or H7.

Incidence of AI in the Netherlands during 1990, 1991, 1992 and 1993

During 1990 and 1991 no suspected outbreaks were reported.
In 1992, 20 healthy breeder flocks (in different regions) were screened serologically by AGPT and HI. No subclinical infections could be diagnosed.

Recently, antibodies against subtype H1 could be demonstrated in blood samples from a layer flock without mortality or signs of the respiratory system.

CURRENT AVIAN INFLUENZA SITUATION IN FRANCE

Michèle Guittet, Jean Paul Picault

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Vaccination

To date, no avian influenza vaccine has been licensed in France.

Incidence Of Avian Influenza During 1990-1993

For several decades no pathogenic avian influenza virus has been isolated in France.

Very few suspected outbreaks occur each year. Details of the two cases since 1990 are shown in Table 1. In each, a non-pathogenic H4N6 virus was isolated, as confirmed by the WHO centre in France.

Beside virus isolation, diagnostic laboratories submitted sera for serological confirmation of AGP positive reactions (Table 2). All were related to egg production problems in turkeys. Two different viruses seem to be involved : H1 and H4.

The turkeys which showed a positive H1 serology were located in the neighbourhood of swine suffering from an H1 influenza episode.

Moreover in 1991, at the request of the Ministry of Agriculture a serological survey was undertaken to assess the presence or not of avian influenza viruses in poultry. It concerned broilers and turkeys, located in seven departments in the North-West part of France. Blood samples were collected at the processing plant, from respectively 32 flocks (640 sera) and 18 flocks (360 sera). No positive reaction was observed. It was concluded, there was no circulation of avian influenza viruses in the territory at that time.

Implementation Of The Accreditation Of Diagnostic Laboratories

See paper on current Newcastle disease situation in France.

Table1 : Isolation of avian influenza viruses in 1990-1993

Ref. of strain	Species	Locality (Department)	Mortality	Diagnostic Laboratory	Virus characteristics		Confirmation by WHO centre
1991					Hemagglutinin	IVPI	
91124	Chicken	Gironde	No mortality	LAV40	H4	nd	H4N6
1993							
93032	Cockatoos	Gironde	No mortality	LAV40	H4	0	H4N6

nd : Not done

Table 2: Serology of avian influenza in 1990-1992

Ref. of serum	Species	Locality (Department)	Month	Signs	Diagnostic Laboratory	Results
1990						
90349	Turkey	Côtes d'Armor	September	Egg drop	LDA22	H1
1991						
91147	Turkey	Côtes d'Armor	June		LDA22	H1
91151	Turkey	Côtes d'Armor	June		LDA22	H1
1992						
92044	Turkey	Vendée	February	Egg drop	LDA22	H4
92070	Turkey	Vendée	February	Egg drop	LDA22	H4
92071	Turkey	Vendée	February	Egg drop	LDA22	H4
92072	Turkey	Vendée	February	Egg drop	LDA22	H4
92034	Turkey	Vendée	September	Egg drop	LDA22	H4
92036	Turkey	Côtes d'Armor	October	Egg drop	LDA22	H1

RECENT OUTBREAKS OF INFLUENZA A IN BIRDS IN IRELAND

G. Campbell and Helen de Geus

Department of Agriculture, Food and Forestry, Veterinary Research Laboratory, Abbotstown, Castleknock, Dublin 15. Ireland

Definition

In Ireland the definition of avian influenza is the same as that in EC Directive 92/40.

Incidence of avian influenza during 1989-1993

No outbreaks of have occurred in this period.

Methods of confirmation of avian influenza A

In Ireland Influenza A in birds is a notifiable disease. Suspect clinical cases are investigated by the Veterinary Research Staff.

Suspicion of infection may arise from positive serology or from finding clinical and/or pathological lesions consistent with avian influenza.

Once the disease is suspected flock is examined clinically. Dead birds, blood samples and cloacal swabs are taken and brought to the laboratory for further examination. Appropriate tissues or swab material are inoculated into 9- to 11-day-old embryonated fowls' eggs from a specific pathogen free [SPF] flock in order to isolate the virus. Eggs with dead or dying embryos, as they arise, and all remaining eggs six days post inoculation are chilled to 4°C and the allantoic fluids are tested for haemagglutinin [HA] activity. At the same time the virus in the allantoic fluid is concentrated and tested in the immunodiffusion test against group specific influenza A antiserum. The HA activity is also checked to see if it is inhibited by monospecific paramyxovirus type 1 [PMV-1] antisera in a haemagglutination inhibition [HI] test. Preliminary subtyping using monospecific antisera to H5 and H7 influenza viruses in HI tests is also done.

If the virus reacts with influenza A antiserum then the virus is confirmed as an influenza A virus. It is then sent to the European Community Reference Laboratory, Weybridge, England for subtyping and virulence testing. If the virus is a pathogenic strain infection with avian influenza

is confirmed. The flock is slaughtered, compensation paid and disease surveillance in accordance with Directive 92/40/EEC is carried out..

Influenza A surveillance programmes

All flocks within a 10 km radius around a confirmed outbreak are examined clinically and screened serologically. This also applies to flocks outside a 10 km radius which had contact with the infected poultry. Flocks within the 3 km protection zone and the 10 km surveillance zone are tested twice serologically for antibodies to influenza A virus of the appropriate serotyped during the period of restriction.

At other times, when there are no suspected infections, all blood samples submitted to the Virology Division from clinical disease outbreaks are routinely tested for antibodies to avian influenza.

Vaccination

Vaccination against influenza A is prohibited in Ireland and such vaccines are not licensed.

Laboratories engaged in surveillance for influenza A

Virus isolation work and serology are performed at the Veterinary Research Laboratory, Abbotstown, Dublin. This is the national reference laboratory for both avian influenza and Newcastle disease. There are six other Regional Veterinary Laboratories located throughout the country. These laboratories investigate disease outbreaks in flocks, perform post-mortem examinations and submit specimens for virological examination.

AVIAN INFLUENZA: CURRENT SITUATION IN GERMANY

Ortrud Werner

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Since 1979 there have not been any outbreaks of highly pathogenic influenza in poultry in Germany.

Additionally, no influenza viruses of low pathogenicity were isolated from poultry flocks in 1992 or 1993.

At present no specific surveillance programmes are being carried out.

THE EPIDEMIOLOGICAL SITUATION OF AVIAN INFLUENZA IN ITALY FROM 1990 TO 1993 IN FERAL BIRD POPULATIONS AND IN BIRDS IN QUARANTINE.

V. Papparella, A. Fioretti and L.F. Menna.

National Reference Centre for Avian Influenza Viruses at the Bird and Rabbit Experimental Centre in Varcaturo, Avian Pathology Section, University of Naples "Federico II", Italy.

The National Reference Centre for the study of avian influenza viruses set up by the Ministry of Health at the Bird and Rabbit Experimental Centre in Varcaturo, associated to the Avian Pathology Section of the Department of Pathology, Disease Prevention and Foodstuff Inspection at the University of Naples "Federico II", has developed and conducted its own control activity through the epidemiological surveillance of feral bird populations that were potential reservoirs of avian influenza viruses. In particular, surveys have been carried out over the last three years on migratory Anatidae and Rallidae found in Tuscany's wildlife parks.

The study of 62 cloacal swabs obtained from as many birds belonging to the *Anas platyrhyncos*, *Anas strepera* and *Fulica atra* species resulted in the isolation of two strains of avian influenza A subtype H5N2. Pathogenicity tests (Intravenous and Intracerebral Pathogenicity Index) showed that both of these were only slightly pathogenic for chickens.

In 1992, following problems encountered in the trapping of feral birds, studies were conducted on a flock of Toulouse geese intensively reared in a location that guaranteed contact with migratory wildfowl. Sampling was performed by cloacal swabs taken on a monthly basis from December 1991 to June 1992. A number of these geese, which were farmed in an open pen with a night shelter, were considered as "sentinel birds" and simple observations showed that contact often took place between migratory birds and the geese reared in the open during the former's passage through the area. During January and February these samples resulted in the isolation of two strains of avian influenza A, subtypes H5N2 and H6N2, both of which were only slightly pathogenic for chicken as evaluated using the Intravenous Pathogenicity Index. At the World Reference Laboratory for Avian Ortho and Paramyxovirus at the Central Veterinary Laboratory in Weybridge (United Kingdom) work was conducted to determine the nucleotide sequencing of the viral genome area corresponding to the haemagglutinin cleavage site. This

excluded the presence of additional basic amino acids in the H5N2 strain examined, thus confirming the substantial apathogenicity of the two isolated strains for domestic poultry.

The sera taken during experimentation and assayed in haemagglutination inhibition tests with the isolated viruses had a constantly negative outcome and showed no immunological response, even in infected birds, as already described in the literature for the Anseriformes order (Kida et al., 1980).

Finally, during 1992-1993 we studied herring gulls (*Larus argentatus*) nesting in colonies in the lesser islands of the Tuscan archipelago. The cloacal swabs taken made it possible to isolate two strains of the avian influenza virus, subtype H7N2, both of which were only slightly pathogenic (Intravenous Pathogenicity Index) and were considered apathogenic due to the lack of additional basic amino acids, determined by nucleotide sequencing of the viral genome area corresponding to the haemagglutinin cleavage site. These subtypes had already been isolated in Italy from gulls in 1987-1988 (Fioretti et al., 1988).

Table 1: Isolation of influenza A viruses from wild birds in Italy during 1990-93.

Year	**Bird species**	**Subtype**	**IVPI[a]**	**ICPI[b]**	**Nucleotide sequence[c]**
1990/91	coot [*Fulica atra*]	H5N2	0	0	not done
1992	mallard [*Anas platyrhyncos*]	H5N2	0	0	not done
1992	geese	H6N2 H5N2	0 0.42	nd nd	no additional basic amino acids
1992/93	gull [*Larus argentatus*]	H7N2 H7N2	0 0.85	nd nd	no additional basic amino acids

[a]IVPI = intravenous pathogenicity index in six-week-old chickens
[b]ICPI = intracerebral pathogenicity index in day-old chicks
[c]Nucleotide sequencing of the part of the genome corresponding to the cleavage site of the haemagglutinin.

Furthermore, during 1991 cloacal swabs were taken in a survey of imported parrots placed in quarantine in the Campania region of Italy. Eight species were analysed and a total of 60 swabs were obtained. These allowed three strains of avian influenza virus A subtype H7N2 to be isolated [Table 2] in addition to a strain of paramyxovirus PMV-1, monoclonal antibody binding group C1.

Table 2: Isolation of influenza A viruses from psittacine caged birds imported into Italy in 1991.

Bird species	Subtype	IVPI[a]
Monk parrot [*Meopsitta monachus*]	H7N2	0.85
Monk parrot [*Meopsitta monachus*]	H7N2	0.45
Cockatiel [*Nymphicus hollandicus*]	H7N2	0.27

a: intravenous pathogenicity index in six-week-old chickens

The virus isolation methods used in SPF embryonated eggs were those suggested in the Report and Recommendations of the Scientific Group on Contagious Diseases of Poultry (Avian Orthomyxoviruses) published by the EEC in 1986, as were the techniques for the Haemagglutination and Haemagglutination Inhibition Tests and the Intravenous and Intracerebral Pathogenicity Index. Finally, the antigenic subtype typing (haemagglutinin and neuraminidase) and the nucleotide sequencing of the viral genome area corresponding to the haemagglutinin cleavage site were performed at the World Reference Laboratory for Avian Ortho and Paramyxovirus at the Central Veterinary Laboratory, Weybridge (United Kingdom).

In conclusion, the epidemiological surveillance studies conducted so far on migratory bird populations have highlighted the two following aspects:

a) There are three subtypes of avian influenza virus A in enzootic form in Italy: H6N2, H7N2 and H5N2, but the first two are isolated more frequently. Furthermore, these strains were isolated several times in the same period from domestic poultry.

b) In Italy, as in Central-Southern Europe in general (Ottis et al., 1980), the exclusive importance of waterfowl as natural reserves of avian influenza virus is less clear than in North America, while the involvement of other feral birds, such as Passeriformes and Laridae might be greater.

Finally, it is worth pointing out that the strict virological control, including tests for avian influenza, of imported exotic birds should be further stepped up.

AVIAN INFLUENZA: CURRENT SITUATION IN GREAT BRITAIN

Dennis J. Alexander

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Introduction

The incidence of infections of chickens or turkeys with avian influenza viruses in Great Britain is low. Between 1959-1990 five outbreaks were reported in chickens and 20 isolates were obtained from turkeys on different farms [1, and unpublished data]. Nevertheless, three of these outbreaks, in 1959 in chickens in Scotland and 1963 and 1979 in turkeys in Norfolk, have been infections with highly pathogenic avian influenza (HPAI) viruses [1].

Avian influenza investigations during 1992 to October 1993

During this period six flocks have been investigated on suspicion of infection with avian influenza viruses [Table 1].

Table 1: Influenza investigations during 1992-1993

	1992	**1993** to date
Suspected cases investigated	2*	4
Influenza virus isolated	1*	1
Confirmed as HPAI	1*	0

* relates to outbreak occurring in late December 1991

Isolations from domestic poultry since 1993

During the 3.75 year period since 1990 only five isolations of influenza virus have been made from different poultry flocks. As shown in Table 2, four of these viruses have given values of 0.00 in intravenous pathogenicity index tests in six-week-old chickens [IVPI]. But one virus relating to an outbreak in turkeys in December 1991 was an HPAI virus giving an index of 3.00. Details of this outbreak have been published [2].

Four of the five outbreaks since 1990 occurred in the county of Norfolk on the east coast of England and this is in keeping with the findings

since 1959. As well as being an important area for turkey and commercial duck rearing, the waterways of Norfolk represent important stop over areas for migratory waterfowl and it is assumed that the viruses are introduced by such birds.

Table 2: Influenza isolations from domestic poultry since 1990.

Date	County	Virus	Subtype	IVPI
1990				
05/90	Lancashire	duck/England/780/90	H4N6	0.00
1991				
02/91	Norfolk	duck/England/1194/91	H4N2	0.00
07/91	Norfolk	goose/England/1440/91	H6N8	0.00
12/91	Norfolk	turkey/England/50-92/91	H5N1	3.00
1992				
1993				
09/93	Norfolk	turkey/England/895/93	H6N8	0.00

Isolations from other birds since 1990

Three influenza virus isolations were made from either feral birds or birds kept in ornamental bird collections, these are listed in Table 3.

Table 3: Isolations from other birds since 1990

Date	County	Virus	Subtype	IVPI
1991				
05/91	Yorkshire	pheasant/England/939/91	H2N9	0.00
10/91	Shropshire	turaco/England/2004/91	H5N2	0.00
1992				
03/92	Hampshire	teal/England/413/92	H11N9	0.00

All viruses showed extremely low virulence in IVPI tests. The 1991 H5N2 isolate from a turaco (*Touraco musophagidae*) was shown to have the amino acid sequence **...PQRETR*GLF...** at the cleavage site of the haemagglutinin [3] i.e. there were no additional basic amino acids at the cleavage site and the virus did not therefore fall within the EC definition of HPAI [4].

Isolations of avian influenza viruses from captive caged birds

Since 1976 captive caged birds imported into Great Britain have been held for a period of at least 35 days under strict quarantine conditions and subjected to virological examination. This procedure has often

resulted in the isolation of influenza viruses. Although the subtypes isolated have been mainly H3 or H4, the frequency of isolation has varied enormously [Table 3] and included a 6.5 year period 1980 to 06.1987 when no isolates at all were obtained from this source, although isolation rates of paramyxoviruses remained constant. Since April 1993, following nearly two years without influenza virus isolations, 4 isolates have been obtained from this source, all were of H4N6 subtype and all were of low pathogenicity in IVPI tests.

Table 3: Isolations of avian influenza viruses from captive caged birds

Date	Subtype	Number
1975*	H4N6	29
1977-06.1977	H3N8	58
07.1977-1978		NONE
1979	H4N6 H10N7 H7N7	2 2 1
1980-06.1987		NONE
1987	H3N8	1
1988	H3N8 H3N6 H4N6	2 3 4
1989	H3N8 H4N3 H4N6	2 14 3
1990	H4N3 H4N8	3 5
01-06.1991	H4N1 H4N8	3 1
07.1991-04.1993		NONE
04.1993-09.1993	H4N6	4

*viruses isolated from dead birds in transit at Heathrow Airport

References

1. Alexander, D.J. (1989). New definitions for avian influenza viruses requiring intervention by Governments - Implications to the situation in Great Britain. State Veterinary Journal 43, 172-187.
2. Alexander, D.J., Lister, S.A., Johnston, M.J., Randall, C.J. & Thomas, P.J. (1993). An outbreak of highly pathogenic avian influenza in turkeys in Great Britain in 1991. Veterinary Record 132: 535-536.

3. Wood, G.W., McCauley, J.W., Bashiruddin, J.B. & Alexander, D.J. (1993). Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Archives of Virology 130: 209-217.
4. Council Directive 92/40/EEC introducing Community measures for the control of avian influenza. Official Journal of the European Communities No. L 167 1-16.

SESSION I - COUNTRY REPORTS

b: NEWCASTLE DISEASE

The representative from **Greece** made a statement that no outbreaks of Newcastle disease occurred in that country during 1992 to 1993.

NEWCASTLE DISEASE: CURRENT SITUATION IN GREAT BRITAIN

Dennis J. Alexander

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Introduction

Historical aspects of Newcastle disease (ND), vaccination policies and the poultry population in Great Britain have been reviewed recently [1].

The last cases of ND in poultry in Great Britain were in 1984 when 23 outbreaks were confirmed, the source of virus was demonstrated to be untreated food contaminated by feral pigeons infected with the pigeon PMV-1 variant of ND virus (PPMV-1) [2]. Similarly, in 1991 in Northern Ireland the six outbreaks confirmed between March and May were all due to infection with PPMV-1 and considered to be a result of feeding food contaminated by infected feral pigeons.

ND in Poultry 1992-1993

Suspect cases in poultry investigated during 1992 and up to 1 October 1993 were:

Table 1: Suspect cases of ND in poultry in Great Britain 1992-93.

	1992	**1993** [to 01.10.93]
Suspect cases investigated	17	11
ND virus isolated	1[a]	3[b]
Confirmed cases	0	0

[a] Intracerebral pathogenicity index [ICPI] =0.22.
[b] ICPI virus 1 = 0.00, ICPI virus 2 = 0.00, ICPI virus 3 = 0.20.

Reactions with monoclonal antibodies (mAbs) confirmed all 4 isolates to be vaccinal viruses.

ND in other birds.

During 1992 ND virus was isolated from birds other than poultry or pigeons on three occasions:

Table 2: Isolations of NDV from "other birds" in Great Britain 1992 to 1993.

Date	Bird	Comments	ICPI	MAb group
03.92	merlin	captive	not done	P
07.92	pheasant	"feral"	0.00	G
12.92	conure	in quarantine	0.00	H

Reactions with a panel of mAbs showed the virus isolated from the captive merlin to be indistinguishable from PPMV-1, while the two other viruses showed mAb binding patterns typical of viruses normally associated with infections of waterfowl.

ND in pigeons.

The incidence of PPMV-1 infections in racing pigeons between 1983 to 1990 and the characterisation of viruses isolated has been the subject of a recent review [3]. Since 1984 confirmation has been allowed on the basis of clinical signs and history alone. Virus isolation attempts are normally carried out only when unusual circumstances exist, although in practice submissions to the regional or central laboratory for such investigations have often occurred when the disease has appeared in an area where it has been absent for sometime. An increase in confirmed cases in pigeons from 188 in 1989 to 324 in 1990 was not sustained and in 1991 only 96 cases were confirmed, the lowest total since 1983 when the disease first reached Great Britain.

Confirmed cases of PPMV-1 in racing pigeons and numbers of viruses isolated (in brackets) during 1992 to 1993 [to 01.07.93] were:

Table 3: PPMV-1 in pigeons in Great Britain 1992-93

Year	Confirmed cases [and isolations] by quarter				
	01-03	04-06	07-09	10-12	TOTAL
1992	4(1)	8(4)	47(13)	98(25)	157(43)
1993	13(5)	20(11)			*33 (16)*

The distribution of cases during 1992 followed the pattern of the previous years with the majority of outbreaks occurring in the last quarter of the year, the close season for racing. The total for 1992 represented an increase of more than 60% on the figure for 1991. This

and the confirmed outbreaks in the first half of 1993 (35), which were nearly three times those in the corresponding period in 1992 (12), indicates the continuing presence of PPMV-1 virus in the racing pigeon population in Great Britain.

References

1. Alexander, D.J. & Thomas, P.J. (1992) Current Newcastle disease situation in the United Kingdom. Proceedings of the CEC Workshop on avian paramyxoviruses, Rauischholzhausen, Germany, 1992 pp 62-67.
2. Alexander, D.J., Wilson, G.W.C., Russell, P.H., Lister, S.A. & Parsons, G. (1985). Newcastle disease outbreaks in fowl in Great Britain during 1984. Veterinary Record 117, 429-434.
3. Alexander, D.J., Parsons, G., Manvell, R.J. & Sayers, A.R. Characterisation of avian paramyxovirus type 1 infections of racing pigeons in Great Britain during 1983 to 1990. Proceedings of the Commission of the European Communities meeting on Virus Diseases of Poultry - New and Evolving Pathogens, Brussels, 1992. *In press.*

NEWCASTLE DISEASE - CURRENT SITUATION IN DENMARK.

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Definition.

The definition of Newcastle Disease (ND) in Denmark will conform to the definition presented in EC Directive 92/66/EEC introducing Community measures for the control of Newcastle Disease.

Diagnostic methods.

The diagnostic methods will strictly follow the recommendations in the above mentioned EC Directive. At present the National Laboratory for ND performs inoculation in embryonated SPF fowl's eggs, HA and HI tests employing reference ND antigen and antiserum and ICPI test on day-old SPF chicks.

Epidemiological situation.

ND has not been diagnosed in fowl and other commercial poultry in Denmark since 1972.

The national surveillance for ND has been based on serological monitoring of breeding parent and grand parent chickens. Since 1 May 1992 the surveillance programme has been reduced with respect to the number of flocks and number of samples per flock examined.

In 1992 a total of 15,200 sera from fowl was tested. Samples from one flock were repeatedly positive, due to the use of an erroneously labelled infectious bronchitis vaccine containing inactivated NDV. The rest of the samples were negative.

From samples submitted for routine diagnostic investigation, material representing 82 flocks of poultry was inoculated in the allantoic cavity of embryonated SPF eggs in 1992. The corresponding figure for the first 6 months of 1993 is 45 flocks. In 1992 no ND virus was isolated. Over the first 6 months of 1993 pigeon paramyxovirus (PPMV-1) was isolated from

2 out of 15 pigeon flocks with suspicion of PPMV-1 infection. The identity of the isolates has been confirmed by the EC Reference Laboratory.

The PPMV-1 isolates originated from pigeons with no contact with commercial fowl production. Vaccination of racing pigeons with an inactivated ND vaccine is compulsory in Denmark.

CURRENT NEWCASTLE DISEASE SITUATION IN IRELAND

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Newcastle Disease Status

Ireland is free from Newcastle Disease (ND) which is a notifiable disease and subject to mandatory control with compensation. A non-vaccination policy is in operation.

Newcastle disease is defined as the infection of poultry with any PMV-1 virus with an intracerebral pathogenicity index (ICPI) greater than 0.7, as specified in EC Directive 92/66/EEC.

Vaccination

Vaccination is prohibited in poultry flocks. The vaccination of racing and breeding pigeons is permitted under direct veterinary supervision with inactivated vaccine only. A certificate of vaccination is required when presenting pigeons for racing. The importation and distribution of the vaccine is under official control. Approximately 90,000 doses of vaccine are used per annum.

Newcastle Disease Surveillance

All breeding flocks are blood-sampled at point of lay and when moulted. If flocks are moved to other holdings during the rearing phase they are also tested. The sampling rate is 10% with a minimum of 50 and a maximum of 250.

All blood samples submitted to the Veterinary Research Laboratory, Abbotstown, Dublin, for routine diagnosis are also tested for the presence of antibodies to PMV-1.

Newcastle Disease Confirmation

Suspected cases of ND are identified by the presence of suspect clinical signs or positive serological reactions during routine monitoring.

In the event of a suspected case, a clinical, epidemiological and pathological investigation of the flock is undertaken. Cloacal and tracheal swabs, appropriate tissues from autopsies and additional blood samples are collected.

Virus isolation is by the inoculation of 10-day-old embryonated specific pathogen free (SPF) chicken eggs. Isolated viruses are identified as PMV-1 by HI test using monospecific antiserum. The ICPI is determined.

In the event of a suspected outbreak being confirmed as Newcastle disease, a stamping out policy is applied. A 3 km protection zone and a 10 km surveillance zone is set up. Epidemiological investigation, serological surveillance, controls on movement and disinfection procedures follow.

All flocks within the 10 km surveillance zone around the outbreak are blood-sampled at least twice during the restriction period.

Where a poultry premises is shown to be infected with a lentogenic strain of PMV-1, movement restrictions and surveillance are implemented and movement for slaughter only is permitted. Movement restrictions are maintained for the life of the flock.

Incidence of Newcastle Disease (ND) in Ireland during 1991 to 1993.

1991

There were 16 poultry flocks seropositive to PMV-1 in 1991.

Newcastle disease was confirmed in two flocks. They were located in different parts of the country (280 km apart). Both had been notified because of suspicious clinical signs. The isolated viruses were shown to be Pigeon PPMV-1 variant, with ICPIs of 1.65.

In a further three seropositive flocks lentogenic virus (ICPIs 0.00; 0.04 and 0.11) were isolated.

PMV-1 (pigeon variant) was isolated from two clinical cases in racing pigeons.

1992

There were 16 poultry flocks seropositive to PMV-1 in 1992.

Newcastle disease was confirmed in one flock. It had been notified because of suspicious clinical signs. The isolated virus was an unusual PPMV-1 variant (ICPI 1.54).

At this time twelve small back-yard flocks within the 10 km surveillance zone were seropositive. No virus was isolated from any of these.

In a further seropositive flock (unrelated to the above outbreak) a lentogenic virus (ICPI 0.11) was isolated.

PMV-1 (pigeon variant) was isolated from four clinical cases in racing pigeons.

1993

There have been three seropositive poultry flocks to PMV-1 to date. Newcastle disease has not been confirmed in any.

PMV-1 (pigeon variant) has been isolated from two clinical cases in racing pigeons.

NEWCASTLE DISEASE AND AVIAN INFLUENZA IN SPAIN

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Incidence of ND and avian influenza in Spain.

In recent years no suspected or confirmed outbreaks of Newcastle disease [ND] or avian influenza [AI] have occurred in Spain.

Surveillance programmes and Diagnosis.

ND and AI are notifiable diseases for which the relevant authority must be informed on suspicion of disease, but there is no special surveillance programme for either disease.

Several breeding flocks are blood sampled and the sera tested using commercial ELISA kits. Random samples from commercial layers and broilers at processing plants are also tested.

In 1993, an epidemiological survey of poultry diseases was started.

Spain has 17 Autonomous Communities and in each there is a Regional Veterinary Laboratory with a poultry department responsible for diagnosis, surveillance and control of poultry disease. the data from each Regional Laboratory are collected at the National Reference Laboratory, where the poultry health service is centred. This laboratory is responsible for maintaining up to date records, and testing vaccines and diagnostic reagents.

The Spanish Government has approved a Royal Decree, No: 1317/1992 of 30th October, which is a reflection of EC Directive 90/539/EEC on animal health conditions governing intra-community trade in, and importation from third countries of, poultry and hatching eggs.

In conclusion, surveillance for ND and AI is on a voluntary basis, but the aforementioned Royal Decree of the Ministry of Agriculture provides a surveillance programme in line with EEC Directive 90/539.

Control measures for ND and AI

The poultry population is concentrated in several areas of Spain. To prevent transmission of ND and AI into Spain, importations of birds and poultry products are strictly regulated and an import licence is obligatory for every import.

Importation of domestic fowl from third countries

All importations of avian stocks are controlled at the external borders. Birds must cone from an ND-free area. An official veterinarian of the exporting country must issue a health certificate for the parents of exported birds. Imported birds are quarantined in specially designed buildings situated at least 2 km from other poultry flocks. Birds imported as day-old chicks are also kept in quarantine.

Importation of other birds

The importation of other birds has also been regulated. They are quarantined in specially designed buildings for two months. All dead birds are sent to an official veterinary laboratory for post mortem examination; virus isolation is attempted when viral disease is suspected. Several methods are used to monitor ND and AI.

Captive caged birds

Ten-day-old chicks are placed in each room as sentinels and sampled for serological examination before the caged birds are released.

Vaccination

In Spain, vaccination is not compulsory. Vaccination with both live and inactivated vaccines is allowed. The programmes used are voluntary. Parent stocks and commercial egg production establishments usually follow a vaccination programme of live and killed vaccines [La Sota, Clone 30] in accordance with breeders', pharmaceutical companies' and veterinarians' recommendations. Most broiler producers do not vaccinate their stocks against ND, due to the absence of the disease since 1982.

The poultry industry in Spain has been thoroughly organised, not only in terms of regulations, but also in practical recommendations for vaccine application and vaccination schedules. a significant part of this control consists of regular monitoring of immune status by blood testing.

Vaccination programmes

Breeder birds and egg producers are vaccinated 3 times during their growing period with live vaccine via drinking water and practically all flocks are boosted by inactivated vaccines:-

Normal vaccination programme for layers

Application	Age of birds	Vaccine	Administration
1	4-14 days	La Sota	drinking water
2	30 days	La Sota	drinking water
{3	8 weeks	live/inactivated	d.w./injection}
4	18 weeks	live/inactivated	d.w./injection

application 3 is not always given

Broiler chickens are vaccinated:-

Normal vaccination programme for broilers

Application	Age of birds	Vaccine	Administration
1	10 days	live	drinking water
2	28 days	live	drinking water

In Spain vaccination is practically 100% in reproduction and laying birds and 15% in broilers.

Pigeons are vaccinated with a killed vaccine containing homologous pigeon PMV-1 virus.

Facilities

Samples for virus isolation are processed in a building used only for the processing of field specimens. The National Reference Laboratory is currently building a facility in which to keep SPF chickens to be used for virus investigations. This facility will consist of 5 departments in which it will be possible to infect simultaneously chickens in independent rooms with different diseases. All biosafety rules for the entrance and exit of animals and materials will be observed.

The new units will provide aseptic and protective separation of animals, environment and personnel with a high degree of confidence and will include ventilation and filtration systems with HEPA filters and automatic vapour sterilisation of the enclosure using peracetic acid or other sterilising agents.

Summary

At present, ND and AI are not a problem in the poultry industry in Spain. Possibly the incidence of disease in the past forced the poultry specialists and the poultry industry to develop and adopt appropriate measures to control these diseases.

The well-structured network of the poultry industry, with the help of the official veterinary service, has helped achieve successful prevention, vaccination and control.

However, it is necessary to maintain this good level with the set up of a well-equipped reference laboratory which should be able to supply standardised reagents and methods.

NEWCASTLE DISEASE SITUATION IN ITALY IN 1993

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The situation of Newcastle disease (ND) in Italy was reviewed at the EC Workshop on Avian Paramyxoviruses held in Rauischholzhausen, Germany in July 1992 [1]. There has been no change in Italian legislation covering ND since that time. In particular no official definition has been stated, no official acknowledgement of Directive 92/66/EEC has been made and no acknowledgement of the designated National Laboratory has been made in the Official Journal of the Italian Republic, although this was done for the Avian Influenza EC National Laboratory by the Health Ministry's Ordinance of 20th May 1991 [2].

As a consequence, the National Laboratory for ND is not yet legally operative. Accordingly, no isolates are sent from Regional Laboratories and no outbreaks are reported directly to the designated EC National Laboratory.

The data presented in this report were the result of requests expressly to the presumptive Regional Laboratories, the Avian Pathology Institutes of the Universities and the Health Ministry. Needless to say, not all the Laboratories addressed sent data as, probably, they did not feel bound to provide them to a Laboratory which has not been officially appointed.

However, the 1993 situation, as drawn from the data received is as follows. Two outbreaks of the disease have been reported, both in March. The first was in hobby pigeons in the province of Rovigo. The second was in feral turtle doves found dead in the province of Reggio Emilia, the same province of the only outbreak recorded in 1991 which was also in pigeons and, incidentally, the same province as the first pigeon variant outbreak to be recorded, in 1981 [3].

Confirmed ND outbreaks in the years 1987 to 1991 have been recorded in the Report of International Disease Surveillance published in 1992 [4]. No outbreaks were recorded in 1991.

Information received on suspect cases investigated was rather scanty and not from all Regions, so it is impossible to present dependable data.

The methods adopted for diagnosis are mainly those listed in the O.I.E. Manual, according to preference and facilities. At least one of the Regional Laboratories makes use of monoclonal antibodies supplied from the EC Reference Laboratory for the characterisation of isolates. The plaque test for assessing pathogenicity is currently used in the National Laboratory.

References

1. D'Aprile P.N. (1992) *In*: Proceedings of the Workshop on Avian Paramyxoviruses, Rauischholzhausen, Germany July 1992 pp 35-37.

2. D.M. 20 maggio 1991 - Gazzetta Ufficiale della Repubblica Italiana, serie Generale n 132 del 7 giugno 1991.

3. Alexander, D.J. et al (1985) Avian Pathology 14: 365-376.

4. International Disease Surveillance (1992) Veterinary Record 130: 151.

SWEDEN: COUNTRY REPORT FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

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Avian influenza

No clinical outbreak of avian influenza has occurred in Sweden.

During 1992 a small scale serological survey was carried out in both breeders and commercial laying flocks. A total of 1200 samples from 60 flocks was tested using the type specific immunodoublediffusion test. No antibodies to influenza A virus were detected in any of the sera.

Newcastle disease

No clinical outbreak of Newcastle disease (ND) has occurred in Sweden in recent years.

During 1992, a comprehensive survey was carried out in the poultry population. A total of 87 parent flocks, 127 broiler flocks and 97 flocks of commercial layers was tested by taking 20 blood samples from each flock. All 6,220 serum samples tested were negative for antibodies to ND virus.

During January to May 1993 a total of 125 flocks was tested by collecting 20 blood samples from each flock. All 2,500 sera were negative for antibodies to ND virus.

PMV-1 infection in pigeons has not occurred since 1990. All racing and show pigeons are vaccinated with killed vaccine.

During 1992-93 blood samples were taken from 82 wild pigeons in the South of Sweden. Seven of these samples had antibodies against PMV-1.

All birds imported in to Sweden are kept in quarantine and tested serologically for antibodies to ND virus. During the last year a batch of ducks and geese in quarantine, destined for a zoological gardens were found serologically positive for ND virus. The birds were re-exported.

NEWCASTLE DISEASE AND AVIAN INFLUENZA: COUNTRY REPORT FROM FINLAND

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Avian influenza

No suspect cases.
No surveillance studies.

Methods used for diagnosis:-

Agar gel precipitin test with avian influenza A group antigen and Avian influenza A positive control serum. Both were obtained from CVL, Weybridge.

Newcastle disease

No suspect cases in domestic poultry.
No outbreaks in domestic poultry.

Surveillance studies for ND.

All flocks that produce hatching eggs from poultry, ducks and geese are tested. Usually 60 blood samples per flock are collected:

Situation up to 15.09.1993:

2519 sera from 49 flocks have been tested for antibodies. These represent about 2/3rd of the total number to be tested. No positive serum has been detected.

Method for HA and HI as Council/Directive 92/66/EEC except:

NDV allantoic-fluid live own strain is used, calibrated with Newcastle disease virus antigen CVL. Cat. No. 0158/03.

Procedure
As Council/Directive 92/66/EEC except:
The test is performed at room temperature.

We use a positive serum that has been calibrated with the Newcastle disease virus antiserum, CVL Cat. No 0015/03.

The sera are pretreated with a 2% chicken RBC suspension on the microplate prior to the assay.

Suspected cases in feral birds

Massive death of Razorbills (*Alca torda*) at the Gulf of Finland occurred in May-June 1992. We tested the organs from 10 birds for HA agents in eggs and inoculation in 4-day-old chicks (i.o. and i.m.). No HA agent was found. The chicks remained healthy.

The cause of the deaths in the razorbills was never determined.

Outbreak of PMV-1 in pigeons in November 1992

About 50 pigeons died after showing central nervous signs in a harbour town on the western coast of Finland.

The National Veterinary and Food Institute received 3 dead pigeons for autopsy and further investigations.

A hemagglutinating agent was isolated from all 3 suspensions from the intestine and from pooled suspensions of brain, liver + spleen, and trachea + lungs. The highest HA titre, 2^9, was from one of the intestine samples and the lowest, no HA, in the first passage, from trachea + lungs. As a rule the HA titre increased in the second egg passage.

Blood samples from the 3 dead birds showed no HI antibodies to NDV.

Allantoic fluid was sent to the CVL, Weybridge who identified the material as "Pigeon Paramyxovirus type I" (PPMV-1). Further characterisation work with monoclonal antibodies at the CVL showed that the isolate was indistinguishable from other PPMV-1 isolates such as pigeon/England/561/83

An ICPI test in day-old chicks, carried out in Helsinki, gave an index of 1.4.

No further suspect cases or evidence of the virus in any species of bird has been reported.

CURRENT NEWCASTLE DISEASE SITUATION IN AUSTRIA

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Definition

Criteria for the official recognition of Newcastle disease [ND] are clinical signs and/or pathological lesions, a high morbidity and mortality, and the isolation of a paramyxovirus [PMV].

Incidence of ND in Austria

ND is seen only occasionally, and up to 1993 Austria had been free of ND for about five years. In 1993 two outbreaks occurred one, in May, in turkeys and the other, in June, in broiler chickens which had not been vaccinated, no further outbreaks have occurred.

The development of the 1993 infections

In the turkey flock clinical signs of respiratory tract infection and green diarrhoea were observed. On post-mortem examination pneumonia and air sacculitis were found but petechial haemorrhagic lesions were seen only rarely. Mortality rose over a three week period up to 35%. Initially, virus isolation in eggs was negative but on the third passage a PMV-1 virus was isolated which was shown to have an intracerebral pathogenicity index in day-old chicks [ICPI] of 1.55.

The disease in the broiler flock was very similar to that seen in the turkeys.

Infections of pigeons and chickens with the "pigeon type" of PMV-1 are seen sometimes in Austria, but these are not classified as ND because the chickens show only drops in egg production.

Methods for diagnosis

The preferred samples for virus isolation are of the central nervous system, the respiratory tract and other organs according to the pathological lesions. Nine- or 10-day-old embryonating eggs from the

laboratory's flock are inoculated into the allantoic cavity. Normally two passages are done, but in strongly suspected cases three or more passages may be performed.

If a haemagglutinating virus is isolated it is identified in haemagglutination inhibition tests using monospecific sera. The mean death time in eggs [MDT] is included in the evaluation of the isolate, but ICPI tests are not performed regularly.

Vaccination

In Austria only inactivated "adsorbate" vaccines and, since 1990, a live vaccine based on Hitchner B1 are licensed. All vaccination is done on a voluntary basis and no special vaccination programmes have been implemented.

CURRENT NEWCASTLE DISEASE SITUATION IN FRANCE

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Vaccination Practice

Vaccination is not compulsory.

Broilers are not vaccinated, with the exception of some free range broiler flocks which may be vaccinated once with live vaccines.

Table egg layers and layer or broiler-breeders are vaccinated with live vaccines during the rearing period and with inactivated vaccines before point of lay.

Incidence Of Newcastle Disease During 1990-1993

From 1976 until November 1992, no pathogenic PMV1 virus (ICPI > 0.7) was isolated from poultry in France, with the exception of the mesogenic strains recovered from pigeons since 1983.

The number of suspected outbreaks of Newcastle disease (ND) in poultry for each of the years 1990, 1991, 1992 and to September 1993 is shown in Tables 1, 2 and 3.

The viruses isolated from fighting cocks in July 1992, were not confirmed as pathogenic strains in spite of the mortality associated with the outbreak. Because these birds are illegal, the isolation of the virus was done late on the surviving birds. In all probability those surviving birds had been vaccinated or in contact with vaccinated birds. According to the results of ICPI tests, sequencing of the cleavage site of the F gene, and monoclonal antibody binding pattern, the virus isolated (92238) appeared similar to those of lentogenic viruses such as Ulster 2C. These results were confirmed by D. Alexander with monoclonal antibodies.

In November 1992, the first isolation of a pathogenic ND virus was made from ornamental fowl. This isolation was followed by others in December (Table 2) and at the begining of 1993 (Table 3). Only ornamental birds

were concerned and all of them were present or in contact with other birds present at the IXth avian international exhibition, in Mulhouse the 21st and 22nd of November 1992. The stamping out policy was applied and strict measures as described in the directive 92/66/EEC were taken in each case.

The virus responsible for this epizootic showed a very high ICPI, and the sequencing of the cleavage site of the F gene confirmed these results. Moreover, D. Alexander has shown with monoclonal antibodies, a similar binding pattern of these French viruses (92338, 92355) and those isolated from the outbreaks in the Netherlands, Belgium and Malta.

Besides virus isolation and on the request of the Ministry of Agriculture a serological survey was undertaken in 1991 to assess the presence or not of PMV1 virus in non-vaccinated poultry. It involved broilers and turkeys located in seven departments in the North West part of France. Blood samples were collected at the processing plant from respectively 32 flocks (640 sera) and 18 flocks (360 sera).

No positive reaction was observed and it was concluded, that there was no circulation of PMV1 viruses in the region at that time.

IMPLEMENTATION OF THE ACCREDITATION OF DIAGNOSTIC LABORATORIES

In the application of the Directives 92/66 and 92/40 EEC, in a few months, local diagnostic laboratories will need to be accredited by the RNE (Réseau National d'Essais = the national network for tests) to obtain agreement of the Ministry of Agriculture for the diagnosis of avian influenza and Newcastle disease. For that purpose, the RNE created a few years ago an Agro-Food sectoral committee which was divided in specialist commissions including one for Animal Health. The last one developed accreditation programs (AP) of which the two following contain tests or analyses concerning avian Influenza and Newcastle disease :

<u>AP N° 109 : Tests and analyses in animal immuno-serology (IS)</u>

- IS280 : Detection of Newcastle HI antibodies
- IS300 : Detection of avian influenza precipitating antibodies

<u>AP N° 112 : Tests and analyses in animal virology (VA)</u>

- VA10 : Isolation of avian myxoviruses on eggs and testing for haemagglutinating activity

- VA20 : Identification of avian influenza viruses of H5 and H7 subtypes by HI

- VA30 : Identification of Newcastle disease virus by HI

Each of these tests on Newcastle disease or avian influenza refers to at least 2 reference papers :

One, for justification, is the corresponding EEC directives.
The other, for technical application, is a French reference technique from the CNEVA-LCRAP Ploufragan for each of the IS or VA tests concerned.

Periodical comparative tests (PCT) are organized by the French reference laboratory. Concerning AP N° 109, one PCT must be run every one or two years. Thus, in 1993, 26 laboratories are candidate for the IS 280-PCT (Newcastle HI antibodies) and 19 laboratories for the IS 300 PCT (Influenza AGP antibodies). Concerning AP N° 112, PCT for avian Influenza or Newcastle disease virology tests have not still been defined.

Table 1 : Isolation of PMV-1 viruses in 1990 and 1991

Ref. of strain	Species	Locality (Department)	Month	Mortality/ Morbidity	Diagnostic Laboratory	Characteristics	
						ICPI	**mAb**
90350	Pigeon	Gironde	July	No mortality nervous signs	LAV40	1.25	mesogenic
90427	Racing Pigeon	Vendée	September	No mortality nervous signs	LDA22	1.59	mesogenic
90431	Racing pigeon	Seine Maritime	September	No mortality	LDA22	nd	mesogenic
90458	Pigeon	Aisne	October		LDA22	nd	mesogenic
90492	Chicken	Vendée	November	No signs	LVD85	nd	lentogenic
91031	Pigeon	Seine Maritime	January	-	LDA22	1.60	mesogenic
91223	Chicken	Deux Sèvres	September	Low mortality	LDA22	0.00	lentogenic

nd : Not done
mAb : Monoclonal antibody

Table 2 : Isolation of PMV-1 viruses in 1992

Ref. of strain	Species	Locality (Department)	Month	Mortality/ Morbidity	Diagnostic laboratory	Characteristics		Remarks
						ICPI	Sequencing F gene cleavage	
92220	Fighting cocks	Nord	June	25 % Mortality	LVA 59	0.14		
92221	Fighting cocks	Nord	June	16 % Mortality	LAV 59	0.00		
92238	Fighting cocks	Nord	June	35 % Mortality	LAV 59	0.08	Ulster/like	
92338	Ornamental fowl	Bas Rhin	November	Mortality	LAV 68 LDA22	1.87	Velogenic	Mulhouse exhibition
92347	Ornamental fowl	Moselle	December	Mortality	LAV 67 LDA22	1.89		Mulhouse exhibition
92348	Ornamental fowl	Haut Rhin	December	Mortality	LAV 68 LDA22	1.87		Mulhouse exhibition
92349	Ornamental cock	Belfort	December	Mortality	LAV 68 LDA22	1.89		Mulhouse exhibition
92350	Ornamental fowl	Haut Rhin	December	Mortality	LAV 68 LDA22	1.88		Mulhouse exhibition
92351	Ornamental fowl	Haut Rhin	December	Mortality	LAV 68 LDA22	1.95		Mulhouse exhibition
92355	Ornamental pigeon	Côtes d'Armor	December	No mortality few signs	LDA22	1.76		Mulhouse exhibition
92360	Ornamental fowl	Bas Rhin	December	Mortality	LDA22	not done		Mulhouse exhibition

Table 3 : Isolation of PMV-1 viruses in 1993

Ref. of strain	Species	Locality (Department)	Month	Mortality/ Morbidity	Diagnostic laboratory	Characteristics	Remarks
						ICPI	
93002	Ornamental fowl	Bas-Rhin	December 1992	Mortality	LDA22	not done	Mulhouse exhibition

NEWCASTLE DISEASE: CURRENT SITUATION IN GERMANY

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Introduction

In the Federal Republic of Germany Newcastle disease (ND) must be notified and controlled. The methods used for prevention and control are federal-wide uniform instructions which are defined in the "Legal Order for Protection against Fowl Plague and Newcastle Disease" version of the 26th July 1985. This legal order is in the process of being altered to comply with Council Directive 92/66/EEC and is almost ready for enactment.

In Germany vaccination is obligatory for chicken flocks with more than 200 birds. In such cases poultry, other than chickens, living on the same farm are also required to be vaccinated. The vaccination of smaller flocks is recommended, and in the event of an outbreak can be enforced by the competent authority.

Outbreaks and isolates in 1992

Three outbreaks of ND were recorded in 1992. Two of these concerned small mixed flocks of commercial poultry with less than 100 birds. The other was in a flock of 237 racing pigeons.

The National Reference Laboratory began its work in the summer of 1992. between July and December 1992 six viruses identified as PMV-1 isolates were received for characterisation [Table 1].

Table1: Newcastle disease virus isolates in 1992

Number	isolated from:	ICPI
1	broiler	0.16
2	pigeon	1.09
3	pigeon	1.46
4	pigeon	0.94
5	pigeon	1.36
6	layer hen	0.00

The two viruses from poultry both gave low values in intracerebral pathogenicity index [ICPI] tests, indicating that they were, most probably, isolations of live vaccine strains of the virus.

Outbreaks and isolates in 1993

From the beginning of 1993 up to 15th September 1993, 98 outbreaks of ND were recorded.

Three of these were in commercial chicken flocks:

1. Bundesland: Mecklenburg-Vorpommern
   flock: 87,740 broilers
   died: 11,695
   slaughtered: 76,045
   ICPI: 1.83
   date confirmed: 15.02.93
   epizootiology: chicks were imported from The Netherlands, not vaccinated due to ill health.

2. Bundesland: Nordrhein-Westfalen
   flock: 12,000 layer
   died: 6,000
   slaughtered: 6,000
   ICPI: 1.89
   date confirmed: 19.02.93
   epizootiology: Source of virus unknown. Clearly vaccination had afforded little protection.

3. Bundesland: Sachsen-Anhalt
   flock: 102,000 broilers
   died: 5,400
   slaughtered: 61,008
   ICPI: 1.26
   date confirmed: 19.04.93
   epizootiology: one-day-old chicks imported from The Netherlands.

The other 95 outbreaks of ND recorded were in small and hobby flocks. These were particularly prevalent in the Bundeslander: Bayern, Thuringen, Sachsen and Brandenburg [Fig 1]. In most cases the outbreaks were traced to the purchase of young hens or hobby animals from travelling tradesmen. The tradesmen tended to work a particular area and this accounts for the distribution of outbreaks seen in Fig. 1.

Without exception, the viruses isolated from the outbreaks associated with the travelling tradesmen were shown to be velogenic. Most of these outbreaks occurred in chickens but turkeys, ducks, geese and pheasants were also affected.

One outbreak of ND occurred in a zoological park. The virus appeared to be similar to the virus infecting pigeons and it was assumed that spread had occurred from wild pigeons.

Diagnosis in Germany

The initial diagnosis of ND is made in the regional diagnostic laboratories, based on the clinical and pathological signs and isolation of the virus in cell cultures or embryonated eggs. In most cases a preliminary differentiation by haemagglutination inhibition test or by direct immunofluorescence is also done.

Confirmatory serological identification of the virus and an intracerebral pathogenicity index test in day-old chicks [ICPI] is done at the National Reference Laboratory.

From the beginning of 1993 to 30th September 1993 128 viruses were received at the National Laboratory for characterisation. Ninety were shown to be velogenic, 30 mesogenic and 8 lentogenic [Table 2]

Table 2: NDV isolates characterised at the National Laboratory during 01.01.1993 to 30.09.1993.

Month	**Number**	**Pathogenicity**			**Isolated from:**		
		velo-genic	meso-genic	lento-genic	poultry	pigeons	exotic birds
January	8	2	2	4	4	2	2
February	6	2	4	0	3	3	0
March	6	2	4	0	2	4	0
April	13	2	9	0	1	2	8
May	9	6	3	0	1	4	4
June	9	8	1	0	8	1	0
July	30	24	4	2	23	7	0
August	21	20	0	1	19	2	0
September	28	24	3	1	23	4	1
Totals	**128**	**90**	**30**	**8**	**84**	**29**	**15**

Since July the number of viruses received has increased enormously, reflecting the increase in outbreaks. Since then 73 isolates have been from chickens, 29 from pigeons and 15 from exotic birds.

The viruses isolated from chickens, turkeys, ducks, geese and pheasants usually had ICPI values of more than 1.5. Whereas the viruses from pigeons were mostly mesogenic [Table 3]. However, in areas with outbreaks of ND in chickens, viruses with ICPI values >1.5 were also isolated from pigeons.

Table 3: NDV isolates from pigeons during 01.01.1993 to 30.09.1993

Ref. no.	Month isolated	Bundesland	ICPI
7	March	Brandenburg	1.04
8	March	Brandenburg	1.09
9	March	Brandenburg	1.44
16	April	Sachsen-Anhalt	1.56
17	April	Sachsen-Anhalt	1.78
22	May	Brandenburg	0.60
26	January	Baden-Wurtemberg	1.12
28	February	Baden-Wurtemberg	1.12
29	March	Hessen	1.24
31	January	Hessen	0.41
33	February	Baden-Wurtemberg	0.98
40	May	Nordrhein-Westfalen	1.30
49	June	Nordrhein-Westfalen	1.43
64	July	Brandenburg	1.45
67	July	Bayern	1.65
73	July	Brandenburg	1.20
74	July	Brandenburg	0.95
75	July	Brandenburg	0.93
76	July	Bayern	1.88
82	July	Bayern	1.88
88	May	Hessen	1.88
90	May	Hessen	1.08
95	August	Bayern	1.88
112	August	Brandenburg	0.44
126	September	Brandenburg	1.66
127	September	Brandenburg	1.80

In exotic birds all forms of pathotypes occurred [Table 4]. Two paramyxoviruses isolated from parrots were identified as PMV-3 viruses which showed little or no pathogenicity for day-old chicks in ICPI tests.

Table 4: Haemagglutinating viruses isolated from exotic birds during 01.01.1993 to 30.09.1993.

Ref. no.	Type of bird	Serotype	ICPI
3	parakeet	PMV-1	1.25
23	amadine	PMV-1	0.00
27	budgerigar	PMV-1	1.13
34	parrot	PMV-1	1.03
35	penguin	PMV-1	1.20
36	penguin	PMV-1	1.05
37	parrot	PMV-3	0.00
38	parrot	PMV-3	0.09
39	parrot	PMV-1	1.08
41	parakeet	PMV-1	0.80
42	parrot	PMV-1	1.19
43	parrot	PMV-1	1.04
89	parakeet	PMV-1	1.88
91	parrot	PMV-1	1.88
92	ibis	PMV-1	1.80
93	parrot	PMV-1	1.78

Further typing and characterisation of the ND isolates has not been possible to date, due to the resources of the National Laboratory being stretched to full capacity dealing with the routine identification and pathogenicity tests associated with the large number of isolates received from the regional laboratories.

REPORT ON THE CURRENT NEWCASTLE DISEASE SITUATION IN THE NETHERLANDS

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Incidence of Newcastle disease [ND] in the Netherlands during 1990 to 1993.

During 1990 no suspected outbreaks were reported. In December 1991 one ND outbreak was diagnosed in backyard poultry.

Between August 1992 and October 1993 60 outbreaks were reported [Table 1].

Table 1: Outbreaks of Newcastle disease in The Netherlands during 1991 to 1992

Year	quarter	backyard poultry	poultry-industry
1990		0	0
1991	(December)	1	0
1992	first	0	0
	second	0	0
	third	0	7
	fourth*	**2**	**30**
1993	first	7	5
	second	1	0
	third	5	1
Total		**16**	**43**

* All commercial and backyard flocks were vaccinated with live ND-vaccine in the first week of October 1992. In November 1992 the vaccination-schedules were strengthened thoroughly for commercial poultry.

Answers to the ND-challenge

- Improvement of hygienic management especially on layer, layer-pullet, and broiler farms.
- An increased level of immunity by improvement of the immunisation procedures (with relation to schedules as well as application). Veterinarians of the Animal Health Services visited many poultry farms to control vaccination procedures.
- Improvements with relation to the monitoring of ND-immunity in all categories of poultry, e.g. all layer farmers are obligated to send eggs (every 8 weeks) to the laboratories of the Animal Health Services.

Changes in vaccination schedules ND (L = live vaccine);

BROILERS

< Nov.'92	**> Nov.'92**
day 1 / L, **or**	day 1 / L, **and**
day 7-12 / L, **or**	day 14-20 / L
incidentally: day 1 / L **and** day 14-20 / L	

LAYERS and BREEDERS

< Nov.'92	**> Nov.'92**	**> June '93**
day 1-10 / L	day1 / L**+OEV**	day 1 / L
		week 3-4 / L
week 5 / L		
	week 8 / L	week 8 / L
		week 12 / L
	week 14 / L	
week 15 / L		
breeders: OEV !	week 16-18 / OEV	week 16-18 / OEV

** During several months farmers, in the south-eastern part of The Netherlands, were obligated to vaccinate their layers and breeders (by their veterinarian) every 6 weeks, starting at 6 weeks after the last vaccination in the rearing-period

Aerosol-application of live-vaccines is **obligatory**, except the vaccination at the hatcheries at one day of age.

TURKEYS

< Nov.'92	**> Nov.'92**
BREEDERS	
day 7 / L	day 1 / L
	week 3 / L
	week 8-10 / L
week 12 / L	
further vaccinations: depending on level of immunity HI-titre < 7,5 revaccination ! (L, or OEV)	further vaccinations: depending on level of immunity HI-titre < 7,5 revaccination ! (L, or OEV)
MEAT-TURKEYS	
day 7 / L	day 1 / L
	week 3 / L
	week 8-10 / L
week 12 / L	
week 16 / L*	week 16 / L*

*if they are slaughtered > 18 weeks

Conclusions

- A sound ND-control management (hygienic management and vaccination-policy, including HI-monitoring) on breeder farms - implemented in the seventies - resulted in only 3 ND-infected breeder flocks with very low mortality and minor respiratory signs.
- The improved vaccination-policy with regard to layer pullets/layers has resulted in an impressive increase of the HI-titres.

- The paucity of knowledge concerning the presence of poultry pathogens in backyard poultry is an economical threat for the poultry-industry.
- As long as it is possible to import pet birds and backyard poultry, the poultry-industry is obligated to ascertain a sound vaccination policy.
- From the hygienic point of view special attention is necessary to specialised poultry-personnel (vaccination, debeaking and "removal"), "between-times-removal" of broilers, and egg trays.
- Good management practices with regard to the prevention of ND on poultry farms depends always and everywhere on strict application of HYGIENIC MEASURES AND VACCINATIONS.

Recommendations

- Principally, it has to be prohibited to import pet/hobby birds, unless one has fulfilled certain conditions, e.g. quarantine
- Poultry farmers should be required to obtain a certificate proving that the hygienic management at their farm is beyond doubt.
- Special attention is required for the possibility of spreading NDV by means of slaughterhouse crates.
- The ND vaccinations have to be performed at each poultry farm using a spray, or aerosol apparatus that is always present on that farm. It should be forbidden to use this apparatus at other poultry farms.
- To adjust the ND-vaccination policy, possibilities for control and sanctions have to be established by law for all categories of poultry included backyard poultry and racing pigeons. It has to be made compulsory that each farmer submits his flocks to regular monitoring with regard to the level of immunity against ND.
- The poultry industry needs a special hygiene policy directed to ND, including detailed instructions for treatment and transport of manure and killed poultry.
- It is necessary to improve and promote diagnostic work with relation to backyard poultry and pet/hobby birds.
- At any moment a ND-control-team/task-force has to be prepared and equipped with the necessary mandate for issuing national measures.

CURRENT SITUATION OF NEWCASTLE DISEASE VIRUS IN BELGIUM

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The first case of velogenic Newcastle disease [ND] in chickens was diagnosed in February 1992. At that time, vaccination against ND was not compulsory. As the disease became rapidly epizootic, compulsory vaccination of all poultry was imposed from the beginning of May 1992 by the Belgian Veterinary Service.

From the end of June until November 1992, this policy seemed to be very effective as no new outbreaks were observed. However, in November two new cases were diagnosed in industrial broiler flocks located in two different provinces: Oost and West Vlaanderen. A further eleven outbreaks were diagnosed in industrial poultry flocks, mainly in broilers, during 1993 [Table 1].

Table 1: Outbreaks of Newcastle disease in industrial poultry in Belgium

Case No.	Date	Province	Type of production	Vaccination
1.	11.11.92	Oost Vlaanderen	broilers 20,000 42 days old	NDW spray day 1
2.	20.11.92	West Vlaanderen	broilers 12,300 46 days old	Hitchner spray day 1
3.	17.01.93	Antwerpen	broilers 46,200 41 days old	NDW spray day 1
4.	22.01.93	Oost Vlaanderen	layers 7,927 41 weeks old	?
5.	22.01.93	Antwerpen	broilers 18,200 38 days old	$^1/_2$ dose NDW day 1
6.	12.02.93	Oost Vlaanderen	broilers 19,500 41 days old	NDW spray day 1

7.	12.02.93	Antwerpen	broilers 18,500 42 days old	NDW spray day 1
8.	19.03.93	Antwerpen	broilers 40,151 40 days old	NDW spray day 1 La Sota clone 30 day 12
9.	04.04.93	Brabant	layers 730 six months to 3 years old	unvaccinated
10.	23.04.93	Limburg	broilers 10,400 28 days old	NDW spray day 1 La Sota clone 30 day 12
11.	23.04.93	Liege	label chickens 7-14 weeks old Guinea fowl 5-8 weeks old	chickens: NDW spray day 1 La Sota clone 30 day 14 Guinea fowl: La Sota clone 30 day 14
12.	11.06.93	Antwerpen	broilers 30,000 28 days old	NDW spray day 1 La Sota clone 30 day 11
13.	30.07.93	West Vlaanderen	broilers 9,208 35 days old	NDW spray day 1 La Sota clone 30 day 18

In all cases a stamping out policy was applied by the Veterinary Services and at the same time blood samples were taken in order to evaluate the vaccination status of the affected chickens. The results of the haemagglutination inhibition tests gave titres which indicated clearly that broilers which had been vaccinated only once, at the hatchery, had insufficient antibody levels at the end of the growing period. Consequently, the existing legislation was modified. A second vaccination of broilers using the La Sota strain given in the drinking water between 10 and 18 days of age was made compulsory from the end of January 1993.

From 1st July 1992 to 30th June 1993, we performed 826 viral examinations. Numerous velogenic ND viruses were isolated from backyard flocks and cage birds. These strains were antigenically indistinguishable from viruses isolated from industrial poultry flocks and indistinguishable from the first isolate obtained in The Netherlands in December 1991 (D.J. Alexander and R.J. Manvell). We conclude that the circulation of pathogenic ND viruses in the hobby sector represents a considerable risk of contamination for the industrial poultry sector.

The pathogenicity characteristics of some of the ND viruses isolated from backyard flocks and cage birds are shown in Table 2.

Table 2: Pathogenicity of some NDV isolates from backyard flocks and cage birds.

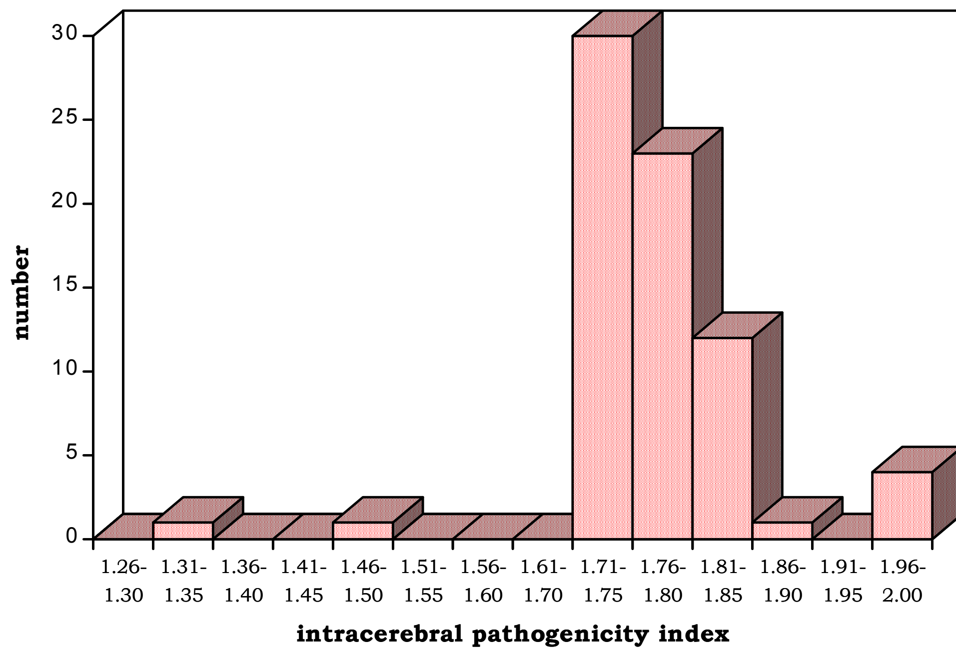
Virus	Species	ICPI	IVPI*
93/101	pheasant	1.76	2.44
93/112	parrot	1.75	N.D.
93/113	parrot	1.76	2.58
93/118	peacock	1.85	N.D.
93/145	peacock	1.75	N.D.
93/159	canary	1.81	N.D.
93/261	pigeon	1.34	0.14
93/257	dwarf-chicken	1.99	2.55
93/236	broilers	2.00	2.64

*determined at the Community Reference Laboratory

We have determined the intracerebral pathogenicity indices (ICPI) of 72 NDV isolates during the present epizootic [Fig. 1]. The distribution of the ICPI values is highly homogenous Only two viruses isolated from pigeons had ICPI values lower than 1.70. The antigenic characterisation of these two viruses using monoclonal antibodies showed that they belonged to the "pigeon variant" type of NDV.

In conclusion, two types of velogenic NDV are still circulating in Belgium: the "pigeon" virus which to date remains restricted to that species and the chicken velogenic virus which has been the aetiologic agent of the current epizootic in industrial poultry and backyard flocks.

Figure 1: ICPI values of 72 NDV strains isolated since the beginning of the epizootic in Belgium



NEWCASTLE DISEASE IN PORTUGAL: SITUATION REPORT - OCTOBER 1993

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The 1982 Newcastle disease outbreak in pigeons was followed by a silent period of the disease that ended in 1990. Four outbreaks occurred that year in chickens in the North region of the country. Several outbreaks have since been reported and confirmed by virus isolation. Despite the efforts of the veterinary services to control the disease, the situation became aggravated in summer 1993. The areas afflicted by the disease include the North and centre of the country where most of the poultry units are located.

In April, 1993 an outbreak was reported in Madeira island. Stamping-out and massive vaccination were applied and in June the situation was controlled. No new outbreaks have since been reported in the island.

The majority of the isolates caused severe symptoms and high mortality. Respiratory, digestive and nervous involvement was observed in layers and broilers, whereas in turkeys and pheasants, paralysis was the most common sign.

All the isolates have been submitted to the International Reference Laboratory for avian Ortho- and Paramyxovirus, CVL - U.K., for antigenic characterisation and assessment of virulence (Tables 1 and 2).

The use of monoclonal antibodies (mAbs) and polyclonal antiserum in haemagglutination inhibition and indirect immuno-peroxidase (IIP) tests revealed that the isolates of 1991, 1992, and 1993 are indistinguishable from each other but distinguishable from the 1990 isolates. The binding patterns with mAbs also showed that the Portuguese viruses are distinct from the viruses currently causing problems in northern Europe. In accord, with the results of the HI and IIP tests, two viruses, labelled 6134/91 and 6334/93, were placed in group E (La Sota).

The intravenous pathogenicity index (IVPI) was determined on six isolates (Table 1). The values obtained ranged from 2.45 to 2.78, indicating that these viruses are highly virulent for chickens. This is in accordance with the severe clinical signs, lesions and mortality observed in the

correspondent outbreaks. The intracerebral pathogenicity index (ICPI) of the isolate 6334/93 (ICPI = 0.125) confirmed that this virus was to be placed in group E. However, respiratory distress, diarrhoea and high mortality (>60%) was observed in this case. Necropsy on these birds revealed pulmonary congestion, enteritis and haemorrhages in the proventriculus. Isolate 6134/91 also placed in group E was obtained from 5- to 6-week-old birds showing marked respiratory lesions (tracheitis and pulmonary congestion) and high mortality (> 50%). In both cases the birds had not been vaccinated, however, La Sota vaccine had previously been used in the poultry unit where isolate 6334/93 was obtained. In this unit, vaccination was abandoned approximately four months before this outbreak was reported. Virus 1800/93 was isolated from broilers showing subclinical symptoms and 7.3% mortality at 45 days.

The origin of the recent outbreaks is yet to be determined. Measures aiming to prevent the spreads of Newcastle disease are been used by the official services.

Table 1: Biological properties of 1990-1993 Portuguese isolates of Newcastle disease virus

Viruses	IVPI	ICPI	Signs	Host	Location
5548/90			severe	Fowl	D.R.E.D.M.
5928/90			severe	Fowl	D.R.E.D.M.
6464/90			severe	Fowl	D.R.E.D.M.
8234/90			severe	Fowl	D.R.E.D.M.
6134/91			severe	Fowl	D.R.E.D.M.
8526/91			severe	Turkey	D.R.A.
8981/91			severe	Fowl	D.R.B.L.
9484/91			severe	Fowl	D.R.E.D.M.
10075/92	2.56		severe	Fowl	D.R.E.D.M.
12154/92	2.65		severe	Fowl	D.R.E.D.M.
113/93	2.50		severe	Fowl	D.R.E.D.M.
1800/93			subclinical	Fowl	D.R.R.O.
2585/93	2.45		severe	Fowl	D.R.E.D.M.
5136/93			severe	Fowl	Madeira
5742/93			severe	Fowl	D. R. B. L.
6042/93			severe	Fowl	D. R. B. L.
6130/93			severe	Fowl	Madeira
6132/93	2.74		severe	Fowl	Madeira
6133/93			severe	Fowl	Madeira
6277/93			severe	Fowl	Madeira
6334/93		0.125	severe	Fowl	D.R.R.O.
6479/93			severe	Fowl	Madeira
6558/93	2.78		severe	Fowl	D.R.E.D.M.
8509/93			severe	Fowl	D.R.R.O.
8546/93			severe	Fowl	D.R.R.O.
8646/93			severe	Fowl	D.R.R.O.
8997/93			severe	Turkey	D.R.R.O.
9140/93			severe	Fowl	D.R.E.D.M.
9186/93			severe	Pheasant	D.R.R.O.
9323/93			severe	Fowl	D.R.R.O.
9324/93			moderate	Fowl	D.R.R.O.

SESSION II - EC COMMISSION AND REFERENCE LABORATORY REPORTS

EC REFERENCE LABORATORIES FOR NEWCASTLE DISEASE AND AVIAN INFLUENZA: COMMISSION REPORT OCTOBER 1993

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Introduction

In this paper I would like to examine the Newcastle Disease situation in recent times from the Community viewpoint and then go on to look at how recent additions to Community legislation will influence how we deal with this disease in the future.

It is particularly appropriate for us to meet to discuss Newcastle disease at this point because 1993 has proved to be the worst year in recent times in terms of outbreaks of this disease. Low disease levels occurred from 1986 to 1991 with the exception of 1988 when 45 outbreaks were confirmed. In 1992 however, 83 cases were reported, and already by this September, 1993 has exceeded that figure.

ND situation 1992-1993

An examination of the monthly outbreaks at the end of 1992 shows an increase in the disease level towards the end of the year. In this period the disease appeared mainly in the Netherlands and Belgium with 12 cases linked to these being identified in France in December. The Commission organised a mission to the Netherlands which agreed with the views of the authorities there, that in spite of a compulsory vaccination policy, the disease had been spreading as a result of poor immunity levels in commercial flocks. In addition, a complacent attitude had developed towards the disease. Following the mission a stricter vaccination regimen was put in place and emphasis was placed on correct vaccination techniques. These measures were accompanied by a drop in the number of outbreaks. Surveys undertaken on the vaccinated flocks in the Netherlands following the implementation of these measures indicate that immunity levels have improved.

Following this, as we entered 1993, disease levels initially dropped until a low was reached in May with only a single case reported. From this point on a major escalation of the disease occurred with this time the outbreaks being concentrated in the Netherlands, Germany, Belgium

and Luxembourg. In August and September, 11 cases were also diagnosed in Portugal. All indications are that the outbreaks in Portugal are a separate occurrence and not related to those in other parts of the Community. So far in 1993, Newcastle disease has been notified in six member states.

Current epidemiological and control problems.

When we look behind these figures however, we see a different picture. If we split the outbreaks up into those affecting commercial and non commercial or hobby flocks we see that the outbreaks in commercial flocks have remained relatively stable. In contrast the number of outbreaks in hobby flocks has shown a considerable increase since May. These have occurred in the Benelux countries and in Germany. Similarly, an examination of the size of the affected flocks shows that almost half (47%) of the outbreaks occurred in flocks of under 100 birds. In contrast 39% of the outbreaks were confirmed in flocks of over 1000 birds.

The purchase of birds was, overwhelmingly, the most common means of transmission with 60% of cases being reported as being due to this source, 36% unknown and 2% each for spread by neighbourhood contact and by fomites.

It is also worth noting that the non-vaccinating parts of the Community have reported no cases so far this year even though, of course, the population there is totally susceptible.

Thus it appears that we have two disease management problems in the Community.

These are:

1 inadequate immunity levels in some vaccinated flocks in the vaccinating part of the Community
2 presence of the virus in non commercial birds

I will deal with each of these in turn but its worth noting that either of these undesirable situations could exist in a Member State without a disease problem becoming evident.
An exception to this obviously exists in the regions where for economic and policy reasons, vaccination is not practised. Here the disease should be quickly apparent because of the presence of the susceptible population. However one would also expect spread to be more rapid.

The first point that we identified was the inadequate protection of flocks. It was surprising that this was identified in the Netherlands where a compulsory vaccination policy was in operation. Inadequate immunity levels could have been due to either:

1. inadequate vaccination regimens
2. inadequate vaccination administration technique
3. factors interfering with the efficacy of the vaccine

The situation in the Netherlands occurred in spite of what was, at that time, the strictest compulsory vaccination programme in the Community. However, following stricter enforcement of their scheme, immunity levels appear to have risen significantly. We still however have to bear in mind the possibility that vaccinated flocks could exist which harbour the disease virus without showing clinical signs. The episode demonstrates that tough regimens or legislation by themselves are not sufficient to tackle this disease effectively. Not only the virus but also complacent attitudes must be attacked.

Secondly, the presence of the disease in non commercial or hobby flocks is a more difficult problem to tackle. In general, these birds are under less control by the official veterinary services, they have less effective vaccination cover, they may not be located in traditional poultry areas and, particularly importantly, they sometimes trade with other hobby flocks, often in small lots of birds of varying ages. Recent work in Belgium has linked the transport and dealing network to the spread of disease and the substantial number of outbreaks where the purchase of birds was given as the source of the outbreak would tend to support this. Thus the continuing presence of virus in this sector must be a cause for concern. A much more unpalatable proposition, and one not supported by the evidence, could be that the virus is present in the commercial flocks, not causing disease because of vaccine cover but yet spilling over into the non commercial sector.

It is essential that we obtain a drastic reduction in the number of outbreaks of this disease. Apart from the disease losses and the additional expenses of stricter vaccination regimens, the disease is causing expense and disruption to the Member States and to the poultry industry.

New control directive

The new Newcastle disease control directive 92/66/EEC is at the time of speaking less than a week in being since it came into force on 1st October 1993. We will be using its provisions to assist the Community and the Member States to deal with the situation.

The directive in general sets down detailed rules and procedures for dealing with an outbreak of the disease. Of particular interest to yourselves are Annexes III, IV and V which set out the diagnostic procedures for the confirmation and differential diagnosis of Newcastle disease and list the national laboratories and specify the duties of the Community Reference Laboratory.

The Directive also requires member states to implement certain measures as regards outbreaks of Newcastle disease. I propose just to mention these since they are all fairly self explanatory. For examples:

- The suspect premises must be restricted pending confirmation of the disease and an epidemiological investigation must be carried out. Upon confirmation, the Directive in general requires the slaughter and destruction of all poultry on the holding. Feed, litter and anything liable to be contaminated must be destroyed. Meat and hatching eggs liable to be produced during the incubation period must be destroyed where they can be traced. The buildings must be cleaned and disinfected and any linked premises must also be restricted. Two zones must be set up around the infected premises, a protection zone of minimum radius of 3km and a surveillance zone of at least 10km. Geographical and ecological etc. factors must be taken into account in drawing up these areas.

- Within the protection zone all flocks must be identified and subjected to a periodic clinical examination. Poultry must be kept indoors and movement of birds and people must be controlled. In general movements of poultry is prohibited except under licence to slaughter and for day old chicks, point of lay pullets and hatching eggs subject to strict limitations. Finally the spreading of poultry manure or litter is banned unless officially authorised and the holding of markets or gatherings of poultry or other birds is prohibited. These measures last for at least 21 days following which the measures in existence in the surveillance zone will apply.

The measures in the surveillance zone are generally the same as in the protection zone except that inspection of flocks is not necessary and less severe prohibitions apply to the movement of poultry. All these requirements are listed in article 9 of the Directive.

From the point of view of dealing with the disease at Community level the following provisions are of interest:

1. Article 21 provides that Member States must submit contingency plans for dealing with the disease to the Commission within six

months. Several have already been received. It is anticipated that these will be examined, amended if necessary and approved by the Standing Veterinary Committee in the coming months. This is to ensure that all Member States have the necessary structure, facilities and organisation in place to combat this disease effectively.

2. Article 17 provides that Member states that have a voluntary or compulsory vaccination programme must submit full details of this programme to the other Member States and to the Commission. This is for information purposes but this information can be acted on under Article 18.

3. Article 18.6 provides that in a disease situation, where non routine vaccination is being practised, the Commission, again through the Standing Veterinary Committee can take additional decisions relating to movement control or to vaccination policy. This disease situation now applies in some parts of the Community. Thus the Community, through the standing Committee, could decide to supplement or amend the vaccination regimen in such an area or to impose additional movement controls on the affected parts of that Member State.

4. The Directive also institutionalises practices which, though not universal, were accepted practice in most but not all of the Community. For example, stamping out must now include all the birds on a holding, both the infected and the in contacts (Art.5). Another example, which concerns yourselves, is that all haemagglutinating agents should now be sent to the Community Reference Laboratory (Annex III, Chapter 3). The need for epizootiological enquiries in the case of outbreaks is emphasised (Art. 7)

5. We also have available, as in the past, the tool of the Safeguard decision in Directive 90/425/EEC which provides for the taking of almost any measures deemed necessary to prevent the spread of serious animal disease between Member States. For other diseases in the past these measures have included movement controls or total bans from regions or indeed entire Member States, additional vaccination or testing requirements or the need for additional veterinary conditions or certification.

Your work, in particular the identification of viruses and their typing, the monitoring of flocks and epizootiological work, are absolutely vital to the task of combating this disease. It is to be hoped that now that we have the legislation and laboratory structure in place at a Community

level we will see progress in the future towards achieving major improvements in this disease in the Community.

REPORT OF THE EUROPEAN COMMUNITY REFERENCE LABORATORIES FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

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Introduction

The Central Veterinary Laboratory, Weybridge was designated the European Community Reference Laboratory for Avian Influenza in Directive 92/40/EEC [1] and the Reference Laboratory for Newcastle disease in Directive 92/66/EEC [2] to come into force on 01.01.93 and 01.10.93, respectively. The Directives also specify the functions of the Laboratory and the role that it will play in the diagnosis and control the diseases in the Community.

The designation of the CVL, Weybridge as these reference laboratories augmented the historical and continuing role CVL, Weybridge has played as a International Reference Laboratory for Avian Influenza and Newcastle disease recognised by both the Food and Agriculture Organisation of the United Nations [FAO] and Office Internationale des Epizooties [OIE].

The functions of these laboratories has been broadly similar to those outlined in the EC directives, but, in fact, the major demand on the reference laboratory has been to receive isolates of haemagglutinating viruses to identify or confirm diagnoses made in the country of origin.

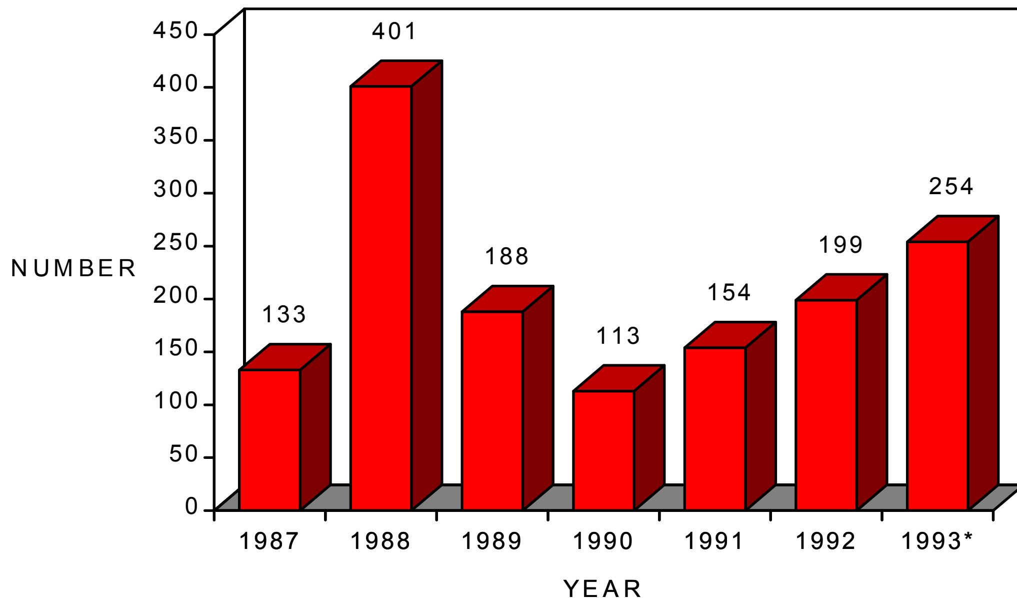
Viruses received by International Reference Laboratory

The number of viruses submitted by all countries to the reference laboratory during the years 1987 to 01.10.1993 is shown in Figure 1. The number of viruses submitted in a particular year does not necessarily mean that the viruses were isolated that year and the Laboratory is often asked to undertake retrospective characterisation of past isolates.

The numbers of viruses received during 1987-1989 were largely those of the so-called pigeon variant PMV-1 virus submitted by laboratories wishing to have confirmation of this. Since 1990 there has been a

gradual increase in viruses received, most of the increase has come from EC countries, reflecting the outbreaks of Newcastle disease in the EC Member States and also the awareness of the role of the Reference Laboratory following the publication of the directives.

Figure 1: Viruses submitted to the International Reference Laboratory.



* to 01.10.93

Influenza viruses submitted during 01.01.1993 to 01.10.93

In the first nine months of 1993 a total of 26 influenza viruses were received at CVL, Weybridge from nine different countries. The countries, number of viruses and subtype characterisation are shown in Table 1.

The two H7N2 viruses received from Italy which had been isolated from feral gulls [3] had low intravenous pathogenicity indices in six-week-old chickens and were subjected to nucleotide sequencing to determine the amino acids at the cleavage site of the haemagglutinin [done by Geoff Wood] as required in Directive 92/40/EEC . For both the sequence was **...PEIPKGR*GLF...** i.e. the viruses do not have additional basic amino acids at the cleavage site and do not fall within the definition of avian influenza for which the EC control policies will be implemented.

Table 1: Submissions of influenza viruses to the International Reference Laboratory during 01.01.93 to 01.10.93

Country	Number	Subtype[s]
Italy	2	H7N2
Ireland	1	H3N8
	2	H9N3
Singapore	1	H4N1
	2	H4N6
	2	H10N6
U.S.A.	1	H7N1
	2	H5N2
Canada	1	H6N8
Cyprus	3	H4N6
South Africa	4	H4N6
Great Britain	4	H4N6
	1	H6N8

not all viruses received had been isolated in 1993

Paramyxoviruses received during 1992 to 01.10.1993 from European Community Countries.

Of the 199 viruses received during 1992, 96 were paramyxoviruses submitted by EC countries and in the first nine months of 1993 119 of the 254 submissions fell into this category. The countries submitting viruses are listed in Table 2. In 1992 all viruses were of PMV-1 serotype except a single PMV-3 virus isolated from birds in quarantine in Great Britain and a PMV-7 virus from pigeons isolated in Ireland. In 1993 three PMV-7 viruses were received which had been isolated from snakes in Germany.

In addition to those figures shown in Table 2, 43 isolates in 1992 and 16 in 1993 from pigeons in Great Britain were identified as the "pigeon PMV-1 variant".

Not all the viruses shown in Table 2 were isolated in the year they were received. For example, 58 of 61 viruses from Germany received in 1993 were isolates made during a survey of waterfowl over the period 1981-1990. Similarly, 14 of the isolates received from Italy in 1992 had been isolated between 1968 and 1984. In addition, a number of the viruses

received proved to be isolations of live vaccines that had been licensed for use in the country of origin.

Table 2: Paramyxoviruses received from EC countries

Country	PMV-1	PMV-3	PMV-7
1992			
Belgium	4		
France	1		
Germany	6		
Great Britain	3	1	
Italy	48		
Ireland	7		1
Netherlands	13		
N. Ireland	5		
Portugal	8		
1993			
Belgium	9		
Denmark	2		
France	3		
Germany	61		3
Great Britain	3		
Italy	25		
Ireland	2		
Netherlands	3		
Portugal	14		

not all viruses received had been isolated in 1992 or 1993

Characterisation of viruses using monoclonal antibodies.

All viruses received during 1992-1993 were characterised by their ability to cause binding of a panel of 28 monoclonal antibodies [mAb] to cell cultures infected with the viruses. For convenience and the need to summarise, the results are shown in mAb groups in Table 3. These groups are based on those described by Russell and Alexander [4,5] using only 9 mAbs. Generally, viruses in the same group show identical binding patterns for the 28 mAbs used. However, some isolates placed in the same group may vary by their ability to react with one or two mAbs.

All viruses placed in the same group tended to share the same biological properties and for convenience the groups have been separated in Table 3 on the basis of virulence for chickens.

The 15 isolates received from Portugal related to the outbreaks occurring during 1991 to 1993, these viruses showed a unique mAb binding pattern and were not grouped with any other viruses [the 5 Italian isolates listed under no group in Table 3 were also distinct from the Portuguese isolates]. Interestingly, the 1992-1993 Portuguese isolates were distinguishable from the viruses causing outbreaks in Portugal in 1990.

Table 3: Antigenic grouping of viruses isolated during 1992-1993.

Country	**Monoclonal antibody binding group**										
	velogenic						**lentogenic**				
	B	**C1**	**D**	**P**	**NE**	**no***	**C2**	**E**	**F**	**G**	**H**
Belgium				1**	12						
Denmark				2							
France					2					2	
Germany	1		1	3	1		2	4		18	37
Gt. Britain				59				4		1	1
N. Ireland										5	
Ireland				6						3	
Italy	8	1	8	4	11	5		26	7	1	
Netherlands					16						
Portugal		4	1			15		2		1	

* binding pattern does not conform to any group
** number of isolates. Not all viruses had been isolated in 1992 or 1993

Viruses placed in group NE [for North European Community] all showed identical unique binding patterns [with the exception of three 1993 isolates from The Netherlands which bound one mAb that the others did not] and were associated with the outbreaks occurring since 1991 in The Netherlands and Belgium. Two viruses associated with outbreaks in France in 1992 were also indistinguishable, as were isolates received from Germany, Italy and Malta.

References

1. CEC 1992a. Council Directive 92/40/EEC introducing Community measures for the control of avian influenza. Official Journal of the European Communities No. L 167 1-16.

2. CEC 1992b. Council directive 92/66/EEC of 14 July 1992 introducing Community measures for the control of Newcastle disease. Off. J. Europ. Commun. L260: 1-20.
3. Papparella, A. Fioretti, L.F. Menna. The epidemiological situation of avian influenza in Italy from 1990 to 1993 in feral bird populations and in birds in quarantine. *These Proceedings.*
4. Russell, P.H. and Alexander, D.J. 1983. Antigeni variation of newcastle disease virus strains detected by monoclonal antibodies. Arch. Virol. 75: 243-253.
5. Russell, P.H. Samson, A.C.R. and Alexander, D.J. 1990. Newcastle disease virus variations. In Applied Virology Research Vol. II edited by Kurstak, E., Marusyk, R.G., Murphy, F.A. & Regenmortel, M.H.V. Plenum: New York pp177-195.

SESSION III - NATIONAL LABORATORY FACILITIES

This session consisted of an open discussion of the facilities, including equipment and reagents, available, currently and ideally, to the National Laboratories and the effect these have on their ability to carry out diagnosis of the two diseases effectively and safely within the demands of the Directives. Several resolutions were drafted for Session V.

SESSION IV - ORIGINAL CONTRIBUTIONS

BLOCKING ELISA TO DETECT GROUP-SPECIFIC ANTIBODIES AGAINST INFLUENZA VIRUS.

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NO MANUSCRIPT RECEIVED

DIFFERENTIATION OF NDV STRAINS OF DIFFERENT VIRULENCE: RECENT DEVELOPMENTS

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NO MANUSCRIPT RECEIVED

QUESTIONS RAISED BY THE HIGHLY PATHOGENIC AVIAN INFLUENZA OUTBREAK IN NORFOLK, ENGLAND IN 1991.

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Introduction

In Council Directive 92/40/EEC introducing measures for the control of avian influenza [1] which came into force on 1st January 1993 the following definition was used for avian influenza for which the control measures would apply:-

"...an infection of poultry caused by any influenza A virus which has an intravenous pathogenicity index in six-week-old chickens greater than 1.2 or any infection with influenza A viruses of H5 or H7 subtype for which nucleotide sequencing has demonstrated the presence of multiple basic amino acids at the cleavage site of the haemagglutinin."

This definition reflects the current understanding of highly pathogenic avian influenza viruses [HPAI]. While, to date, all HPAI viruses have been of H5 or H7 subtype, not all viruses of these subtypes are HPAI. In addition, it has been shown that influenza virus particles are only infectious after post-translation cleavage of the precursor H0 haemagglutinin molecule to H1 and H2 by host enzyme[s] has taken place and that while avirulent viruses are restricted to cleavage by trypsin-like enzymes, there appears to be a ubiquitous enzyme[s] capable of cleaving HPAI viruses. This difference appears to be related to the number of basic amino acids at the cleavage site, the HPAI viruses having, either by apparent insertion or apparent substitution, multiple basic amino acids at that site [2]. The cleavage sites for H5 viruses of high and low pathogenicity are shown in Table 1 and demonstrate this point.

An exception to the rule was the low virulence of virus chicken/Pennsylvania/1/83 which had the sequence P-Q-K-K-K-R at the cleavage site. Retrospective analysis showed that this was due to a glycosylation site sequentially distant but structurally close to the cleavage site, which, it was assumed, on glycosylation resulted in a carbohydrate chain which interfered with access to the cleavage site by

the ubiquitous enzyme [3]. Loss of this glycosylation site by point mutation occurred in the field and resulted in the emergence of HPAI virus with unchanged amino acids at the cleavage site. [Table 1]. Subsequent work by Kawaoka et al [4] and Klenk et al [5] has shown that glycosylation at that site does not affect the pathogenicity of viruses which, unlike Pennsylvania/83, have more than the minimum number of amino acids at the cleavage site.

Table 1: Deduced amino acid sequences at the haemagglutinin cleavage sites of influenza A subtype H5 viruses

H5 viruses		
Virus	Pathogenicity for chickens	Amino acid sequence at the H cleavage site
chicken/Scotland/59	high	PQ**RKK**....**R***GLF
tern/S.Africa/61	high	PQ**R**ET**RR**Q**KR***GLF
turkey/Ireland/83	high	PQ**RKRKK**..**R***GLF
turkey/Ontario/66	high	PQ**RRKK**...**R***GLF
SEVEN ISOLATES	low	PQ**R**ET....**R***GLF
chicken/Penn./1/83	low	PQ**KKK**....**R***GLF
chicken/Penn./1370/83	high	PQ**KKK**....**R***GLF

basic amino acids are shown in bold. * represents the cleavage site

Isolates from the Norfolk 1991 outbreak

The outbreak in Norfolk, England occurred during December 1991 and in a six day period leading up to 24th December over 7,000 deaths occurred in a flock of 18-week-old turkeys leaving about 600 survivors [6].

A total of four influenza viruses was isolated from material available from the outbreak. These viruses and their source are detailed in Table 2.

Table 2. Influenza viruses isolated from the 1991 HPAI outbreak in Norfolk, England.

Isolate	**Source**	**Subtype**	**IVPI**	**Haemagglutinin cleavage site sequence**
50-92	tissues	H5N1	3.00	PQ**RKRK**T**R***GLF
87-92FB	frozen brain	H5N1	3.00	PQ**RKRK**T**R***GLF
87-92RT	respiratory tract of frozen carcase	H5N1	3.00	PQ**RKRK**T**R***GLF

87-92BFC	brain of frozen carcase	H5N1	0.00	PQ**RKRK**T**R***GLF

One isolate, 50-92, was obtained from tissue samples inoculated into embryonated eggs at the time of the outbreak, the others from frozen material, kept several weeks before virus isolation attempts. All viruses were of H5N1 subtype, but, whereas the original virus, a virus isolated from a frozen brain sample and another from the respiratory tract of a frozen carcase gave maximum IVPI values of 3.00, the fourth virus, from brain material from the same carcase as the respiratory tract virus, produced the lowest possible IVPI value of 0.00.

The deduced amino acid sequence at the cleavage site of the haemagglutinin obtained by nucleotide sequencing was identical for all four viruses [Table 2].

The sequence obtained was unusual in that while 5/6 of the amino acids at the H1 C-terminus were basic, the amino acid at position -2, next to the arginine at the cleavage site, was threonine, which is similar to all H5 viruses of low pathogenicity tested to date, but unlike other HPAI viruses which have either arginine or lysine at -2 [7].

Sequencing the H gene of Norfolk isolates and their clones.

Wood et al [8] produced a panel of clones from highly pathogenic isolate 50-92 by plaque-picking which showed a range of values in IVPI tests from 0.00 to 3.00. These authors sequenced the entire H gene of 11 isolates and clones from the Norfolk outbreak and partially sequenced the H gene of a further five clones. These sequences revealed variation at one or more of five nucleotide and four amino acid sites. However, there was no evidence that any of these site variations correlated with pathogenicity for chickens.

All viruses and clones examined had a potential glycosylation site at amino acid residue 26 which corresponds to the glycosylation site that was so important in masking the potential virulence of the virus responsible for the Pennsylvania 1983 epizootic. Its failure to influence the pathogenicity of the Norfolk isolates is in keeping with the presence of more than the minimum number of amino acids at the H cleavage site, as discussed above.

Discussion

The outbreak occurring in Norfolk in 1991 raised several important questions. The onset of the disease was sudden, mainly without clinical signs, and resulted in the death of more than 7000 birds over a six day

period, and yet about 600 birds survived, even though there was serological evidence that they had been infected. One possible explanation of this was that the virus originally infecting the birds was similar to the isolate 87-92BFC, but the potential virulence was realised on passage through the flock and those birds already infected with the virus of low pathogenicity were protected against the consequences of subsequent infection with the HPAI virus. However, it is difficult to explain why a virus of low pathogenicity should be isolated from the carcase of a bird dying in the outbreak and even more difficult to explain why this virus was present in the brain.

The viruses isolated from birds dying in the Norfolk outbreak also showed an unusual deduced amino acid sequence at the H cleavage site. Despite the presence of three basic amino acids close to the cleavage site, representing a nett gain of two amino acids, the Norfolk isolates were unlike all other HPAI viruses in that they possessed a threonine in the -2 postion and not a basic amino acid [Table 1]. This conflicts with the proposed minimum amino acid motif suggested by Vey et al [9]. These authors in a study of haemagglutinin mutants of the HPAI virus chicken/Germany/34 (H7N1) which varied in their ability to be cleaved when expressed in cell culture using an SV40 vector, concluded that for HPAI viruses the minimum amino acid motif leading to the HA1 terminus was **R-X-K/R-R** where X represented any amino acid and K/R either lysine or arginine. They further emphasised the positional importance of the motif, since viruses with the same motif moved even one amino acid residue upstream were unlikely to prove to be HPAI. This may require some modification to **R/K-X-K/R-R** since the chicken/Pennsylvania/83 viruses have the motif **K-K-K-R**. But the Norfolk viruses have the sequence **R-K-R-K-T-R** leading up to the cleavage site which suggests further understanding of the mechanism of pathogenicity is necessary before the minimum amino acid requirement can be stated definitively.

The subsequent findings by Wood et al [8] that variations in the pathogenicity of the Norfolk isolates did not appear to be related to any variation in the haemagglutinin the amino acid sequence suggests that other factors may play an important role in modulating the ostensible pathogenicity of potentially virulent viruses.

References

1. CEC (1992), Council directive 92/40/EEC introducing Community measures for the control of avian influenza. Off. J. Europ. Commun. 167: 1-15.
2. ROTT, R. (1992) The pathogenic determinant of influenza virus. Vet Microbiol. 33: 303-310.

3. KAWAOKA, Y., NAEVE, CW., WEBSTER, RG. (1984). Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the haemagglutinin? Virology 139: 303-316.
4. KAWAOKA, Y., WALKER, JA, WEBSTER, RG (1992) Origin and molecular marker of avian influenza viruses. Proc. 3rd Int. Symp. Avian influenza. Univ. Wisconsin Press: Madison. pp175-181.
5. KLENK, H-D, OHUCHI, M., OHUCHI, R., KONIG, G., ANGLIKER, H., SHAW, E., STIENEKE-GROBER, A., VEY, M., GARTEN, W. (1992). Proteolytic activation as a determinant for the pathogenicity of influenza viruses: substrates and proteases.Proc. 3rd Int. Symp. Avian influenza. Univ. Wisconsin Press: Madison. pp182-198.
6. ALEXANDER, D.J. (1987). Avian influenza - Historical aspects. Proceedings of the 2nd International Symposium on Avian Influenza, Athens 1986. University of Wisconsin : Madison pp 4-13.
7. WOOD, G.W., McCAULEY, J.W., BASHIRUDDIN, J.B. and ALEXANDER, D.J. (1993). Deduced amino acid sequences at the haemagglutinin cleavage site of avian influenza A viruses of H5 and H7 subtypes. Archiv. Virol. 130: 209-217.
8. WOOD, G.W., BANKS, J. McCAULEY, J.W., and ALEXANDER, D.J. (1994). Deduced amino acid sequences of the haemagglutinin of H5N1 avian influenza virus isolates from an outbreak in turkeys in Norfolk, England. Archiv. Virol. in press.
9. VEY M., ORLICH, M., ADLER, S., KLENK, H-D., ROTT, R. and GARTEN, W. (1992). Haemagglutinin activation of pathogenic avian influenza viruses of serotype H7 requires the protease recognition motif R-X-R/K-R. Virology 188: 408-413.

DIRECT IDENTIFICATION AND CHARACTERIZATION OF A-PMV1 FROM SUSPICIOUS ORGANS BY NESTED PCR AND AUTOMATED SEQUENCING

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Summary

A nested PCR test was employed to amplify a 175 bp product centred on the part of the gene encoding the cleavage site of the F protein of Newcastle disease viruses (NDV). Viral RNA was extracted from individual suspect organs (trachea, spleen, brain) or from pools of several suspect organs, using glass milk and guanidine isothiocyanate.

The organs had been collected from chickens infected experimentally as well as from conventional birds submitted to a diagnostic laboratory; both types of organ samples were inoculated in the allantoic cavity of embryonating fowl's eggs and the growth of virus detected by hemagglutinating activity. Very strict conditions were observed in order to avoid contamination of samples during the PCR process.

5/5 samples that gave a positive NDV isolation at the first passage, were positive by PCR. 5/5 samples from which no NDV virus could be recovered after 3 passages, were negative by PCR. 2/2 samples from which NDV was isolated, but only at the second passage, were negative by PCR.

The dye terminator method was used for automated sequencing. Only preliminary results are presented. However the motif of the Essex strain at the cleavage site was confirmed, when compared with published data.

Introduction

The amino acid sequence at the cleavage site of the F protein of Newcastle disease virus [NDV] is different according to the virulence of the strain (2, 6, 8). Consequently, it appeared useful to adapt a technique so that this property could be exploited with the goal of characterising every a-PMV1 isolate. For this reason we first developed a

PCR test in order to amplify the fragment of the gene encoding the cleavage site (3); further, a good correlation between intracerebral pathogenicity index [ICPI] and the sequence obtained for the PCR product was shown (1, 4, 5). These results were obtained employing isolates grown in the allantoic cavity of embryonating fowl's eggs.

Despite the fact that these tests could be carried out without having to use isolators and other experimental animal facilities, the time required to achieve such techniques was not competitive in comparison with ICPI tests (5); thus their systematic use was excluded. In addition, in these studies only one strand of the PCR product was sequenced (1, 4, 5) and that is not completely satisfactory as sequencing both strands would improve reliability and accuracy.

In order : 1) to reduce the time required by the previous technique; 2) to characterise the representative viral population in the organs of avian species without any shift due to culture conditions, we have attempted to improve the method so that it could be applied to amplify viral RNA extracted from suspected organs. Moreover, it was decided to try to sequence both strands to increase accuracy. At the same time, the availability of an automated sequencer prompted us to adapt the technique to incorporate this equipment.

Materials and Methods

Samples

All the organs examined had been stored at -20°C for at least 6 months and up to three years. Two kinds of samples were used :

1 - trachea that had been collected from experimental 40- to 42-day-old chickens challenged with the Essex '70 NDV strain and maintained in strict isolation; four individual samples were tested, two had been collected 5 days after the challenge, in such a way that RNAse contamination was limited, and stored for 16 months. The others had been collected 6 days after the challenge, without any precautions with regard to RNAse contamination, and stored for 3 years as supernatants of 10 % suspensions of the ground organs in RNAse free medium. From these organs, NDV was recovered at the first passage in the allantoic cavity of SPF embryonating eggs.

2 - various organs that had been collected since the last quarter of 1992 until the first quarter of 1993, without any precautions with regard to RNAse contamination, from several species of domestic birds, and submitted to a French diagnostic laboratory (LDA22 Ploufragan), were analysed. They are listed in Table 1; the related results of NDV isolation are also shown. These organs were stored as supernatants

of 10% suspensions of ground organs in medium which was not specifically RNAse free.

RNA extraction and PCR

In order to extract RNA from organs, the technique of McCaustland et al. (7) was carried out, using glass milk (Bio 101) and guanidine isothiocyanate 6M (stratagene). The cDNA preparation and the first PCR, were done in the same tube with thermostable reverse transcriptase (Perkin Elmer), as described (4). However, for each sample, concentrations of RNA ranging from 100 to 200 ng were used and a new set of primers, selected with the "Oligo program", was employed: 5' ACACCTCATCCCAGACAG 3' (A) for reverse transcription and for PCR (upper primer), 5' TCTTCCCAACTGCCACTG 3' (B) as the lower reverse primer of PCR; in addition, after 5 min at 95°C, thirty five cycles at 95°C (20 sec.), 54°C (30 sec.), 70°C (15 sec.) were carried out and followed by a final step of 6 min at 75°C in a DNA thermal cycler Gene Amp PCR system 9600 (Perkin Elmer). Then, nested PCR was carried out with a second set of primers using initially concentrations of the first PCR product : undiluted, diluted log -1, and -2, and subsequently only the -1 dilution; the upper primer (C) was as described (3) :- 5'CTTTGCTCACCCCCCTTGG3', the lower reverse primer (D) was : 5'GCATTTTGTTTGGCTTGTA3'; otherwise the concentrations of the reactives required for the PCR were identical to those used previously (3) and the conditions of thermal DNA amplification were similar to those detailed above for the first PCR.

To circumvent risks of contamination during the PCR process, very strict conditions were employed, such as using separate equipment for each step, positive displacement pipettes and multiple negative controls. Only one source of samples was processed at the same time.

The final PCR products were electrophoresed on ethidium bromide (EB) agarose gel as described (3). To the extent that all negative controls were correct (including negative controls of the first PCR, amplified again with the nested PCR), the results were valid. Thus, for a given sample under investigation, in case of a positive nested PCR result for at least one of the conditions tested (depending on the RNA and the first PCR product concentrations), the nested PCR was started again using these optimal parameters in such a way that 200 to 300 µl of the final PCR product was obtained. This PCR product was further processed by purifying it on a DNA spun column (Pharmacia) as described (4) and quantifying it on EB agarose gel by comparison with a gamut of DNA marker VI (Boehringer Mannheim) concentrations.

Automated sequencing of the PCR product

The purified PCR product was processed by the dye terminator method, using dye-labelled ddNTPs and DNA sequencer model 373 A (Applied Biosystems), according to the recommendations of the supplier. Briefly, 30 to 50 ng of each purified PCR product were reacted with primer C or D (3.2 pM in 1 µl) and 9.5 µl of the terminator premix including Taq DNA polymerase (Dye Deoxy TM Terminator Cycle Sequencing kit, Applied biosystems), in a final volume of 20 µl for twenty five cycles at 96°C (10 s), 50°C (5 s), 60°C (4 mn) in the DNA thermal cycler mentioned above. Once the dideoxy sequencing reactions achieved, the ddNTP in excess were eliminated by a phenol chloroform extraction; the purified product was precipitated and dried and the pellet was solubilized with formamide EDTA 50 mM 5/1. The final product was loaded onto a 0.1 mm thick 6 % acrylamide gel containing urea 8.3 M in TBE pH 8.3. Electrophoresis was carried out at 30 W for 12 h. Sequence was automatically read at a separation distance of 24 cm. Sequence data were collected and analysed using the softwares "Data Collection" and "Analysis" (Applied Biosystems) on the microcomputer belonging to the system.

The latter technique was applied for sequencing the nested PCR product obtained from the trachea of a chicken experimentally challenged with NDV Essex strain, 6 days earlier.

Results

Detection of NDV gene in organs

Taking into account the different combinations of concentrations for RNA and the first PCR product that were tested, as well as the reproduction of the nested PCR optimal conditions, the mean of repetitions for each sample was 3.1.

The tracheas collected from 4 chickens infected experimentally with NDV Essex '70 gave a positive result by nested PCR. The results are shown (Fig. 1) for trachea collected 5 days after challenge. The results concerning organs collected from conventional birds are given in Table 1.

Automated sequencing of PCR product

The deduced amino acid sequence at the cleavage site [*] of the PCR product obtained from the trachea of a chicken challenged with NDV Essex strain 6 days before, was :

....SGGRRQKR * FI.....

employing primer D only. In fact, several previous attempts did not give satisfactory results, using primer C, because some nucleotides at the cleavage site could not be determined.

Discussion

Before carrying out the technique presently described for RNA extraction from organs, several unsuccessful attempts had been made using other techniques including the one already detailed concerning NDV strains and isolates obtained as allantoïc fluids (3). Thus, techniques employing only guanidine isothiocyate or techniques consisting in the simple lysis of the sample preparation with detergents such as NP_{40} and tween 20 under alkaline conditions, were not efficient in our hands.

The quality of the viral RNA according to the conditions of sample collection and storage had to be considered. For this reason we tested a range of disadvantageous conditions taking into account the practical conditions that could exist in the case of an outbreak on the field. In fact this parameter did not appear as a limiting element. The fact that our PCR test involves a very short fragment of the viral RNA might be an explanation.

The sensitivity of the present test may appear insufficient since it is not able to detect NDV in organs from which virus was isolated at the second passage in embryonating eggs. Further investigations have to be done to confirm the positivity of organs and to exclude any cross contamination during viral isolation process. In this case the sensitivity of our PCR test will have to be increased further, though available means are becoming fewer since the present technique of RNA extraction and nested PCR contribute greatly to this goal. The thermostable reverse transcriptase rTth presently used gives lower cDNA efficiency than M-MLV reverse transcriptase (MMLVRT) (Perkin Elmer personal communication). We confirmed this observation with NDV RNA extracted from allantoïc fluids since we were able to detect RNA from virulent viruses with a simple PCR test using MMLVRT (3) whereas a further amplification with nested PCR was required using rTth (5). However the use of the latter appears in other respects as an improvement in comparison with the previous technique (3) since it suppresses manipulation of a highly toxic chemical methylmercuric hydroxide, it eliminates a further step with mercaptoethanol and it permits limited opening of the tubes and consequently risks of contamination. Nevertheless, efficiency may be higher using reverse primer B instead of primer A for cDNA preparations. This point is to be investigated.

In order to settle the test several RNA and first PCR product concentrations were analysed. From the results obtained it appeared possible to simplify by first amplifying two RNA concentrations : 100 and 200 ng, for each sample, then amplifying again, by nested PCR, the corresponding first PCR product that had been diluted log -1 only. It

should be advisable in the future, in case of a suspicion in the field, to adopt these modifications and to carry out the nested PCR under a large volume directly after the first PCR, without the need to determine the optimal conditions; this should consume more reactives but should suppress one step and could save 4 hour time.

From experimentally challenged chickens only data corresponding to organs collected 5 and 6 days after the challenge are presented. From a practical point of view there is a need to know whether or not the test is able to detect the NDV gene consistently and sooner, namely as soon as clinical signs appear. However in the case of backyard flocks, it may be a few days, even more, before the poultry are suspected of being infected.

It is to be outlined that nested PCR is a very dangerous test from the point of view of risks of contamination and therefore is difficult to succeed. Despite all the precautions listed above, contamination occurred and obliged us to start again which is consuming in both time and money. Thus, we have to consider whether specialised diagnostic laboratories could apply this procedure if sequencing of PCR products was recognised officially as an alternative to ICPI.

The present sequencing results are very limited but they demonstrate the feasibility of the technique. The deduced amino acid sequence presently obtained at the cleavage site agrees with the one published (1) for the Essex strain. In order to validate our sequencing technique more substantially, it is necessary to compare further sequence data of RNA extracted from organs and RNA extracted from the corresponding allantoic fluids, then to relate this to ICPI results. This is the goal of our present investigations.

One difference is to be notified between our sequencing results and those previously published (1). The deduced amino terminus of the F_1 fragment is : FI…or FV, according respectively to our present results or those of Collins et al (1). It is questionable whether it is the result of our procedure or whether it corresponds to a different evolution of the strain in each laboratory.

The choice of the present technical parameters is not definitive and we are intending to test other methods (dye-primer method, sequenase …). We did not succeed in sequencing correctly both strands. In fact sequencing results obtained using primer C were not satisfactory because of some undetermined nucleotides. This may be explained by the fact that primer C is too near to the cleavage site. However the use of this primer as a confirmation of results given by primer D, is not to be excluded.

Presently, the sequencing result could not be delivered at best before the 5th day, once the procedure of RNA extraction started and only one origin of samples was tested at the same time. In the future, provided that all the steps follow each other, this time should be reduced by one day. Therefore, this time appears quite favourable in comparison with the animal test required by the official procedure (ICPI).

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References

1 Collins M.S., Bashiruddin J.B. and Alexander D.J., 1993. Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. Arch. Virol., 128, 363-370.

2 Glickman R.L., Syddall R.J., Iorio R.M., Sheehan J.P., and Bratt M.A., 1988. Quantitative basic residue requirements in the cleavage activation site of the fusion glycoprotein as a determinant of virulence for Newcastle disease virus. J. Virol., 62, 354-356.

3 Jestin V. and Jestin A., 1991. Detection of Newcastle disease virus RNA in infected allantoïc fluids by in vitro enzymatic amplification (PCR). Arch. Virol., 118, 151-161.

4 Jestin V. and Cherbonnel M., 1992. Monoclonal antibodies and gene amplification (PCR) for the characterization of a-PMV_1 strains. Proceedings of the CEC workshop on avian paramyxoviruses, Rauischholzhausen, Germany July 27-29, p. 157-166.

5 Jestin V. and Cherbonnel M., 1993. Epidémiologie moléculaire des infections à paramyxovirus aviaires de type 1. L'Aviculteur, 546, 57-59.

6 Le Long, Brasseur R., Wemers C., Meulemans G., and Burny A., 1988. Fusion (F) protein gene of Newcastle disease virus : sequence and hydrophobicity, comparative analysis between virulent and avirulent strains. Virus Genes 1 : 4, 333-350.

7 McCaustland K.A., Bi S., Purdy M.A. and Bradley D.W., 1991. Application of two RNA extraction methods prior to amplification of

hepatitis E virus nucleic acid by the polymerase chain reaction. J. Virol. Meth., 35, 331-342.

8 Toyoda T., Sakaguchi T., Imai K., Inocencio N.M., Gotoh B., Hamaguchi M., and Nagai Y., 1987. Structural comparison of the cleavage-activation site of the fusion glycoprotein between virulent and avirulent strains of Newcastle disease virus. Virol., 157, 242-247.

Figure 1 : Detection of NDV in trachea collected from experimental chickens, using nested PCR

A and D are negative controls.

B and C correspond to the tracheas collected from two SPF chickens challenged 5 days before with the NDV Essex strain.

M is the DNA marker VI (Boehringer Mannheim).

U, -1, - 2 : respectively the undiluted, diluted -1, diluted -2 PCR products obtained after the first amplification, were amplified again by nested PCR.

Table 1 : Detection of NDV in organs collected from conventional birds using nested PCR

Specimen	Passage	Host	PCR results
Viral isolation (NDV) negative*			
Brain + liver + spleen		Fowl	PCR -ve
Pool of several organs		Fowl	PCR -ve
Spleen		Red partridge	PCR -ve
Spleen		Pheasant	PCR -ve
Brain		Pigeon	PCR -ve
Viral isolation (NDV) positive			
Brain	+ P1**	Fowl	PCR +ve
Brain	+ P2**	Fowl	PCR -ve
Brain	+ P2	Fowl	PCR -ve

* no recovery of NDV following 3 passages into the allantoïc cavity of embryonating fowl's eggs.

** Viral isolation positive at the first (P1) or second (P2) passage in the allantoic cavity of embryonating fowl's eggs.

ASSESSMENT OF THE MOLECULAR BASIS OF VIRULENCE OF THE "PIGEON VARIANT PMV1 VIRUS"

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Introduction

The so-called "pigeon variant" strain (PPMV-1) of Newcastle disease virus (NDV) which has been present in racing and show pigeons in Europe since 1981[1] has been usually identified by the antigenic homogeneity of the isolates and their antigenic variance from more classical NDV (or PMV-1) strains detected using monoclonal antibodies (mAbs) [2].

Assessment of the virulence of viruses identified as PPMV-1 isolates for chickens using standard intracerebral and intravenous pathogenicity index (ICPI and IVPI) tests [3], has produced unusually variable results. Most isolates obtained from pigeons have had ICPI values of about 1.4 and low or zero values in IVPI tests, although some isolates have produced ICPI values lower than 0.7 [4]. However, most PPMV-1 isolates obtained from natural infections of chickens [5] have had high indices in both tests and Alexander and Parsons [6] showed that for some isolates the IVPI values obtained in tests were increased to >2 after experimental passage through chickens [Table 1].

Table 1: Effect of passage of PPMV-1 isolates in chickens on pathogenicity for chickens.

Passage level	**Viruses**			
	isolate 2024/85		isolate 2270/85	
	ICPI	IVPI	ICPI	IVPI
chick embryo 1			1.38	0.34
chick embryo 2	1.47	0.57	1.46	0.72
chicken 3	1.68	2.03	1.75	2.27

Molecular basis for pathogenicity

Studies, mainly by Rott, Klenk and co-workers [7], have established that infectious NDV particles are only produced following post-translational cleavage of the fusion protein F0 to F1 and F2 and the ability of NDV isolates to cause death and severe disease in chickens is governed by this process being mediated by a ubiquitous host protease[s]. Viruses which are susceptible to cleavage by the putative ubiquitous protease may spread throughout the animal causing severe disease and death whereas those that cannot are cleaved only by trypsin-like enzymes and therefore restricted to the respiratory and/or intestinal tracts. At a molecular level the susceptibility of the F0 protein has been shown to be related to the amino acid sequence at the cleavage site. Collins et al [8] reported that of 26 PMV-1 viruses which had been sequenced at the F1/F2 cleavage site all 14 viruses pathogenic for chickens showed the amino acid sequence: **^{112}R/K-R-Q-K/R-R^{116}** at the C-terminus of the F2 protein and had **F** [phenylalanine] at position 117 the N-terminus of F1. In contrast, the 11 viruses of low virulence showed the sequence in the same region: **^{112}G/E-K/R-Q-G/E-R^{116}** and **L** [leucine] at residue 117.

Sequencing PPMV-1 viruses

Collins et al [8] examined the amino acids at the F0 cleavage site of a single PPMV-1 virus with ICPI and IVPI values of 1.47 and 0.00 respectively. This virus had the deduced amino acid sequence of **^{112}G-R-Q-K-R-F^{117}** which was similar to the sequence of virulent viruses but with a single pair of basic amino acids, at 115 and 116 instead of a double pair at 112/113 and 115/116. Jestin and Cherbonnel [9] reported the F cleavage site sequence of two isolates from chickens but identified as PPMV-1 viruses as ^{112}G-R-Q-K-R^{116}.

The obvious questions to arise from these findings were whether or not differences in the amino acids at the cleavage site, when comparing PPMV-1 viruses with more classical virulent NDV isolates, were responsible for the unusual virulence of the PPMV-1 viruses and whether PPMV-1 virus showing apparently enhanced virulence following passage in chickens exhibited changes at the cleavage site.

Collins et al [10] examined 14 isolates which had been confirmed as typical PPMV-1 viruses using monoclonal antibodies. These viruses showed considerable variation in pathogenicity for chickens as measured by ICPI and IVPI tests. Three of the viruses were isolated from natural outbreaks occurring in chickens and had high pathogenicity indices. One isolate, 760/83, was examined at two passage levels, the

first was as close as possible to initial isolation from pigeons and had an ICPI value of 1.48 and an IVPI value of 0.28, the second was following three passages in chickens which increased the ICPI and IVPI values to 1.54 and 2.01 respectively.

Sequencing of the nucleotides coding for amino acid residues 109 to 119 of the F gene of each virus detected variation in only 3/15 viruses. One virus had a single change of GGG to GGA in the codon for residue 112 which was silent. Two other viruses, 340/91 and 1606/91, showed three identical nucleotide differences:- i. in the codon for residue 110, GGA to GGG, which was silent, ii. GGA to GAA resulting in a change from glycine to glutamic acid at residue 111, iii GGG to AGG resulting in a change from glycine to arginine at position 112.

Table 2: Deduced amino acid sequences at the F0 cleavage site for PPMV-1 viruses, adapted from Collins et al [10]

Viruses	amino acid sequence	ICPI	IVPI
9 pigeon isolates	^{109}SGGGRQKR-FIG^{119}	1.36*	0.32*
3 chicken isolates	^{109}SGGGRQKR-FIG^{119}	1.67*	2.26*
760/83bp3	^{109}SGGGRQKR-FIG^{119}	1.54	2.01
340/91	^{109}SGERRQKR-FIG^{119}	0.66	0.22
1606/91	^{109}SGERRQKR-FIG^{119}	1.36	0.44

* mean values

Thus 13 viruses, including 760/83 and 760/83bp3, had the amino acid sequence ^{109}SGGGRQKR-FIG^{119} and the two viruses showing variation from this had the sequence ^{109}SGERRQKR-FIG^{119} [Table 2].

In view of the high pathogenicity for chickens of some of the viruses examined these results confirmed that the minimum requirement for pathogenicity was a motif at the cleavage site of RQKRF and that a double pair of basic amino acids at residues 112 and 113 and 115 and 116 were not a prerequisite for virulence. However, it is also clear from the apparent low pathogenicity of some of the isolates tested, that other factors may affect the virulence of PMV-1 viruses in pathogenicty tests. In this context it is worth noting that isolate 340/91 which had the lowest ICPI of the viruses tested, 0.66, did have a double pair of basic amino acids at the F cleavage site.

Discussion

The work by Collins et al [10] failed to show any molecular basis associated with the F2/F1 cleavage site which would account for the apparent low pathogenicity of PMV-1 viruses isolated from pigeons in

ICPI and IVPI tests on initial isolation. It is possible that some host adaptation is required before some PMV-1 viruses can realise their potential virulence for chickens. This may not be restricted to pigeons as other NDV isolates from unusual hosts received at the International Reference Laboratory, Weybridge have also shown pathogenicity indices on initial testing which are lower than expected and below their demonstrable potential.

The fact that the PPMV-1 viruses show increased pathogenicity on passage and the high indices recorded for most isolates obtained from natural infections of poultry indicates their importance as serious poultry pathogens. Nevertheless, some, e.g. 340/91, have shown such low indices in pathogenicity index tests that they do not come within the EC definition of NDV infections for which the control measures will be applied [3] i.e. they have an ICPI value less than 0.7. This raises the possibility that such viruses could be introduced into a member state infecting a non-poultry host, and, despite detection and isolation, be allowed to circulate uncontrolled until entering the poultry population and causing serious disease.

To date, definitions of NDV for which control measures should be taken put forward by international agencies such as the EC have relied on determination by in vivo tests such as the ICPI test. The finding that viruses, isolated from unusual hosts, may not show their potential virulence for chickens in such tests despite having the necessary genetic capacity for high pathogenicity may bring that reliance into question. Perhaps an additional alternative test, possibly nucleotide sequencing of the F gene at the region coding for the cleavage site of the F0 protein, should be used routinely in the diagnostic assessment of ND viruses from non-poultry hosts to detect those viruses of ostensibly low virulence which may have the potential to show full virulence on passage through poultry hosts.

References

1. Vindevogel H, Duchatel JP (1988) Panzootic Newcastle disease virus in pigeons. In: Alexander DJ (ed) Newcastle disease. Kluwer, Norwell, pp184-196
2. Alexander DJ, Manvell RJ, Kemp PA, Parsons G, Collins MS, Brockman S, Russell PH, Lister SA (1987) Use of monoclonal antibodies in the characterisation of avian paramyxovirus type 1 (Newcastle disease virus) isolates submitted to an international reference laboratory. Avian Path. 16: 553-565.
3. Council of the European Communities (1992) Council directive 92/66/EEC introducing Community measures for the control of Newcastle disease. Off. J. Europ. Commun. 35: 1-20

4. Alexander DJ, Parsons G, Manvell RJ, Sayers AR, Characterisation of avian paramyxovirus type 1 infections of racing pigeons in Great Britain during 1983 to 1990. Proceedings of the Commission of the European Communities Meeting on Virus Diseases of Poultry - New and Evolving Pathogens, Brussels, 1992 in press.
5. Alexander DJ, Wilson GWC, Russell PH, Lister SA, Parsons G (1985) Newcastle disease outbreaks in fowl in Great Britain during 1984. Vet. Rec. 117: 429-434.
6. Alexander DJ, Parsons G (1986) Pathogenicity for chickens of avian paramyxovirus type 1 isolates obtained from pigeons in Great Britain during 1983-85. Avian Path. 15: 487-493.
7. Rott R, Klenk H-D (1988) Molecular basis of infectivity and pathogenicity of Newcastle disease virus. In: Alexander DJ (ed) Newcastle disease. Kluwer, Norwell, pp98-112
8. Collins MS, Bashiruddin JB, Alexander DJ (1993) Deduced amino acid sequences at the fusion protein cleavage site of Newcastle disease viruses showing variation in antigenicity and pathogenicity. Arch. Virol. 128: 363-370.
9. Jestin V, Cherbonnel M (1992) Use of monoclonal antibodies and gene amplification (PCR) for characterisation of a-PMV-1 strains. Proc. CEC Workshop on Avian Paramyxoviruses, Rauischholhausen, 1992. Inst. Geflugelkrankheiten, Giessen pp157-166
10. Collins, MS, Strong, I., Alexander DJ (1994) Evaluation of the molecular basis of pathogenicity of the variant Newcastle disease viruses termed "pigeon PMV-1 viruses. Archives of Virology *in press*.

SESSION V - CONCLUSIONS AND REC0MMENDATIONS

The topics, listed from the morning's preliminary discussion were covered and the following Conclusions and Recommendations made:

1. The Meeting recommends that the Commission investigates to what extent member countries have implemented the requirement to establish National Laboratories as specified in Articles 14 of Directives 92/40 and 92/66.

2. The Meeting considers that the functions and goals of the National Laboratories as listed in Articles 14 of Directives 92/40 and 92/66 could be clarified, and recommends that the Commission lists these in greater detail.

3. The Meeting recommends that the Commission organises a series of visits by their representatives to each of the National Laboratories with the aim of assessing their ability, in terms of staff, facilities and other resources, to carry out the agreed diagnostic functions.

4. The Meeting recommends that third country trading partners are required to set up National Laboratories comparable to those of the EC countries and capable of carrying out the same functions.

5. The Meeting considered that the identified problem of disease in non-commercial poultry [i.e. hobby, show birds etc.] poses an extremely serious threat to the poultry industry and current legislation covering trade in and movement of such birds should be reviewed.

6. The Meeting was alarmed at the anecdotal reports from the representatives of several countries of the reluctance of farmers and veterinarians to report Newcastle disease to the proper authority. The Meeting suggested that the Commission should tackle this by publicity and education stressing the risks involved in such practice.

7. The Meeting concluded that it would be extremely helpful if it were possible to distinguish between ND antibody titres produced by

vaccination and those produced by field infection. Research leading to this situation, such as vaccines based on the fusion protein alone should be encouraged.

8. The Meeting discussed apparent differences in the diagnostic tests and protocols used in different laboratories, despite the recommendations of Directives 92/40 and 92/66. There is a need for greater standardisation and quality control and moves towards obtaining this should be accelerated.

9. Some National Laboratories had reported difficulty in carrying out pathogenicity index tests on isolates since national animal welfare legislation made such testing difficult. Currently, such tests are, by far, the most reliable method for assessing virulence and they are specified as the tests that will be used in Directives 92/40 and 92/66, signed by the Ministers of all Member States, and, therefore, all National Laboratories. should be in a position to carry out such tests. The Meeting agreed that such tests should be phased out as soon as it was practicable to do so following the development of an alternative *in vitro* test of equal reliability.

10. The Meeting emphasised the high risk of introducing Newcastle disease or avian influenza by the importation of exotic birds. The Meeting recommended that all such birds should be subjected to quarantine [*period not specified -Ed*], during which there is at least one attempt to isolate virus from faecal swabs, or collected faeces, from the exotic birds in addition to isolation attempts from birds dying in quarantine. It was further felt that sentinel antibody negative chickens should be placed in the same air space and examined for seroconversion. Birds entering quarantine should not have been vaccinated against Newcastle disease.

11. The Meeting felt that, in general, the importance of practising good biosecurity measures aimed at the prevention of introducing Newcastle disease virus was being ignored in preference to vaccination and recommended that greater emphasis should be placed on this aspect of disease control. In addition it was felt that the Commission should maintain a register of disinfectants that had been tested and shown to be effective against ND virus for use in the field.

12. The Meeting recommended to the Commission that future annual meetings should, whenever possible, be held in a different country each year. Proposals/volunteers were invited for the venue for the next meeting, in 1994.

JOINT ANNUAL MEETINGS OF EEC NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES, BRUSSELS 1993

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