PROCEEDINGS OF THE JOINT THIRD ANNUAL MEETINGS OF THE NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES OF COUNTRIES OF THE EUROPEAN UNION

HELD IN BRUSSELS, 30th-31st OCTOBER 1995

Edited by Dennis J. Alexander

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JOINT ANNUAL MEETINGS OF EUROPEAN UNION NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES 1995

PROGRAMME

Monday 30th October 1995

10.30: Welcome and introduction.

10.45: SESSION I **COUNTRY REPORTS - 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
15. Norway

SESSION II **COUNTRY REPORTS - NEWCASTLE DISEASE**

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

12.45: LUNCH

14.00: SESSION II continued

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

15.15: SHORT BREAK

15.30: SESSION III **EC REPORTS**

15.30: Report of the EC Reference Laboratory for avian influenza and Newcastle disease. *Dennis Alexander*

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

16.30: General Discussion of Current Situation in European Union Countries

Tuesday 31st October 1995

10.00: SESSION IV **ORIGINAL CONTRIBUTIONS**

10.00: *B. Engstrom, G. Czifra and G. Koch.* Comparison of haemagglutination inhibition titres, monoclonal antibody blocking ELISA titres and neutralisation indices of ostrich sera.

10.30: *A. Oberdörfer and O. Werner:* Estimation of the pathogenicity of Newcastle disease virus isolates by hybridisation with digoxigenin-tailed oligonucleotides.

11.15: SHORT BREAK

11.30: *D.J. Alexander*: Newcastle disease haemagglutination inhibition tests in National Laboratories - Results of the comparison of test reproducibility in different laboratories.

12.45: LUNCH

14.00: 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. In particular we will discuss what we have learnt from the ND HI exercise and what future quality control tests should be organised. 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.

15.45: CLOSING REMARKS

16.00: CLOSE

SESSION I - COUNTRY REPORTS: AVIAN INFLUENZA

Representatives of the following countries made statements that no outbreaks of avian influenza had occurred in their countries since the last meeting:

Belgium/Luxembourg
Spain
Portugal
Denmark
The Netherlands
Ireland
Sweden
Finland
Austria
Norway

AVIAN INFLUENZA : CURRENT SITUATION IN GREECE

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Definition

The definition of avian influenza in Greece follows that presented in EU Directive 92/40/EEC introducing Community measures for controlling this disease.
The Greek Government has adopted the Directive into the National Legislation through the 31/95 Presidential Decree.

Diagnostic methods

The diagnostic methods for AI applied in Greece conform in detail to the recommendations of the Directive, namely: inoculation of SPF embryonated eggs, IVPI test in SPF chickens and HA/HI tests employing reference antisera and antigens.

Epidemiological situation

AI has never been diagnosed in Greece and no suspected outbreaks have been reported during the year of 1995.

Surveillance programme

There is no compulsory serological routine programme for AI.
When there are suspected cases, blood samples or specimens are sent to the National Laboratory for AI for further investigation.

Vaccination

Vaccination against AI is prohibited in Greece and no vaccine is licensed.

AVIAN INFLUENZA : SITUATION IN FRANCE FROM OCTOBER 1994 TO SEPTEMBER 1995

Michèle Guittet & Jean-Paul Picault

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The incidence of avian influenza virus infections was very low during the relevant period.

Serological survey
In some departments serological investigations were undertaken on a total of 145 flocks of fowls or turkeys. Only two flocks of turkeys were found positive by using the agar gel precipitation test, these flocks were located in the departments of Vendée (85) and Côtes d'Armor (22). Both were related to egg production problems. One of them was confirmed by the inhibition haemagglutination test as H6 subtype (Table 1).

Isolation of avian influenza viruses
Two investigations were done. The first, on a breeder flock of turkeys which showed a drop in egg production located in Finistère ; a virus of H6 subtype with an intravenous pathogenicity index in six-week-old chickens (IVPI) of 0.00 was isolated. The second, in a cage bird at the airport of Roissy; the virus isolated was of H9 subtype with a IVPI of 0.00.

These two viruses were submitted to the WHO Centre and were characterised as H6N5 and H9N8 respectively.

Table 1: Avian influenza serological survey (data received from diagnostic laboratories)

Department	N° of Flocks	Species	Results	
			AGP	HI
56	12	turkey	-	
22	1	turkey	+	
49	4	turkey	-	
72	4	turkey	-	
85	1	turkey	+	H6
22	100	fowl/turkey	-	
72	15	fowl/turkey	-	
56	7	fowl	-	
59	1	cage bird	-	

AVIAN INFLUENZA: CURRENT SITUATION IN GERMANY

Ortrud Werner

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No outbreaks of highly pathogenic influenza have occurred since 1979. In addition, no influenza virus of low pathogenicity was isolated from poultry flocks from 1992 until 1994.

In 1995 we received 3 influenza virus isolates (Table 1). They came from different Bundeslanders and from various species (chicken, turkey, duck). In each of the three cases mixed backyard poultry flocks were involved and investigated as suspicious of Newcastle disease. All birds were killed before the laboratory diagnosis was made.

In the Regional Laboratory responsible in each case a haemagglutinating virus was isolated and submitted to the National Laboratory.

We identified the isolates as influenza viruses and in all of the three cases the haemagglutinin subtype was H9. The neuraminidase subtype(s) have not yet been identified.

The pathogenicity of the isolates was low. The first two isolates had an ICPI of 0, but the third showed some pathogenicity for one-day-old chicks by this route. In contrast eight-week-old chickens did not fall ill after intravenous infection. Therefore the viruses do not fall within the EU definition of highly pathogenic avian influenza.

Table 1. Isolation of avian influenza viruses in Germany in 1995

Date	Land	Virus	Subtype	ICPI	IVPI
08/95	Bavaria	chicken/Germany/90/95	H9	0	n.d.
09/95	Rhineland-Palatinate	turkey/Germany/106/95	H9	0	n.d.
09/95	Thuringia	duck/Germany/113/95	H9	0.95	0

THE EPIDEMIOLOGICAL SITUATION OF AVIAN INFLUENZA IN ITALY DURING 1995

V. Papparella, A. Fioretti, L.F. Menna, & M. Calabria

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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 population that were potential reservoirs of avian influenza viruses. In particular, surveys have been carried out over the last two years (1994-1995) on migratory Anatidae found in Veneto's lagoon. The study of 80 cloacal swabs obtained from migratory waterfowl that were trapped (unfortunately the species were not determined), resulted in the isolation of two strains of avian influenza A virus but due to technical problems the subtype(s) have not yet been determined.

During 1995 we have also obtained 60 tracheal swabs from a broiler flock that had slight respiratory problems. From these swabs we have isolated two strains of avian influenza type A that were both shown to be of H3N2 subtype at the World Reference Laboratory for Avian Ortho and Paramyxovirus at the Central Veterinary Laboratory in Weybridge (United Kingdom). The IVPI determined in the same Laboratory was 0.47 for both strains. The interest of these isolations are correlated to the problem of interspecific transmission of influenza, in fact the subtype is the same as isolated recently in swine in Italy. A group of researchers at Istituto Superiore di Sanità of Rome have carried out recently some interesting work about the genetic reassortment between avian and human influenza A virus in pigs and the results of this work provide the first evidence of a genetic reassortment between human and avian viruses in a natural swine environment. In fact during the last years there has been a lot of antigenic shifts in Italian pigs, but in the last 10 years we have not isolated from birds (feral or domestic) any viruses belonged to the H3N2 subtype. The phylogenetic studies and the sequence analysis are in progress in Istituto Superiore di Sanità of Roma (Italy) and also in World Reference Centre, Weybridge, and the results could be very interesting in understanding the origins of human strains (swine or avian).

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 tests.

It is important to remember that in Italy the use of inactivated vaccine for Avian Influenza is officially allowed only for ring vaccination, but a lot of turkey farms vaccinate breeder flocks with inactivated vaccine containing the enzootic influenza viruses of H6N2 and H9N2 subtypes.

No influenza viruses were isolated during the survey of imported exotic birds in quarantine in Campania region of Italy, but the number of these birds admitted was very low.

AVIAN INFLUENZA: SITUATION IN GREAT BRITAIN OCTOBER 1994 TO OCTOBER 1995

Dennis J. Alexander & Ruth J. Manvell

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Avian influenza investigations

The incidence of avian influenza (AI) virus infections of turkeys and chickens in Great Britain remains extremely low (Table 1).

Table 1. Avian influenza investigations in poultry during 1992-1995

	1992	1993	1994	1995[a]
Suspect cases investigated:	2[b]	4	1	2
Influenza virus isolated:	1[b]	1	1	1
Confirmed HPAI:	1[b]	0	0	0

[a]to 30th October 1995 [b]outbreak occurred at the end of December 1991

Isolations of avian influenza viruses from domestic poultry

Only one isolate was obtained from domestic poultry during the period. This was an H4N6 virus with IVPI 0.15 obtained from muscovy ducks (Table 2).

Table 2. Isolations of avian influenza viruses from poultry 1990-1995

Date	County	Virus	Subtype	IVPI
1990				
05/90	Lancashire	duck/England/780/90	H4N6	0.00
1991				
02/91(x2)	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**
1993				
09/93	Norfolk	turkey/England/895/93	H6N8	0.00
1994				
06/94	Norfolk	duck/England/611/94	H4N2	0.00
1995				
10/95	Avon	muscovy/England/1131/95	H4N6	0.15

Influenza isolations from other birds

During the year two influenza isolates were obtained from birds other than domestic poultry (Table 3). Both were obtained from caged birds and were of the same subtype as the virus isolated from a sun conure (*Aratinga soltitialis*) during an investigation of mortalities amongst psittacine species at a caged bird suppliers earlier in 1994. The viruses proved to have an IVPI of 0.00, but since they were of H7N1 subtype nucleotide sequencing was done to determine the deduced amino acid sequences at the cleavage site of the haemagglutinin molecules. The sequences obtained were the same for both viruses and the earlier isolate, **...PEIPKGR*GLF...**, and meant the viruses did not fall within the EU definition of highly pathogenic avian influenza [1].

Table 3. Influenza isolations from other birds 1990-1995.

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
1994				
07/94	Staffordshire	sun conure/England/766/94	H7N1	0.00
08/94	Norfolk	duck/England/935/94	H4N6	0.42
10/94	N. Yorks	parrot/England/1174/95	H7N1	0.00
10/94	N. Yorks	painted/conure/1234/94	H7N1	0.00
1995				
		none to 10.10.95		

Isolations of influenza viruses from birds in quarantine.

No influenza viruses were obtained from this source during the period under report.

Reference

1. CEC (1992) 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 II - COUNTRY REPORTS: NEWCASTLE DISEASE

The participants from the following countries made statements that no outbreaks of Newcastle disease had occurred in their countries since the last meeting:

Ireland
Finland
Austria

NEWCASTLE DISEASE: SITUATION IN GREAT BRITAIN DURING OCTOBER 1994 TO OCTOBER 1995

Dennis J. Alexander & Ruth J. Manvell

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Investigations of Newcastle disease in poultry during 1995.
During 1995 a total of 11 suspected cases of Newcastle disease (ND) was investigated in poultry. Viruses were isolated from two of the investigations (Table 1).

Table 1. Newcastle disease investigations in poultry during 1992-1995.

	1992	1993	1994	1995[a]
Cases investigated	17	16	22	11
NDV isolated	1	4	11	2
Confirmed cases	0	0	0	0

[a]to 30.09.95

The two viruses isolated were subjected to intracerebral pathogenicity index (ICPI) tests and antigenic characterisation using a panel of 28 monoclonal antibodies (mAbs), the results obtained are shown in Table 2. Both viruses showed a "group E" mAb binding pattern. Isolate 649/95 had an ICPI of 0.05 and was not inhibited in haemagglutination inhibition tests by mAb 7D4, 094/95 had an ICPI of 0.50 and was inhibited by mAb 7D4. These results are consistent with ND vaccine strains B1 and La Sota [including clone 30] respectively, although the ICPI value for isolate 094/95 is higher than expected.

Table 2. Characterisation of ND viruses isolated from poultry in Great Britain during 1995.

Reference	Type of bird	mAb binding	ICPI	Conclusion
094/95	chickens	E 7D4 +ve	0.50	La Sota vaccine [?clone 30]
649/95	turkeys	E 7D4 -ve	0.05	B1 vaccine

ND in racing pigeons
Infections of racing pigeons with the variant PPMV-1 virus may be confirmed in Great Britain by clinical signs alone, serology, virus isolation or a combination of

these. The numbers of confirmed cases during 1992-1995 in Great Britain are shown in Table 3. The total of 36 confirmed outbreaks recorded in 1994 represented the lowest in one year in the 12 year history of the disease in Great Britain. Although in other years the largest number of cases were recorded in the last quarter this was not the case in 1994, presumably as a result of the enforcement of the requirement to vaccinate birds taking part in races, so it is to be hoped that when the figures for the final quarter of 1995 are available these will show a continued decline in the total for the year.

Table 3: PPMV-1 outbreaks in pigeons in Great Britain 1992-1995.

	Confirmed cases by quarter				
Year	**01-03**	**04-06**	**07-09**	**10-12**	**Total**
1992	4	8	47	98	157
1993	13	20	33	34	100
1994	5	16	11	4	36
1995	4	9	9		*22*

ND in other birds

No isolations of NDV were made from other captive, domestic, quarantined or feral birds in Great Britain during the period October 1993 to December 1994.

NEWCASTLE DISEASE: CURRENT SITUATION IN GREECE

Athanasios Katsibras

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Epidemiological situation:
During the year of 1995 no Newcastle disease (ND) outbreaks have been reported in Greece.

The last outbreaks in Greece occurred during the years of 1984 and 1985, when the source of infection was attributed to back-yard poultry. Since then, no ND cases have been diagnosed in the country.

Diagnosis and Control
The diagnosis and control measures follow those in EU Directive of 92/66/EEC. The Greek Government has adopted the Directive into the National Legislation through a Presidential Decree which will come into force within two months.

Surveillance Programme
There is no compulsory serological surveillance programme for ND in Greece. Some serology and virology work is being performed by the regional or National laboratories on a voluntary basis.

Vaccination
There is no compulsory vaccination programme for ND, but practically all the flocks (broilers, layers and breeders) are vaccinated with live or inactivated vaccines. Emergency vaccination may be imposed by the Ministry of Agriculture, in case of an outbreak.

NEWCASTLE DISEASE - CURRENT SITUATION IN DENMARK, 1994/95

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Definition.

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

Diagnostic methods.

The diagnostic methods will follow the recommendations in the above mentioned EC Directive. No alterations of the diagnostic procedures have been made since the last meeting.

Epidemiological situation.

A total of 115 samples from poultry, submitted for routine diagnostic investigation, was inoculated in to the allantoic cavity of embryonated SPF eggs in 1994. The corresponding figure for the first 9 months of 1995 is 81 samples.

In July 1995 pigeon paramyxovirus (PPMV-1) was isolated from a non commercial pigeon flock with suspicion of PPMV-1 infection. The isolate was been identified in the national reference laboratory by use of monoclonal antibodies (U86 and 161/617) supplied by the EU Reference Laboratory.

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

In October 1995 paramyxovirus serotype 1 (PMV-1) was isolated from a flock of back yard poultry with a history of respiratory illness and approximately 10% mortality in chickens. The flock consisted of 34 hens, 12 cocks, 21 turkeys and 10 geese. The virus isolate was further identified by means of monoclonal antibodies (U86 and 161/617) as PMV-1. The ICPI of the isolate was 1.64. The flock was destroyed and measures according to the EU directive 92/66/EEC were initiated. Serological testing of contact poultry flocks indicated until Oct. 15. the presence of PMV-1 infection in 4 back yard flocks. Birds from these flocks are at

present tested virologically but until now no virus has been isolated. The epidemiological investigations are still in progress.

In addition to routine diagnosis by virus isolation in embryonated eggs, national surveillance for ND in fowl is based on serological monitoring of parent stock.

In 1994 a total of 11,272 sera from fowl were tested. Of these, 111 samples from an illegally imported of a flock of pullets were positive while the rest were negative. As mentioned in last year's report investigations revealed that the flock had been vaccinated in the country of origin. No PMV could be isolated from the flock. The number of samples tested 1994 was increased in order to prove freedom of ND in Denmark.

During the first 9 months of 1995 approximately 5600 serum samples from fowl were investigated with negative results. During the first 2 weeks of October, 31 serum samples, representing 4 back yard poultry flocks, were positive in HI tests. As mentioned above, these flocks were contacts of the ND outbreak.

NEWCASTLE DISEASE: CURRENT SITUATION IN ITALY FROM SEPTEMBER 1994 TO OCTOBER 1995

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In 1994 41 outbreaks of disease caused by velogenic PMV-1 strains and by pigeon PMV-1 strains were diagnosed. In contrast during the last 12 months only one PMV-1 isolate was submitted to the Department of Virology of our Institute, which acts unofficially as a National Laboratory for diagnosis or confirmation of Newcastle disease.

We use the plaque test and the mean death time of the minimum lethal dose in eggs for the first assessment of the virus virulence; as it is impossible at the moment to perform ICPI and IVPI tests in our laboratory. The final typing of the isolates is carried out at the European Union Reference Laboratory, Weybridge.

The above mentioned isolate was from a pigeon of a hobby flock in the province of Teramo (central Italy); it was placed in the mAb group P with an ICPI of 1.14 ad an IVPI of 0.00.

In the last fortnight, however, three other PMV-1 isolates were obtained from commercial flocks: two broiler flocks (one in province of Udine and one in province of Vicenza, in the north of Italy) with mild respiratory symptoms and without unusual mortality; and one flock of 15- to 20-day-old chickens with malabsorption syndrome (in province of Treviso, northern Italy).

In all three cases the preliminary typing carried out in our laboratory, suggests they are lentogenic; these isolates will be obviously sent to the EU Reference Laboratory for confirmation.

The General Direction of Veterinary Services of the Italian Ministry of Health recently informed us that the 92/66 EEC directive will be officially acknowledged very soon.

CURRENT AVIAN INFLUENZA AND NEWCASTLE DISEASE SITUATION IN SWEDEN

Björn Engström

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Avian Influenza

No clinical outbreak of avian influenza has occurred in Sweden.
All imported poultry flocks have been tested with an immuno-precipitation test. No antibodies against AIV have been detected in any flock.

Newcastle disease

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

Serological surveys

All breeder flocks are now tested with 60 serum samples taken at point of lay.
No antibodies against NDV have been detected in any flock.

Vaccination

All racing pigeons and pigeons attending shows have to be vaccinated with a killed PMV-1 vaccine.

Survey of migrating wild birds

The haemagglutination agent isolated from migrating birds, reported last year, has now been typed.
It was a virus belonging to the PMV-1 subtype. The bird was a black headed gull (*Larus ridibundus*). The isolate had an ICPI of 0.00. Immunoperoxidase binding test revealed a pattern only seen recently with an isolate from Northern Ireland.

NEWCASTLE DISEASE : SITUATION IN FRANCE FROM OCTOBER 1994 TO SEPTEMBER 1995

Michèle Guittet and Jean-Paul Picault

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NATIONAL TERRITORY

Investigation of Newcastle disease in poultry

A total of 17 suspected cases of Newcastle disease (ND) was investigated by different diagnostic laboratories. No precise case histories were provided (Table 1).

From 7 suspected cases in turkey flocks, only one virus was isolated and was subjected to intracerebral pathogenicity index (ICPI) at the National Reference Laboratory : the result obtained was 0.13 ;

From 10 suspected cases in fowl, one virus was isolated, the ICPI was 0.0.

Investigation in pigeons

Eight suspected cases were studied but no virus was isolated (Table 2).

Investigation in other birds

No virus was isolated (Table 2).

OVERSEAS TERRITORIES

Martinique

Three fighting cocks were analysed, no virus was isolated.

Ile de la Réunion

A total of three viruses was isolated from broilers. The ICPI values obtained were: 1.90, 1.78 and 1.88. These three viruses were submitted to the European Union Reference Laboratory for characterisation. All of them showed a similar binding pattern with the viruses isolated from the outbreaks in North Europe in the last few years [pattern NE].

Table 1. Newcastle disease - Suspect cases investigated in poultry

Department or Country	Number of suspect cases	Species	Results	Virus characterisation
22	2		-	
22	1		PMV-1	ICPI = 0.13
10	1	turkeys	-	
85	1		-	
02	1		-	
56	1		-	
2	2		-	
32	1		-	
27	1		PMV-1	ICPI = 0
14	1	fowl	-	
77	2		-	
56	1		-	
40	2		-	
Réunion	3	fowl	PMV-1	ICPI = 1.78-1.90

Table 2. Newcastle disease - Suspect cases investigated in pigeons and other birds

Department or Country	Number of suspect cases	Species	Results
67	1		-
22	1		-
10	1	pigeons	-
23	1		-
88	1		-
40	2		-
33	1		-
77	1	pheasant	-
69	1	canary	-
33	1	cage bird	-
40	1	parrot	-
40	1	penguin	-
40	1	mule	-
40	1	heron	-
40	1	cormorant	-
40	1	coot	-
Martinique	3	fighting cock	-

THE CURRENT NEWCASTLE DISEASE SITUATION IN NORWAY 1994-1995

Johan Krogsrud

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Flocks with antibodies against Newcastle disease have been recorded in a few layer parent farms since 1971. No clinical signs of Newcastle disease (ND) have ever been recorded in such flocks. Occasionally seroreactors have been found in hobby flocks and in goose units.

As reported last year all the parent flocks and the pullet rearing flocks in the country were tested serologically in 1994. Flocks with seroreactors were recorded in four farms. In November 1994 a paramyxovirus-1 (PMV-1) was isolated from seroconverting chicks from one of these farms. When characterised at the EU Reference Laboratory, Weybridge the virus was found to have mainly the same properties as an isolate from 1993 from another of the four farms. The intracerebral pathogenicity test gave an index of 0.41. There was no haemagglutination inhibition with monoclonal antibodies (mAbs) specific for the pigeon panzootic virus and for F and La Sota vaccine strains, respectively, Immunoperoxidase binding tests with a panel of mAbs placed this virus together with the 1993-isolate in Weybridge group C2, i.e. viruses of low pathogenicity mainly isolated from feral ducks and geese.

From mid-October 1994 159 poultry flocks have been tested serologically, 92 of them within the yearly testing scheme of the breeding stock in connection with a health certification programme. In addition 67 imported flocks in quarantine have been tested. Altogether approximately 9500 samples have been tested (60 samples per flock). Since November 1994, when seroreactors last were found on three of the four mentioned farms, no antibodies against NDV have been detected in any flock, except one quarantine flock which later proved to have been vaccinated.

In order to eliminate the virus on the infected farms sanitation programmes based on isolation of the units from each other and on hygienic measures have been in force since the autumn 1994. So far no new flocks with seroreactors have been recorded, but the programmes have not yet been finalised.

CURRENT SITUATION OF NDV IN BELGIUM.

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The Newcastle disease (ND) situation in Belgium is significantly different from that observed in 1994. Indeed, ND was absent in industrial poultry flocks and in backyard birds during the whole year 1994.

New cases of ND were diagnosed in backyard flocks at the end of the month of August 1995 and today, 11 different cases have been identified (Table 1 and Figure 1).

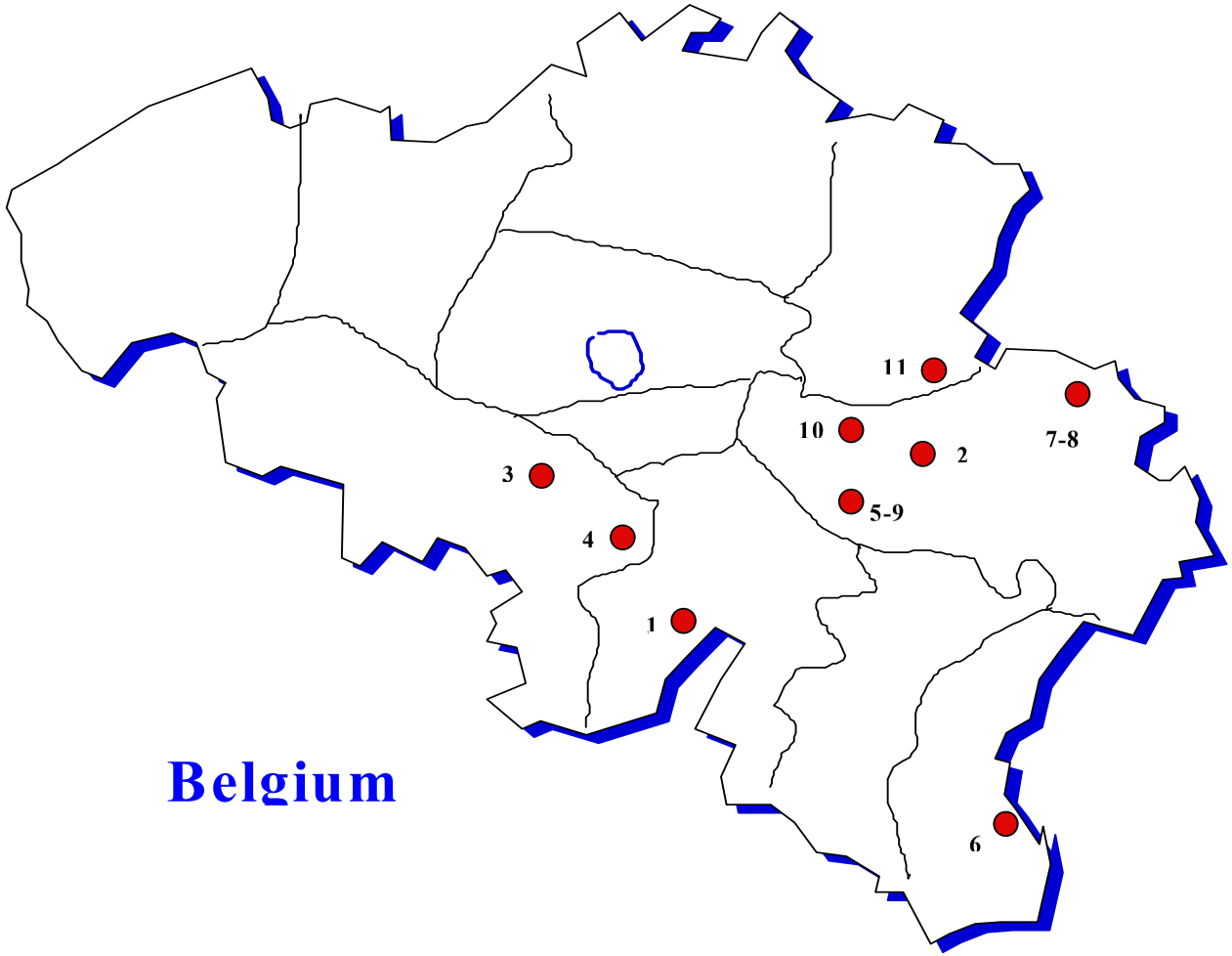
All ND viruses were characterised as velogenic by their reactivity in haemagglutination inhibition tests with monoclonal antibodies. The ICPI values were determined for the two first isolates and reached 2.0 for strain 251VB and 1.87 for strain 260VB. The IVPI value of strain 251VB was also determined and shown to be 2.47.

The biological and antigenic characterisation of the different viral isolates is still in progress.

Table 1: Outbreaks of Newcastle Disease in Belgium in 1995.

Case number	Reference number	Date 1995	Location	Province	Type of production	Number of chickens	Source of infection
95/01	251VB	29/08	Flavion	Namur	backyard	?	purchase of turkeys at the market
95/02	260VB	08/09	Stockay	Liège	backyard	?	purchase of guinea-fowl at the market
95/03	283VB	22/09	Ecaussines	Hainaut	backyard	50	
95/04	286VB	22/09	Gougnie	Hainaut	backyard	21	
95/05	288VB	22/09	Stree	Liège	backyard	?	
95/06	296VB	25/09	Canech	G D du Luxembourg	backyard	25	
95/07	300VB	25/09	Gemmenich	Liège	backyard	?	
95/08	301VB	25/09	Gemmenich	Liège	backyard	50	purchase of a hen at the market
95/09	308VB	28/09	Stree	Liège	backyard	30	
95/10	317VB	02/10	Faimes	Liège	backyard	?	purchase of a turkey at the market
95/11	338VB	13/10	Borgloon	Limbourg	backyard	?	

Figure 1: Location of ND outbreaks.



NEWCASTLE DISEASE: CURRENT SITUATION IN PORTUGAL

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In 1995 the number of clinical outbreaks of Newcastle disease (ND) decreased when compared to 1994. Seven isolates were obtained from backyard flocks and only two outbreaks occurred in the poultry industry. One Paramyxovirus type 7 virus was isolated from a racing pigeon belonging to a pigeon-loft where vaccination against ND is regularly administered. Pigeons showed depression and performed poorly in the races. The symptoms disappeared within two weeks and no casualties were registered.

The biological and antigenic characteristics of the isolates were studied at the EU Reference Laboratory. They produced a IIP binding pattern identical to the NDV isolated in previous years in our country. Virulence tests were done with three isolates giving high ICPI values. The MDT/MLD and plaque assay was carried out in our laboratory. The majority of the isolates showed a MDT < 60h and with the exception of isolate 2781 (PMV-7) all viruses produced plaques on MDBK cells in the absence of trypsin (Table 1).

Vaccination against ND is compulsory in Portugal. Broilers are vaccinated at day-old and revaccination, though not imposed, is practised on many farms.

Table 1. Portuguese isolates of NDV and their biological properties

Virus	Bird	ICPI	PF	MDT
2781	pigeon[1]		no	>72h
2988	broilers		yes	<60h
3159	broilers		yes	<60h
3160	broilers		yes	<60h
3444	broilers		yes	>60h
3620	broilers		yes	<60h
5199	chicken	1.97	yes	>60h
5228	chicken		yes	<60h
5408	chicken		yes	<60h
6063	chicken		yes	<60h
8245	pheasants		yes	<60h
8570	chicken	1.875	yes	<60h
8595	chicken		yes	<60h
8691	chicken		yes	<60h
8769	chicken		yes	60h
9001	chicken		yes	<60h
8506	pigeon[2]	1.25	yes	>60h
9002	pigeon		yes	<60h
9300	chicken		yes	<60h
9839	broilers		yes	<60h
10304	chicken		yes	<60h

PF - plaque formation on MDBK cells in the absence of trypsin
nt - not tested
[1] PMV-7 [2] pigeon PMV-1

REPORT ON THE NEWCASTLE DISEASE INCIDENCE IN THE NETHERLANDS SINCE OCTOBER 1994.

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During the period October 1994 to October 1995, 3 outbreaks occurred in the Netherlands. In one case virulent virus was isolated from broiler parents in November 1994 and in two cases from backyard poultry in June 1995 and October 1995.

In November 1994, organs were send to our institute from which virulent virus with an ICPI of 1.76 was isolated. The organs were collected from parent broilers with increased mortality. The virus isolated reacted as a virulent virus with 4 mAbs that are used to differentiate virulent from non-virulent virus. However, the reaction patterns of the new isolate and the earlier Dutch isolates were different. The farm was located in Ruinerswold in the province Drente in the Northern part of the Netherlands and was, therefore, the first outbreak to occur above the large rivers that traverse the Netherlands.

Possibly the virus may had been transmitted from the Northern part of Germany since two cases from which virulent NDV was isolated had occurred in August and October 1994 in the lower Saxony region. Samples of these viruses were received from Prof. Kaden and their reaction pattern with the 4 mAbs was the same suggesting that the virus was transmitted from North Germany. Although one of the collaborators of the farm admitted that he had traversed the region of both outbreaks in north Germany, the way the virus was transmitted could not be established.

In June 1995, high mortality occurred among a group of 15 Orpington chickens at a backyard farm in Laren in the Province North-Holland. The animals were first treated with antibiotics and two dead animals were sent for macroscopic examination to the Department of Pathology of the Veterinary Faculty in Utrecht. After dissection organs were collected and sent to the Health Service 5 days later. The Health service isolated a haemagglutinating virus that caused embryo death within 48 hours after infection. Allantoic fluid was sent to us at 13 days after the actual outbreak was manifest. On the premises another 6 chickens and 21 ducks were kept. All chickens were kept in close contact whereas the ducks were

separated from the chickens by a fence only. These animals, which were without any clinical symptoms, were sent to our institute for macroscopic and virological examination. Clinical signs of Newcastle disease were not observed and no virus could be isolated from faeces or other organs collected. Strangely enough, no antibodies to Newcastle disease could be detected.

On October 23 1995, brains, tracheas and livers of 4 bantam chickens were sent to our institute by the Animal Health Service of East Netherlands after mortality occurred. The chickens originated from a backyard farm located in Bleiswijk near Rotterdam. The Animal Health Service was called in because of respiratory problems and mortality in the backyard flock by the neighbour, who kept 15,000 laying chickens. Virulent virus was detected within 24 h using the PCR technique. The next day the PCR result was confirmed by virus isolation and the inhibition by mAbs. The determination of the ICPI was still in progress at the time of writing. The backyard owner had purchased two bantam chicks from a trader in backyard poultry. Three days later these animals died and shortly thereafter mortality and respiratory problems occurred in the other bantams chicks. Two laying chickens were without clinical signs. These chickens were obtained at one point from the neighbouring laying flock, because they escaped from the batteries. These chickens probably still had protective immunity as a result of the vaccinations programme of laying hens. Unfortunately, these two chickens were killed just before the outbreak was confirmed in the laboratory. The backyard trader was visited by the Dutch Veterinary Service, 7 suspected animals were collected and sent to the reference laboratory for examination. At post mortem examination no specific NDV lesions were observed. All seven chickens had haemagglutinating antibodies. These may have resulted from vaccination or infection with field viruses. Virus isolation is in progress. Also an attempt to isolate virus will be performed on swabs taken from all animals kept by the trader and 60 swabs taken from the laying flock. Because, the laying flock was re-vaccinated one to one and half week earlier, just after the problems at the backyard farm started, it is unlikely that virulent virus will be isolated *[Note added in press: Virulent virus was not isolated]*.

NEWCASTLE DISEASE - CURRENT SITUATION IN GERMANY 1994 - 1995

Ortrud Werner

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After a long absence of velogenic NDV in Germany there have been many outbreaks of ND in recent years (Table 1). The first cases of velogenic NDV in chickens were diagnosed near the border with The Netherlands at the end of 1992. In 1993 the virus spread over all of Germany. In 1994 179 cases were reported. Almost all Bundeslanders were affected, although a stamping out policy was applied.

The outbreaks occurred primarily in small and hobby flocks. More than 90 % of the diseased flocks were smaller than 200 birds (Table 2). For these small flocks vaccination against ND was not compulsory until the end of 1994.

In 1994 the National Reference Laboratory received 306 virus isolates or suspicious material for further investigation. 287 of these were identified as PMV-1. One virus isolate from parakeets was PMV-3. It was not pathogenic for chickens.

All isolates were passaged once in SPF-eggs, identified serologically with polyclonal antisera against PMV-1 and PMV-3 and also with monoclonal antibodies specific to pigeon-type PMV-1 and to the current epizootic NDV. Their virulence was determined by the intracerebral pathogenicity test in one-day-old chicks. In addition all isolates were sent to the EU Reference Laboratory in Weybridge.

The pathotype of the viruses is shown in Table 3. Most isolates from chickens, in fact 174 out of 190, were of velogenic pathotype. They were classified as NE viruses in Weybridge. Two mesogenic viruses from chickens were identified by monoclonal antibodies as pigeon-type PMV-1 [PPMV-1]. The lentogenic virus isolates from poultry were mostly vaccine viruses.

From pigeons mainly PPMV-1 were isolated, but in 9 cases velogenic viruses were obtained. The latter isolates were identified as the current epizootic viruses, which normally occurred in chicken flocks. This leads to the conclusion, that pigeons might be involved in the spread of the virus.

Last year the German "Legal Decree for Protection against Fowl Plague and Newcastle Disease" were altered to comply with Council Directive 92/66/EEC. In the new version from 21st December 1994 vaccination against ND is made compulsory for all chickens, regardless of the size of the flock. If chickens are kept together with other poultry in the same flock, all birds have to be vaccinated. This leads to new difficulties, because, in Germany, most vaccines are licensed only for chickens or chickens and turkeys, but not for commercial waterfowl.

The compulsory vaccination policy for all chickens has improved the situation of ND in Germany and we have observed a noticeable reduction of outbreaks. Up to now only 18 cases of ND have been confirmed in 1995. Most cases are located in the north-east of Germany in Mecklenburg- Western Pomerania and in Rhineland- Palatinate (Figure 1).

Table 4 gives a summary of all outbreaks in 1995. Only small hobby flocks with ornamental fowls and backyard poultry were affected. Therefore the number of birds involved in the outbreaks is small. Nearly all of the viruses isolated are velogenic. They show the typical reaction pattern of the current epizootic virus with our monoclonal antibodies. I think they will be sorted into the NE group in the EU Reference Laboratory. Only one virus isolate from an ornamental fowl has a mesogenic ICPI. It is characterised with monoclonal antibodies as PPMV-1.

As last year, the most common means of transmission was the purchase of birds. But in some cases it was considered that the virus was spread by neighbourhood contact and also by contact with free-living birds. No outbreaks of ND occurred in industrial poultry flocks this year. They are controlled by strict hygienic management and intensive compulsory vaccination policy.

Table 1. Outbreaks of Newcastle disease in Germany

Year	Number of outbreaks
1992	2
1993	181
1994	179
1995 Jan-Sept	18

Table 2. Size of the flocks with ND outbreaks in 1994

Size (Number of animals)	Outbreaks Number	%	Vaccination
1 - 9	5		
10 - 99	139	91.6	none
100 - 199	20		
200 - 999	7		
1000 - 9999	5	8.4	compulsory
> 10000	3		

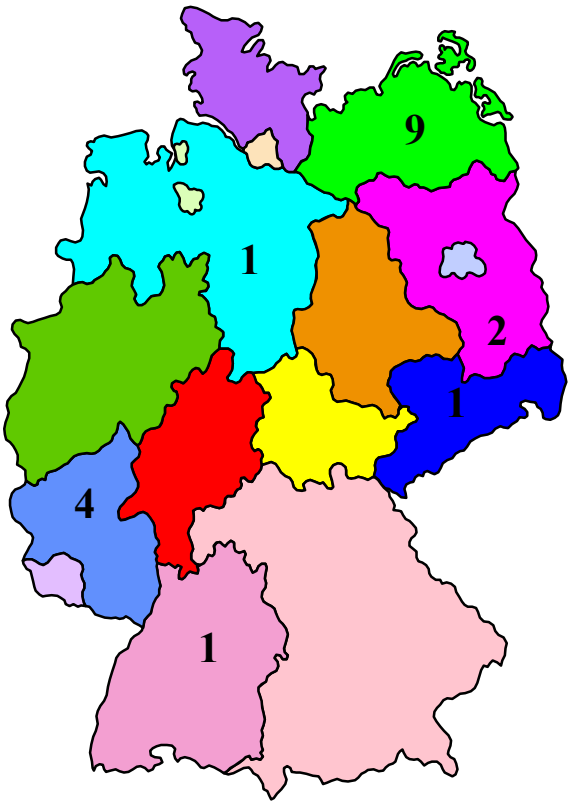
Table 3. Pathotypes of NDV isolates 1994

Number	Origin	I C P I		
		< 0.7 lentogenic	**0.7 - 1.5 mesogenic**	**> 1.5 velogenic**
190	chicken	14	2	174
5	turkey	1	-	4
4	duck	1	-	3
3	pheasant	-	-	3
1	partridge	1	-	-
1	goose	-	-	1
204	**all poultry**	**17**	**2**	**185**
58	pigeon	12	37	9
13	exotic bird	12	-	1
12	deep frozen duck	-	-	12
287	**Total**	**41**	**39**	**207**

Table 4. Newcastle disease outbreaks in Germany in 1995

Outbreak reference	Locality	Month	Species	Flock size	Characteristics of Virus		Origin of infection
					ICPI	mAb	
95/1	MV	Jan	ornam.fowl	23	1.75	epizootic	neighbouring contact
95/02	RP	Jan	laying hen	101	1.79	epizootic	purchase of animals
95/03	MV	Jan	ornam.fowl	26	1.75	epizootic	neighbouring contact
95/04	Sa	Jan	ornam.fowl	60	1.83	epizootic	unknown
95/05	RP	Febr	laying hen	28	1.88	epizootic	purchase of animals
95/06	MV	Febr	laying hen	20	1.83	epizootic	unknown
95/07	Br	Febr	laying hen	23	1.86	epizootic	waste food feeding
95/08	BW	Febr	laying hen	61	1.88	epizootic	unknown
95/09	MV	April	laying hen	18	1.88	epizootic	unknown
95/10	RP	May	ornam.fowl	16	1.88	epizootic	unknown
95/11	MV	June	laying hen	15	1.83	epizootic	unknown
95/12	NS	June	laying hen	20	1.88	epizootic	contact with free living birds
95/13	RP	Aug	ornam.fowl	124	0.84	pigeon-type	contact with free living birds
95/14	MV	Sept	laying hen	7	1.88	epizootic	purchase
95/15	MV	Sept	laying hen	30	1.85	epizootic	purchase
95/16	MV	Sept	laying hen	60	1.89	epizootic	purchase
95/17	MV	Sept	laying hen	36	1.89	epizootic	purchase
95/18	Br	Sept	laying hen	34	1.88	epizootic	unknown

Outbreaks of Newcastle disease in Germany
01.01.95 to 15.10.95



SESSION III - EC REPORTS

REPORT OF THE EUROPEAN UNION REFERENCE LABORATORIES FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE 1995

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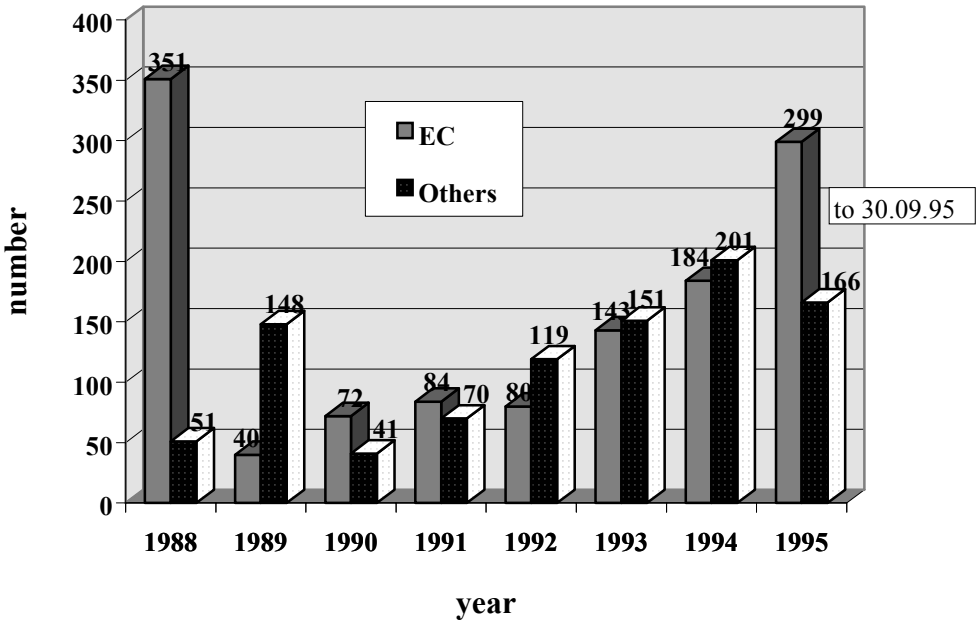
Viruses received by International Reference Laboratory

The number of viruses submitted by all countries to the reference laboratory during the years 1988 to 1995 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 characterise isolates retrospectively.

Once more there has been an increase in the number of viruses submitted to the reference laboratory; 294 viruses were received in 1993, 369 viruses for 1994 and in the first nine months of 1995 465 isolates were submitted. This was largely due to the rise in outbreaks in several countries. In the 12 month period from 1.10.94 to 30.9.95 a total of 520 viruses were received. Unfortunately 70 of these isolates failed to produce any haemagglutinating activity after two passages in embryonated fowls' eggs.



Influenza viruses submitted during 1.10.94 to 30.9.95

During the 12 month period from October 94 to September 95 a total of 17 influenza viruses was received at CVL, Weybridge from 7 different countries. The countries, number of viruses, their pathogenicity and subtype characterisation are shown in Table 1.

Table 1: Influenza A viruses submitted to the International Reference Laboratory during 01.10.94 to 30.09.95*.

Country	Number	Host	Subtypes	IVPI
Zimbabwe	6	ostriches	**H5N2**	0.00
Zimbabwe	1	parrot	**H5N2**	0.00
Germany	1	turkeys	H9N2	0.00
South Africa	1	ostriches	H9N2	0.00
Italy	1	chickens	H3N2	0.00
Austria	1	swine	H3N2	0.00
UK	2	caged birds	**H7N1**	0.00
Pakistan	3	chickens	**H7N3**	**2.68-2.89**
Australia	1	chickens	**H7N3**	**3.00**

*Not all viruses received had been isolated within this period.

The total of 17 viruses shows a slight increase from the 15 viruses recorded in the same period in 1993-1994

All but two viruses had low intravenous pathogenicity indices in six-week-old chickens. Seven viruses, from Zimbabwe, were of H5N2 subtype, 6 were isolated from ostriches and 1 from a parrot. Two viruses, one from ostriches in South Africa and one from an outbreak in turkeys from Germany were of H9N2 subtype. Two viruses were of H3N2 subtype, one from Italy and one from Austria and two H7N1 viruses from the UK isolated from parrots were received.

Four isolates of high pathogenicity one from Australia and three from Pakistan were all typed as H7N3, although nucleotide sequencing at the area corresponding to the cleavage site of the haemagglutinin showed differences in the amino acid sequence for viruses from the two countries. All viruses of H5 or H7 subtypes were subjected to nucleotide sequencing to determine the amino acids at the cleavage site of the haemagglutinin as required in the Directive 92/40/EEC. The results obtained are shown in Table 2. It can be seen that only those viruses from Pakistan and Australia which showed high IVPI values had multiple basic amino acids at the haemagglutinin cleavage site.

Table 2. Amino acid sequence at the HA cleavage site of H5 and H7 viruses submitted to the International Reference Laboratory.

Virus	Cleavage site sequence
A/parrot/England/1174/94 (H7N1)	P-E-I-P-**K**-G-**R***G-L-F
A/conure/England/1234/94 (H7N1)	P-E-I-P-**K**-G-**R***G-L-F
A/ostrich/S.Africa/95 (H5N2)	N-V-P-Q-**R**-E-T-**R***G-L-F
A/parrot/S.Africa/95 (H5N2)	N-V-P-Q-**R**-E-T-**R***G-L-F
A/chicken/Pakistan/95 (H7N3)	P-E-T-P-**K-R-K-R-K-R***G-L-F
A/chicken/Australia/95 (H7N3)	P-E-I-P-**R-K-R-K-R***G-L-F

Paramyxoviruses received during 1.10.94 to 30.9.95

During the twelve month period October 1994 to September 1995 429 paramyxoviruses were received at CVL, Weybridge. Of these 316 were received from nine different countries in the EU of which 308 were PMV 1 (Table 3). The other eight viruses being four isolates of PMV-3 and four of PMV-7 (one from a racing pigeon in Portugal and three from snakes in Germany).

Table 3: European Union countries submitting PMV-1 viruses during 01.10.1994 to 30.09.1995*.

Country	PMV-1 submissions
UK	16
Germany	266
Norway	1
Ireland	2
Portugal	13
Italy	2
Sweden	1
Denmark	4
Austria	3
Total	**308**

*Not all viruses received had been isolated within the twelve month period

A total of 113 viruses all of which were typed as PMV-1 viruses (Table 4), was received from 12 countries from outside the EU representing a wide cross section from different continents and geographical areas.

Table 4: Other countries submitting PMV-1 viruses during 01.10.1994 to 30.9.1995*.

Country	PMV-1
Zimbabwe	12
South Africa	29
Bolivia	2
India	9
Zambia	4
Kenya	1
Peru	5
Turkey	16
Nepal	1
Saudi Arabia	8
Bulgaria	25
Croatia	1
Total	113

*Not all viruses received had been isolated within the twelve month period.

Antigenic grouping of Newcastle disease virus [PMV-1] isolates.
All PMV-1 viruses received during September 1994 to October 1995 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 Tables 5 and 6. Generally, viruses in the same group show identical binding patterns for all 28 mAbs used. However, some isolates 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 Tables 5 and 6 on the basis of virulence for chickens.

Viruses were received from 9 member states; from 7 of these, viruses were either the pigeon variant PMV-1 (PPMV-1) [mAb group P], live vaccine viruses [group E] or lentogenic viruses usually associated with feral birds especially waterfowl.

The virulent viruses from Portugal were antigenically indistinguishable from those isolated since 1991 in that country. Interestingly the six viruses received from Turkey which were not grouped (Table 6) showed very similar binding patterns to the Portugal isolates, varying by only 1/28 mAbs.

The majority of isolates received were from Germany, reflecting the number of outbreaks seen there, 53 were vaccine virus, 44 virulent viruses showing the NE pattern seen in previous years and 29 PPMV-1, from both pigeons and poultry. The five virulent viruses showing pattern B had been isolated from imported duck meat.

Table 5. Antigenic grouping of Newcastle disease virus isolates from EU and EFTA countries submitted during 01.10.94 to 30.09.95

Country	Number of isolates in monoclonal antibody binding group									
	velogenic				*lentogenic*					not done
	B	P	NE	Port	C2	E	F	G	L	
UK		9				6				
Germany	5	29	44			53	1			134
Norway					1					
Italy		1				1				
Portugal				13						
Ireland								1		
N.Ireland									1	
Sweden									1	
Denmark		4								
Austria		3								

The virulent viruses isolated from countries outside the EU showed groups A, B and C1 mAb binding which is consistent with past viruses received from different countries around the world.

The single virus from Nepal was unusual in that while it showed mAb binding pattern G which is usually associated with avirulent viruses similar to Ulster 2C and Queensland V4 it had an ICPI of 1.62 which is indicative of a virulent virus. Assessment of whether or not this represents a mixture is currently under investigation.

Table 6. Antigenic grouping of Newcastle disease virus isolates from other countries submitted during 01.10.94 to 30.09.95

Country	Number of isolates in monoclonal antibody binding group									
	velogenic						*lentogenic*			**not**
	C1	**A**	**B**	**?[a]**	**D**	**P**	**F**	**E**	**G**	
Zimbabwe	2			1				9		
S. Africa	20		1	3				4		1
Bolivia								2		
India	4		1	3	1					
Zambia								4		
Kenya								1		
Peru		1	3					1		
Croatia			1							
Turkey				6		9		1		
Nepal									1*	
S. Arabia			6						2	
Bulgaria	9		9		1		2	4		

[a]not grouped - viruses placed in this category did not necessarily show the same mAb pattern. *ICPI 1.62

Acknowledgements
We thank Karen Frost her assistance. The nucleotide sequencing of the influenza viruses was done by Jill Banks and Geoff Wood.

AVIAN INFLUENZA AND NEWCASTLE DISEASE IN THE EUROPEAN COMMUNITY: THE SANITARY SITUATION AND LEGISLATIVE DEVELOPMENTS IN 1995 TO DATE

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Sanitary situation

Avian Influenza
No outbreaks of avian influenza were confirmed in the Community in the past year.

Newcastle Disease
The situation with regards to Newcastle disease [ND] was not satisfactory in both 1993 and 1994. The reported position in the 12 months of 1994 was as follows:

Country	Outbreaks
Germany	181
Italy	41
Portugal	12
Netherlands	8
Belgium	1

giving a total of 243 outbreaks.

There has been, however, a considerable improvement in the number of outbreaks of the disease reported to the Commission in 1995. So far the position is as follows:

Country	Outbreaks
Germany	20
Italy	2
Portugal	2
Netherlands	1
Luxembourg	1

The total so far in 1995 is 26 outbreaks. Since 1994, the issue of Newcastle disease has featured as a permanent item on the agenda of the Standing Veterinary Committee. All outbreaks reported and actions taken are described and discussed by all the Member States.

As last year, the majority of the outbreaks occurred in Germany and were almost all in small non commercial poultry flocks located in the former East Germany.

A high proportion (95%) of last years cases these have been attributed to the purchase of hens from a small number of dealers. In August 1994, in an effort to counteract the situation, the German authorities introduced new provisions by which the Landers could make vaccination compulsory in all flocks, no matter how small. In addition poultry dealers were required to register and their own flocks are subjected to checks.

Once again, the situation in Portugal is giving cause for concern. Though only two flocks have been reported as being affected, these have been commercial flocks and have been infected with the same virus (not seen elsewhere in the EU) as in former years. This could indicate that the virus is persisting in Portugal, either in backyard flocks or in inadequately vaccinated commercial flocks.

The has been an improvement from last year in the situation in the Benelux countries, though unfortunately marked by a return of the disease to Luxembourg. In this case two small flocks were involved, one of which was made up of racing pigeons. These pigeons had returned from a race and had not been vaccinated as required by the Newcastle disease Control Directive (92/66/EEC). It is also worth mentioning that several complaints have been received about the implementation of these rules by the Member States. These complaints are under investigation by the legal infractions section of the Commission.

In the other case in Luxembourg, the purchase of birds and market contacts were implicated in the spread of the disease. This should remind us of the outbreaks in the Benelux countries in 1992 and 1993 where markets and sales of hobby birds were strongly implicated. Vigilance is still required in this sector.

Two outbreaks have occurred in Denmark, also in small backyard, mixed species flocks. These have been the only outbreaks so far this year in the non vaccinating part of the community.

As last year, the decrease, not only in the number of outbreaks, but also in their size has continued. So far this year a total of 65,000 birds have been, slaughtered compared to 165,000 in the same period last year and to over one million in the same period of 1993. This drop obviously reduced the costs to the industry, the

Member States and the Community in dealing with the disease. It may indicate improved vaccine protection and disease security measures on commercial farms or perhaps a lower level of circulating virus. We must, however, guard against complacency which could result in a rapid increase in disease levels, losses and trade disruption.

1995 also saw the enlargement of the Community with the accession of Sweden, Finland and Austria. Of the three new members, Sweden and Finland both continued their non vaccination policy and were recognised as such by Commission decisions. These three countries as well as European France, Spain, Italy, Greece, Ireland, United Kingdom and Belgium have also not reported any disease so far in 1995.

Legislative developments

As in previous years legislation has been enacted dealing with disease control, trade and non vaccination areas. Other topics are also under discussion with legislation planned for later this year or early 1996.

1. Disease control

- Two decisions were adopted to provide for the funding of the Community Reference Laboratories for Newcastle disease and Avian Influenza for 1995 - decisions 94/878/EC and 94/859/EC respectively. These provided that the Community Reference Laboratory for both diseases would be the Central Veterinary Laboratory (Weybridge), Addlestone United Kingdom. We also anticipate renewing this contract for 1996.
- Several decisions concerning the funding of measures resulting from the confirmation of Newcastle disease in 1993 and 1994 were adopted. These involved the payment of 50% of the costs of slaughter and cleaning up following outbreaks of the disease in Portugal, in both years, and in Spain and Germany in 1993. It is likely that further decisions for Germany and the Netherlands will be taken before the end of the year.
- A decision was adopted (Decision 95/117/EC) on the conditions by which poultry meat originating in a farm in a surveillance zone for Newcastle disease but outside the protection zone may receive the EC health mark. Clinical inspection, epidemiological examination of the farm and virological examination of the birds is required. The decision involves taking 60 swabs for virological examination. These are evenly split between cloacal and tracheal swabs.
- Another important development is that decision 93/152/EC came into force on 1 January 1995. This decision sets out the criteria that must be met by vaccines used for routine vaccination in the community. The decision effectively provides that vaccines based on virulent strains of Newcastle disease virus are not used in the Community.

2. Trade in live poultry and poultry meat

- The plans for veterinary monitoring of establishments in the new member states have been examined and approved by Commission decision in the case of Finland and Sweden (Decisions 94/964/EC and 95/141/EC respectively). The plans met both the criteria contained in the annexes to Directive 90/539/EEC and the Commission guidelines on the subject. A decision has not yet been taken on Austria, because of delays in the transposition of the Directive into Austrian law.
- Decision 94/85/EC which sets out a third country list for the importation of poultry meat has been extended to include Namibia, Slovenia and Malta. This list is a list "in principle" only and any importations would also have to meet additional animal and public health requirements.
- Commission Decision 94/984/EC laying down animal health conditions and veterinary certificates for the importation of fresh poultry meat from certain third countries was adopted last December and came in to force on 1 May 1995. This decision contains a list of countries from where importation is allowed. Two types of certification are specified, Type A from disease free countries which do not use "hot" ND vaccines and which do not have outbreaks in non commercial flocks and Type B certificates for countries which do not meet these two criteria. Additional virological testing is specified for countries using the type B certificate.
- Technical agreement between the member states has just been reached for a Commission decision on the list and certification requirements for the importation of live poultry from third countries.

3. Non vaccinating areas

- Decisions on the Newcastle disease status of the new member states were taken earlier in the year. Two of the new Members Sweden and Finland both met the criteria for non vaccinating status against Newcastle disease. The decisions are Commission Decision 95/98/EC and 94/963/EC respectively.

4. Legislation under discussion

- A proposal to amend Directives 90/539/EEC and 91/494/EEC is being forwarded to Council. Discussions in Council and Parliament are expected in the first half of 1996. The proposals may be summarised as follows;

Amendments to 90/539/EEC

1. Exclusion of ratites from the non commercial exemption in Article 11.
2. amendment of the rules concerning trade into the non vaccinating part of the community in response to the introduction of a

harmonized standard for ND vaccine for routine use in the Community.

The proposed changes are

a. removal of the restriction that live vaccines could not be used on hens producing eggs for hatching in the 60 days before lay.
b. addition that the chicks themselves must not be vaccinated (possible at day old stage)
c. Poultry for slaughter - removal of the requirement that live vaccines must not have been used in the 30 days prior to slaughter - otherwise it remains the same.

3. Amendment to give the Commission the power to authorise the importation of poultry and hatching eggs following a regime of quarantine both in the third country of origin and following entry into the Community. This applies to poultry and hatching eggs coming from countries which might not ordinarily be permitted to export to the community.

Amendment to 91/494/EEC

This amendment proposes to remove the requirement that the poultry that produced the meat have not been vaccinated with a live vaccine in the 30 days prior to slaughter. This is consistent with the amendments to 90/539/EEC and with the harmonized standards in vaccine use referred to above.

- Discussions are also underway on the introduction of a quarantine regime for the importation of non poultry species from third countries.

5. Forthcoming legislation

In addition to those detailed above, the following legislative developments are likely in the near future:

- Ratites
  Amendments are likely to the annexes of Directive 90/539/EEC to reflect the special situation as regards ratites. These changes should follow the finalisation of the Scientific Veterinary Committee's report on ratites. Following the adoption of this report and any necessary changes to the annexes, further amendments to each member states surveillance plans will also be necessary to implement any new regime.

- Safeguard decisions
  Following from the adoption of the harmonized rules for the importation of poultry meat into the Community, any third country that suffers an

outbreak of Newcastle disease or Avian influenza will be automatically prohibited from exporting product to the European Community (the veterinary certificate cannot be signed). This prohibition will last for a period of six months unless certain possibilities are deemed appropriate and are adopted by the Standing Veterinary Committee. These possibilities are as follows;

1. Do nothing and allow the prohibition to stand
2. Regionalise the Third Country, allowing trade to continue from the unaffected part.
3. Authorise the third country to use certificate B instead of A, requiring additional testing, where the outbreak has been in backyard birds.
4. Accept that the measures implemented the third Country are equivalent to Community disease control legislation and continue trade.

Given the level of Newcastle disease worldwide, it is anticipated that this situation will occur in the next 12 months.

SESSION IV - ORIGINAL CONTRIBUTIONS

COMPARISON OF PMV-1 HAEMAGGLUTINATION INHIBITION TITRES, NEUTRALISATION INDICES AND PMV-1 SPECIFIC MONOCLONAL ANTIBODY BLOCKING ELISA TITRES OF OSTRICH SERA.

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Introduction

A PMV-1 specific monoclonal antibody blocking ELISA (B-ELISA) was developed and evaluated with the help of NDV-positive and negative chicken and turkey sera (2). As indicated, it is possible to test sera or egg yolk from any species without any modification of the test system.

In the present study, 211 ostrich sera, previously tested with virus neutralisation (VN) and haemagglutination inhibition (HI) tests, were examined by B-ELISA. There was no other evidence of the presence of NDV in the tested birds, except the serological results obtained by the different methods. VN test is considered as the most reliable method, therefore it was used as reference test when estimating the sensitivity and specificity of the HI and B-ELISA tests.

Materials and methods

Ostrich sera

One hundred and forty-one sera previously examined by HI and VN methods (4) were retested with mAb ELISA. Additionally, 70 ostrich sera from 5 Swedish farms were tested by all three methods.

No clinical signs of disease were observed in any of the bird tested. There was no information available about possible vaccinations, or the owners declared their ostrich flocks as non-vaccinated.

Serological methods
The virus neutralisation (VN) test was performed as described (4) with the mixture of 1:10 diluted ostrich sera and 10^7 EID_{50} of virulent NDV, PMV-1/Ch/NI/152608. The mixture was subsequently diluted on monolayers of chicken embryo related (CER) cell line. Monolayers were checked for CPE at 48 hours after infection. The neutralisation index (NI) is a difference between the $\log_{10}$ titre of the virus in the absence and presence of serum.

The haemagglutination inhibition (HI) test was performed as described in the Council/Directive/92/66/EEC (1) using 8 haemagglutination (HA) units, sera were not pre-absorbed. Titres of $\log_2$ 3, or greater, were regarded as positive.

The monoclonal antibody blocking ELISA (B-ELISA) test was performed as described (2), using the commercially available Newcastle disease-AB EIA Kit (Svanova Biotech, Uppsala, Sweden). The working dilution of sera was 1:10; all sera showing > 50 percent inhibition (PI) were considered as positives. The B-ELISA titres were expressed as the highest twofold dilution of the starting 1:10 dilution still showing PI>50%.

Statistical analyses
Sensitivity was defined here as the proportion of VN-positives that were correctly identified also by the tested method (HI or B-ELISA), whereas *specificity* showed the proportion of the correctly identified VN negative samples. They are expressed in percents.

Predictive values showed the proportion of sera resembling VN results to all sera examined. The maximum value is 1; the higher the value, the higher the probability that examination of the same sample with the reference method would give similar results.

Correlation coefficients were calculated in order to measure the strength of associations between VN index, HI titre and B-ELISA titre. To evaluate whether a possible linear association reflects good agreement between two methods, measure of agreement (kappa) was also calculated (3).

Results
Of 211 ostrich sera, 140 contained NDV-specific neutralising antibodies as shown by the VN test, whereas 130 sera had positive HI titres and 122 were positive by B-ELISA. The relationships between the different tests are shown in Table 1. The sensitivity, specificity and predictive accuracy of HI and the B-ELISA were similar compared to the VN test (Table 2).

When B-ELISA was compared to HI using the latter as the reference test, the sensitivity was 91% and specificity was 96%. The probability that any of the B-ELISA-positive samples would be also HI-positive was found to be 0.97 (positive predictive value), whereas the probability of a concordant negative result was 0.87 (negative predictive value).

Table 1. Ostrich sera examined by 3 serological methods.

1a). B-ELISA compared to virus neutralisation [VN]

		VN		
		+	-	**total**
B-ELISA	+	116	6	122
	-	24	65	89
	total	140	71	211

1b). HI compared to VN

		VN		
		+	-	**total**
HI	+	121	9	130
	-	19	62	81
	total	140	71	211

1c). B-ELISA compared to HI

		HI		
		+	-	**total**
B-ELISA	+	119	3	122
	-	11	78	89
	total	130	81	211

Table 2.: Relative sensitivity, specificity and predictive accuracy of HI and B-ELISA tests compared to the VN test.

	HI compared to VN	B-ELISA compared to VN
Sensitivity (%)	86	82
Specificity (%)	87	91
Positive predictive value	0.93	0.95
Negative predictive value	0.76	0.73

A close correlation (r=0.71) was found between B-ELISA titres and VN indices as shown in Figure 1. Similar correlation coefficients (r=0.72) characterised both the B-ELISA and HI, and the VN and HI relationships.

The degrees of agreements and the kappa values are summarised in Table 3.

Table 3. Proportional agreements and kappa values of the three tests.

	Observed proportional agreement	kappa
VN and HI	86.7%	0.70
VN and B-ELISA	85.8%.	0.70
HI and B-ELISA	93.3%.	0.85

Discussion

In a previous comparison VN-positive ostrich sera were examined with HI test (4). The results showed that the HI test was nearly as sensitive as the reference method (92%). Additionally, ostrich sera originating from Swedish farms were tested and proved to be negative with both methods. Summarising these results, the overall sensitivity of HI test relative to VN test was 86%, the specificity was 87%.

The same set of ostrich sera were also retested with B-ELISA. The sensitivity of the recently introduced method was 82%, whereas the specificity was 91% relative to the VN test. However, ostrich sera were examined in tenfold dilutions instead of the default twofold dilution, and the use of higher dilutions decreases the sensitivity of the B-ELISA (2).

The concordant results and the close relationship between HI and B-ELISA (kappa =0.85) suggest that the two methods are interchangeable and both are measuring the same thing; the amount of NDV-specific antibodies.

The presence of NDV-specific antibodies in traded ostriches raises many questions concerning diagnosis. There is a definite need for a standardised, reliable serological method which is relatively easy to perform with sera from these exotic birds.

The VN test would be the most accurate and most reliable test for this purpose but it is very laborious and needs special skills, therefore it is not widely used routinely. Serological examination of ostrich sera by HI tests can be also difficult due to the non-specific agglutination of chicken red blood cells. The number of non-specific reactions can be reduced by heat inactivation and absorption, but these procedures seem to decrease the sensitivity of the method as well.

The B-ELISA, showing similar sensitivity and specificity to HI test in this study, could be the method of choice when sera from exotic birds must be tested serologically.

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n =211
r=0.71
P<0.001

ELISA log_{10}		0	1	2	3	4	5	6	7
positive	4					2	4	3	1
	3		1	3	4	5	4		
	2	1	1	10	11	11	8	1	
	1		3	14	20	14	1		
negative	0	17	48	21		2	1		
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
		negative		**positive**					

NI log_{10}

Figure 1. *Correlation between mAb ELISA titres and virus neutralisation indices (NI).*

ESTIMATION OF THE PATHOGENICITY OF NDV-ISOLATES BY HYBRIDISATION WITH DIGOXIGENIN-TAILED OLIGONUCLEOTIDES

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Introduction

In Germany in the last two years there was a large number of Newcastle disease (ND) outbreaks, mostly in backyard flocks. According to Directive 92/66/EEC it is compulsory to determine the ICPI of each virus isolated, which means not only a considerable amount of work, but also a large number of animal experiments.

The knowledge of the relationship between pathogenicity and the sequence of the cleavage site of the F0 protein made it possible to develop a new method to determine the pathogenicity without doing animal experiments [1,2]. Amplification of a desired nucleic acid fragment is a common method today and further characterisation of this fragment by hybridisation enabled us to distinguish between isolates of different pathogenicity.

Material and methods

The method of choice for isolating RNA was the acid guanidiniumthiocyanate-phenol- chloroform extraction, reported by Chomczynski and Sacchi [3]. It needs only 50 µl allantoic fluid for the RNA-preparation.

The following PCR was done with primers 3 and 4 (Table 1) according to the protocol described previously [4] and the resulting 362 bp fragment was transferred to a nylon membrane.

It was necessary to design pathogen specific oligonucleotides for hybridisation. The chosen oligonucleotides are listed in Table 1, they are designed from determined sequences of PCR fragments of viruses isolated recently in Germany.

Table 1: Sequences of the primers and oligonucleotides used.

name	specificity	nucleotide sequence
PCR primer 3	all	TTGATGGCAGGCCTCTTGC
PCR primer 4	all	GGAGGATGTTGGCAGCATT
oligo 1	velogenic	TCCACGCCTGGGGGAAGGAGACAGAAA
oligo 2	mesogenic	TCCACATCAGAAGTAAGGAGGAAGAAG
oligo 3	mesogenic	TCCACATCAGGAGGAGGAAGGCAGAAG
oligo 4	lentogenic	ACTACATCTGGAGGGGGGAGACAGGGG

The designed oligonucleotide probes are enzymatically, but nonradioactively, labelled at their 3' end with terminal transferase by addition of a longer nucleotide tail (protocol by Boehringer Mannheim). For the generation of tailed oligonucleotide probes, a mixture of deoxynucleotidetriphospate (dNTP) and digoxigenin-dUTP (DIG-dUTP) is applied and added in a template-independent reaction. DIG-labelled oligonucleotide probes are detected after hybridisation to target nucleic acids by enzyme linked immunoassay using an antibody conjugate. A subsequent enzyme catalysed colour reaction with 3-bromo-4-chloro-3-indolylphosphate (X-phosphate) and nitro blue tetrazolium salt (NBT) produces an insoluble blue precipitate, which visualises the hybrid molecule.

The hybridisation of oligonucleotides requires conditions adapted to the length and nucleotide composition of the oligonucleotides. It is usually carried out at a temperature 5 to 10 °C below the T_m-value.

Results & Discussion

All potentially pathogen-specific oligonucleotide probes were labelled and different NDV PCR products were applied to the nylon membrane. A typical dot-blot-hybridisation result is shown in Figure 1 and the results compared with ICPI values in Table 2. PCR fragments of different pathogenic NDV isolates are hybridised with three pathogen-specific, DIG-labelled oligonucleotide probes. The reactions of both the velogen-specific and the lentogen-specific oligonucleotides are very selective. There were some small cross reactions with the mesogenic probe.

Results of hybridisation reactions with isolates from chickens and pigeons from 1993 and 1994 are listed in Table 3. Most are in an agreement with the determined ICPI. For a few cases where the hybridisation result differs from the ICPI it is necessary to repeat the hybridisation and possibly to sequence the PCR fragment of such an isolate. There is always a possibility of changes in the sequence of the cleavage site, as published for different velogenic strains [5,6].

Table 2: Comparison of the hybridisation technique and ICPI results

	Hybridisation/ICPI		
	1	**2**	**3**
A	velogen/1.9	lentogen/0.1	lentogen/0.4
B	velogen/1.9	velogen/1.8	lentogen/0.2
C	velogen/1.9	velogen/1.9	mesogen/1.2
D	velogen/1.9	lentogen/0.02	relevant+control
E	velogen/1.9	velogen/1.8	
F	velogen/1.9	velogen/1.9	
G	velogen/1.9	velogen/1.9	

Figure 1: Dot-blot hybridisation with three pathogen specific dig-labelled oligonucleotides

Table 3: Comparison of the hybridisation technique and ICPI results for isolates made in 1993-1994.

isolate	bird	hybridisation result	ICPI	isolate	bird	hybridisation result	ICPI
1/93	chicken	velogen	1.8	180/94	chicken	lentogen	0.01
6/93	"	mesogen	1.2	203/94	"	velogen	1.9
14/93	"	velogen	1.9	206/94	"	"	1.9
18/93	"	mesogen	1.3	215/94	"	lentogen	0.1
101/93	"	velogen	1.9	217/94	"	velogen	1.9
118/93	"	"	1.7	218/94	"	lentogen	0.02
121/93	"	"	1.8	223/94	"	velogen	1.9
134/93	"	"	1.8	234/94	"	lentogen	0.02
143/93	"	"	1.6	252/94	"	velogen	1.9
144/93	"	"	1.8	293/94	"	"	1.9
145/93	"	"	1.9	299/94	"	"	1.9
148/93	"	"	1.9	306/94	"	"	1.9
149/93	"	lentogen	0.3	309/94	"	"	1.9
159/93	"	velogen	1.8	314/94	"	"	1.8
163/93	"	"	1.7	317/94	"	"	1.8
169/93	"	"	1.8	*64/94*	*"*	*velogen/lentogen*	*1.9*
1/94	"	"	1.9	*296/94*	*"*	*velogen?*	*1.9*
2/94	"	lentogen	0.2	33/93	pigeon	mesogen	1.0
8/94	"	mesogen	1.0	135/93	"	"	1.1
20/94	"	velogen	1.9	3/94	"	"	1.3
29/94	"	"	1.9	5/94	"	"	1.0
68/94	"	lentogen	0.4	16/94	"	lentogen	0.2
114/94	"	velogen	1.9	*146/94*	*"*	*mesogen*	*1.7*
150/94	"	"	1.9	185/94	"	velogen	1.7
177/94	"	"	1.8	*265/94*	*"*	*mesogen/velogen*	*0.8*

The investigation of ND isolates from imported ducks by PCR and following hybridisation with pathogen-specific DIG-labelled oligonucleotides wasn't successful. The PCR worked very well, but in hybridisation reaction there was no positive signal with any oligoprobe. Sequencing of the PCR product and comparison of the obtained sequence with sequences in the Genebank results in a 100 % homology of the nucleotide sequence with the strain Italien. Hybridisation was repeated with an oligoprobe designed from strain Italien for confirmation of this homology and we obtained a clearly positive signal.

In summary, PCR in connection with hybridisation is a suitable method to identify velogenic isolates without performing animal experiments. The

advantages of this method based on molecular structure of the virus to determine the pathogenicity are:

- quickness
- only small amounts of investigation material are necessary
- the method is practicable while omitting animal experiments.

Mutations of strains near the cleavage site of the fusion protein are seldom seen during an epidemic period, as we have observed in Germany since 1993. Thus oligonucleotides designed in such a way can be used for screening.

In order to limit or to replace the determination of the pathogenicity of NDV isolates by ICPI it will be necessary to evaluate far more samples.

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DIFFERENTIATION OF VIRULENT AND NON-VIRULENT STRAINS OF NEWCASTLE DISEASE VIRUS BY USING POLYMERASE CHAIN REACTION

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Introduction

Diagnosis of a Newcastle disease virus (NDV) infection is routinely done by virus isolation from tissue homogenates from birds of suspected flocks using embryonated eggs as substrate. A preliminary differentiation of virulent and non-virulent strains can be obtained using the haemagglutination inhibition (HI) test and monoclonal antibodies (mAbs). Finally, the virulence of isolates is measured by determination of the intracerebral pathogenicity index (ICPI). The whole procedure will take at least five days.

Another problem is the lack of mAbs that are specific for virulent strains. The preliminary differentiation by HI tests depends thus on the inhibition by mAbs specific for non-virulent strains. Therefore, samples that contain a mixture of virulent and non-virulent virus may result in the same reaction pattern with mAbs in HI tests as non-virulent viruses.

We attempted to overcome these problems by using the polymerase chain reaction (PCR). The differences in the nucleotide sequence around the splicing site of the fusion protein are the basis for the differentiation of virulent and non-virulent strains. The RT-PCR developed can be used both to detect and differentiate NDV using RNA isolated from tissue homogenates. The RT-PCR was specific and sensitive and could be completed within 24 h.

Materials and Methods

RNA isolation

In preliminary experiments the isolation of RNA was performed as described by Chomczynski and Sachhi (1), Yamada *et al*. (2) or McCaustland *et al*. (3). Routinely, RNA was isolated from clarified allantoic fluids and tissue homogenates using RNeasy spin columns (Qiagen). The procedure is as follows. One hundred µl samples were lysed with 350 µl lysis buffer. After adding 250 µl 100% ethanol, the samples were loaded on RNeasy spin columns. The columns were centrifuged and subsequently washed once with washing buffer 1 and twice

with washing buffer 2. The bound RNA was eluted with 30 µl water.

RT-PCR and hybridisation

The code, location and sequence of the oligonucleotides used as primers for the RT-PCR or used as probes for hybridisation are shown in Table 1. Eight µl of the eluate was denatured with methylmercury-hydroxide in the presence of primer A. Subsequently, c-DNA was synthesised with Superscript H^- reverse transcriptase (BRL). The reaction mixture was used to perform three different PCRs using three primer pairs A+B, A+C and A+D, and Taq DNA polymerase (Perkin Elmer). After denaturation for 4 min at 94°C, the PCR consisted of 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. Samples were analysed by electrophoresis on 2% agarose gels and blotted onto Hybond N^+ membranes (Amersham). Probes K, L and M were 3'-end labelled with digoxigenin (DIG) (Boehringer) and used for hybridisation. Hybridisation was visualised by incubation with anti-DIG immunoglobulin conjugated to alkaline phosphatase and CSPD^R as substrate (Boehringer).

Table 1. Primers used for RT-PCR and probes used for hybridisation

Code (orientation)	Location*	Sequence
A (forward)	141-159	5′ TTGATGGCAGGCCTCTTGC
B (reverse)	503-485	5′ GGAGGATGTTGGCAGCATT
C (reverse)	395-380	5′ AGCGTCTCTGTCTCCT[#] T
D (reverse)	395-380	5′ GACGACCCTGTCTCCC G T T
K	315-333	5′ CTTTGCTCACCCCCCTTGG
L	296-336	5′ TACAACAGGACATTGACTACTTTGCTCACCCCCCTTGGTGA C C
M	296-336	5′ TATAACAGAACACTGACTACCTTGCTCACTCCCCTTGGCGA

*Numbering according to Toyoda *et al*. (4)

[#] Differences between primer C and D and between probe K, L and M are underlined.

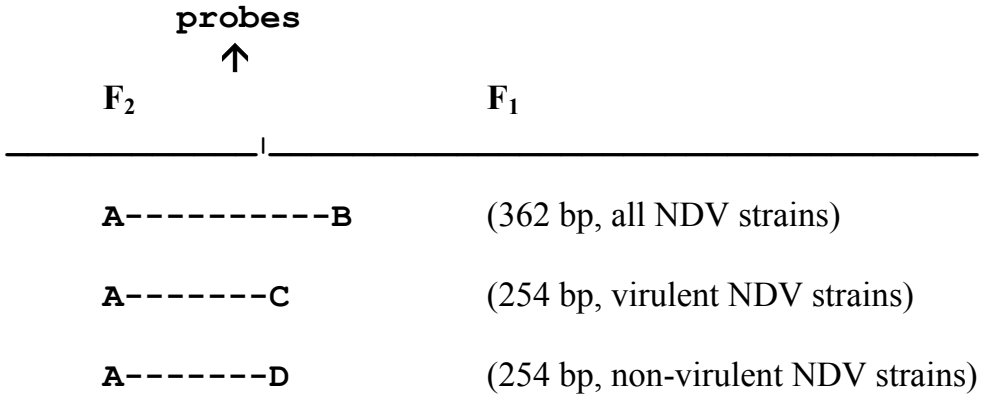
Results

RNA isolation

Several RNA isolation procedures were tested using allantoic fluids containing different strains of NDV. The procedure of Chomczynski and Sachhi (1) proved to be laborious. The amplification was poor using RNA isolated according to Yamada *et al*. (2) and McCaustland *et al*. (3) (data not shown). The best results were obtained when RNA was isolated according to the RNeasy protocol. This procedure was used in all further experiments reported.

RT-PCR and hybridisation
The RT-PCRs using primer pairs A+B, A+C and A+D resulted in fragments with sizes that were in agreement with those summarised in Figure 1.

Figure 1. Outline of PCR and hybridisation.



The fragments were not clearly visible in all cases in the EtBr stained agarose gel. In particular, this was the case when RNA isolated from tissue homogenates, was used for cDNA synthesis and amplification. To overcome this problem we decided to complement the assay with blotting and hybridisation. Probes K or L showed a strong signal after hybridisation of the amplification products of most strains. However, a weak signal was observed with products of recent virulent isolates and three virulent strains isolated by others (Florida, PMV-1/NL/56 and Houbraken).

Nucleotide sequencing of the region complementary to the probes showed that the recent isolate PMV-1/ch/Nl/1992/152608 (Nl/92) had five mutations compared to other sequences. Probe M represents the Nl/92 sequence. Subsequently, a mixture of probes L and M was used for hybridisation.

Sensitivity
To determine the sensitivity of the assay allantoic fluid from the non-virulent La Sota (Las) strain ($10^{9.5}ELD_{50}$/ml) and from the virulent Nl/1992 isolate ($10^{8.5}ELD_{50}$/ml) was diluted 10^2, 10^4, 10^6 and 10^8 fold and RNA was isolated. RT-PCR and hybridisation were as described in the Materials and Methods section.

Products of the expected size were observed with both strains until the 10^4 dilution using primer pair A+B. Thus, the minimum amount of NDV which can be detected with the RT-PCR is about 10^5ELD_{50}/ml. The same concentration appeared to be necessary with La Sota using primer pair A+D and with Nl/1992 using primer pair A+C. Blotting and hybridisation did not increase the sensitivity significantly when compared to EtBr staining.

Specificity
The specificity of the assay was verified using allantoic fluids containing different strains of NDV. Some other haemagglutinating avian viruses were also tested. Results are summarised in Table 2.

Almost all NDV strains were differentiated according to ICPI values. However, with 2/4 batches of strain Hitchner, obtained from different laboratories, specific products were obtained not only when the primer pairs A+B and A+D were used, but also when A+C was used. This might be caused by contamination of these batches with virulent virus or by heterogeneity of the original Hitchner isolate.

More or less the same result was obtained with Texas GB and two Herts batches. A Herts batch of low passage level reacted strongly using primer pair A+C and weakly using primer pair A+D, in contrast to an other batch of higher passage level where the result was reversed.

No specific amplification was observed using RNA of any of the haemagglutinating avian viruses other than NDV.

Tissue homogenates
Obviously the speed of the diagnosis can be significantly increased when the RT-PCR can be performed on tissue homogenates without prior virus isolation. Therefore the RT-PCR was performed on RNA isolated from homogenates of brain, trachea, lung and spleen from chickens of 13 flocks suspected of Newcastle disease. The homogenates were also used for routine virus isolation and differentiation by using mAbs in HI tests and by determination of the ICPI.

The isolated virus was characterised as velogenic in 7/13, mesogenic in 1/13 and lentogenic in 2/13 flocks. From the homogenates of three flocks, no virus could be isolated.

The results of the RT-PCR were, with one exception, in agreement with the virus isolation and characterisation. No specific products were observed using the homogenates of organs of 1/2 flocks from which lentogenic virus was isolated. However, isolation of this virus needed two passages to get specific haemagglutination, suggesting that the virus concentration in the original samples was very low.

Table 2. Differentiation of virulent and non-virulent NDV strains using PCR

Virus	Virulence[#]	PCR results with primers		
		A+B	A+C	A+D
Beaudette	Velo	+[@]	+	-
Bogor	Velo	+	+	-
California	Velo	+	+	-
Florida	Velo	+	+	-
Herts(L)[*]	Velo	+	+	±
Herts(H)	Velo	+	±	+
Milano	Velo	+	+	-
Mukteswar	Velo	+	+	-
PMV_1/NL/56	Velo	+	+	-
PMV_1/NL/Houbraken/75	Velo	+	+	-
PMV_1/NL/152608/92	Velo	+	+	-
Texas GB	Velo	+	+	±
Victoria	Velo	+	+	-
Komarov	Meso	+	-	+
Roakin	Meso	+	+	-
Hitchner(1,2)[**]	Meso/Lento	+	+	+
Hitchner(3,4)	Meso/Lento	+	-	+
F	Lento	+	-	+
La Sota	Lento	+	-	+
Ulster	Lento	+	-	+
PMV-3		-	-	-
PMV-6		-	-	-
PMV-7		-	-	-
Infl. A/Parakeet/H3N8		-	-	-
Control AAF		-	-	-

[#] Virulence based on ICPI values

[@] + denotes specific product, ± only small amount of specific product and - no specific product was found

[*] Two batches of strain Herts were used one with low (L) and one with high (H) passage level

[**]Four batches of strain Hitchner, obtained from different laboratories, were used

In 3/7 cases from which velogenic virus was isolated, also lentogenic La Sota strain virus was isolated from the trachea homogenates and only this latter virus was indicated by the mAb reaction pattern in HI tests. Using the same samples for the RT-PCR, specific products were observed with all the three primer pairs. This demonstrated clearly that both virulent and lentogenic NDV were present in these samples.

Discussion

Differentiation between virulent and non-virulent NDV strains is possible by using the PCR technique and primers with the nucleotide sequences of the region around the splicing site of the fusion protein.

The assay is specific, sensitive and has several advantages. The sensitivity is about $10^5 ELD_{50}$/ml of NDV, which seems to be high enough to detect virulent virus in homogenates of tissues of chickens suspected of Newcastle disease. The result of the RT-PCR may be obtained within 24 h of arrival of samples. Confirmation of the test by hybridisation takes another 24 h. Another advantage of the RT-PCR is that it enables the detection of virulent virus in the presence of non-virulent strains. Virulent virus can not be detected in the HI when also lentogenic virus is present because no mAbs are available that react with virulent strains only.

Some NDV strains could not be differentiated according to ICPI values, specific products were obtained with both primer pairs A+C and A+D. This finding can be explained when those strains are contaminated with respectively virulent or non-virulent NDV, or when sequence differences, other than those published, exist in the region of primer C and D leading to annealing and subsequently amplification. We will be able to distinguish between both possibilities by either plaque purification or sequencing the primer region of those strains.

Further validation of the assay is needed by testing more recent isolates. In particular, isolates from pigeons that exhibit high ICPI values only after several passages in chickens should be analysed by the RT-PCR described.

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PROPOSAL FOR A SIMPLIFIED DETERMINATION OF VIRULENCE OF PMV-1 ISOLATES

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For the current definition of Newcastle disease in the European Union it is necessary to determine if the intracerebral pathogenicity index [ICPI] is greater than 0.7, it is, therefore, not necessary to determine the exact ICPI value for each isolate. In fact velogenic viruses show much higher values than 0.7. Because of the recent epidemiological situation in Germany it was considered necessary to seek alternative, quicker estimations of virulence.

In practice, diagnosticians with laboratory experience were able to recognise velogenic NDV by the death time of inoculated embryos and the growth of virus in cell cultures.

In our experience during 1994, nearly all viruses causing the death of all inoculated embryos within two days of inoculation at a single 10^{-2} dilution proved to be velogenic [Table 1].

Table 1: Death time of the 287 PMV-1 isolates which were investigated in the National Reference Laboratory in 1994.

Number of isolates for which all inoculated eggs* were dead:		ICPI	Pathotype
at 2 d p.i.	later than 2 d p.i.		
202	5	>1.5	velogenic
2	37	>0.7 <1.5	mesogenic
0	41	<0.7	lentogenic

* Eggs were inoculated with 10^{-2} diluted cell culture or egg allantoic fluid.

In addition, we routinely inoculate chick embryo cell cultures with each isolate and test each isolate with our own monoclonal antibody panel.

We have therefore adopted the procedure that if an isolate fulfils the following criteria:

- **it causes the death of all inoculated embryos within 2 days and**
- **it grows in chick embryo cell cultures without added trypsin with plaque formation or a strong cytopathic effect with syncytial formation and**
- **shows a typical reaction pattern for the current epizootic virus with our monoclonal antibody panel**

we do not carry out an ICPI test since we can be sure that the ICPI will be greater than 0.7. We give the diagnosis:- **PMV-1 with an ICPI above 0.7**.

For all isolates that fail to fulfil these criteria we determine the ICPI value by the intracerebral inoculation of day-old chicks.

NEWCASTLE DISEASE HAEMAGGLUTINATION INHIBITION TESTS IN NATIONAL LABORATORIES - RESULTS OF THE COMPARISON OF TEST REPRODUCIBILITY IN DIFFERENT LABORATORIES

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Introduction

One of the functions and duties of the Community reference laboratory for Newcastle disease is to organise "periodical comparative tests in diagnostic procedures at Community level". To fulfil this duty and following discussions at the 1994 meeting of the National Laboratories, a simple test of the reproducibility in the National Laboratories of the haemagglutination inhibition [HI] test for the detection of Newcastle disease virus antibodies was organised.

Materials and methods

Sera

A series of sera, **A-J**, designed to give a range of haemagglutination inhibition titres was prepared at the reference laboratory and freeze-dried.

The sera had the following histories:

A. pooled sera from SPF chickens;
B. pooled sera from chickens primed with Hitchner B1 and then challenged twice with Herts 33;
C. pooled sera from chickens primed with Hitchner B1 and challenged once with Herts 33 prepared June 1995;
D. pooled sera from SPF chickens [different from A];
E. pooled sera from chickens primed with Hitchner B1 and challenged once with Herts 33 prepared January 1995;
F. pooled antibody negative duck sera;
G. pooled sera from chickens primed with Hitchner B1 and challenged once with Herts 33 prepared January 1987;
H. pooled sera from chickens challenged twice with PMV-1/pigeon/England/561/83;
I. pooled sera from chickens challenged twice with PMV-3 - turkey/1087;
J. pooled sera from chickens primed with Hitchner B1 and challenged once with Herts 33 prepared May 1988.

Antigen
The Ulster 2C strain of NDV was grown in SPF hens' eggs, the harvested infective allantoic fluid was inactivated with 1/1000 formalin overnight and freeze-dried.

Test Methodology
The sera and antigen were dispatched to the National Laboratory of each EU country and Northern Ireland with the following instructions:

Accompanying this letter you will find 11 vials. Ten of these, labelled A-J, contain chicken antisera. The other contains Ulster 2C antigen and is labelled accordingly. Vials B, D and Ulster 2C antigen should be reconstituted by adding one ml of distilled water, all other vials should be reconstituted by adding 0.5ml distilled water. After reconstitution store at 4 ^{o}C.

The tests we would like you to carry out are:-

1. *Do haemagglutination inhibition (HI) tests on the antisera A-J using the antigen used in your laboratory and the method you would normally use.*

2. *Do a haemagglutination (HA) test on the Ulster 2C antigen using your normal technique.*

3. *Following the procedures in Annex I Chapters 5 and 6 of Directive 92/66/EEC (enclosed) prepare 4 HA units of the Ulster 2C antigen and use in HI tests with sera A-J following the 92/66/EEC protocol for HI tests.*

If your normal method is to follow the 92/66/EEC protocol exactly it is still necessary to do two lots of tests using your antigen [even if it is Ulster 2C] and the antigen supplied.

Results and Discussion
Results using the protocols in Directive 92/66/EC
The titres supplied by each laboratory are summarised in Table 1 The titres obtained in HA tests with the antigen supplied were important as the HA titre obtained relative to the other laboratories could influence the HI titres obtained with the sera. It was expected that the HA titre should be close to 512 and this was achieved by eight of the laboratories [10 if the laboratories starting with a 1:5 or 1:10 dilution, B and D, are included] and three laboratories were within one dilution of this titre. Laboratory NI recorded a titre of 2048 and Fi 4096. The effect of a high HA titre for the antigen used should be to under estimate 4HAU, relative to other laboratories, and this would result in higher HI titres with the sera. The higher value obtained by the Northern Ireland laboratory did not seem to greatly affect their HI titres relative to the other laboratories [Tables 2-5], but

the tendency for the sera titres obtained by Finland to be higher than other laboratories, seen best in Table 3, may have resulted from the high HA result.

It was decided not to do detailed statistical analyses on the titres produced by the different laboratories, but simple comparisons of the titres are shown in Tables 2-5. Comparisons of titres for the sera compared with the geometric mean titres showed good correlation with 12/16 laboratories having one or no sera outside one doubling dilution of the mean. If the nearest possible titre to the geometric mean is taken the only results outside one dilution of those were obtained with Serum C by Greece, serum D by France and Sweden, serum F by France and Greece, serum G by Austria and serum I by France [Table 2].

The geometric mean titre may not be the best way to make comparisons as it is influenced by titres outside the normal range. In Tables 3 and 4 the results have been compared to the titres obtained by the majority of the laboratories, i.e. the consensus titres, for each serum. The number of laboratories recording each titre is shown in Table 3. Since the test allows variation by one dilution only titres outside the limits are shown in Table 4; of 160 individual tests only 12 results fell outside the allowable limits.

The relationship between titres for each laboratory can be determined from Table 1. In Table 5 the overall agreement for each laboratory with each of the other laboratories is summarised by comparing the number of sera with two or more dilutions different. In this type of comparison a single aberrant result, for examples the results obtained with serum D by Sweden and F by Greece, will greatly influence the number of differences recorded. Because of this it is difficult to assess what level represents significant discrepancies between two laboratories. If an arbitrary level of 4 or more sera showing differences of two or more dilutions is taken, it can be seen from the highlighted numbers in Table 5 that all differences of this magnitude are associated with three laboratories: Finland which reached this level with 3 other laboratories, Italy with 5 laboratories and France with six laboratories.

Results obtained by National Laboratories using their normal procedure

The results supplied by each laboratory using their normal techniques and antigen are shown in Table 6. At the Reference Laboratory it was noticed that the standard operating procedure for the GB National Laboratory had erroneously omitted the incubation period after adding virus to the diluted serum. Although in practice this was routinely done by the National Laboratory it was decided to omit this step to see the effect on titres obtained. By comparing RL results in Tables 1 and 6 it can be seen that by omitting the incubation period the results obtained were often two to three dilutions lower. RL results were not used in the comparisons summarised in Table 7-9.

Interestingly, in terms of the overall differences, discrepancies and aberrant results there was marked similarity to the results obtained using the protocol of Directive 92/66/EEC. For example, 11 results fell outside one dilution of the consensus titres [Table 8] compared to 12 using the 92/66 protocol. Similarly, the number of times four or more sera with titres two or more dilutions different were seen in comparing each of the laboratories was 14 [Table 9] compared to 13 [Table 5] using the protocol in Directive 92/66. However, apart from Italy, the laboratories showing this level of disagreement were quite different from those using the EU protocol.

The main differences between the protocol in 92/66/EEC and those used routinely at National Laboratories are summarised in Table 10. Sweden, Spain., Portugal, Italy, Great Britain and Denmark all report using the protocol in Directive 92/66/EEC exactly, Northern Ireland and Ireland report the minor variation of starting with a 1/8 dilution of serum. Seven laboratories did not routinely use Ulster 2C as antigen in HI tests.

Conclusions

Overall the results were encouraging. Whether using the protocol in Directive 92/66/EEC or the National Laboratory's own method there were no widely varying results obtained by any one laboratory. The three negative sera, A, D and F were reported as showing titre <16 by all laboratories except for a titre of 16 recorded by Sweden with serum D [Table 6]. Sera A and F had been chosen because they had been shown to be particularly negative at the Reference Laboratory so it was slightly surprising that two laboratories reported titres of 8 with serum F [Table 1]. All the positive sera were reported as solidly positive by all laboratories, although there was some significant variation as mentioned above. Similarly, all laboratories detected cross reactive antibodies in serum I from chickens challenged with a PMV-3 virus although not always as 16 or greater. Taken as a whole the results obtained should allow confidence in ND HI testing at the different laboratories.

One disturbing finding was that 7 of the 15 laboratories were normally using protocols that were substantially different from that recommended in Directive 92/66/EEC. While this did not appear to influence the relative results obtained in this study, the adoption of Directive 92/66/EEC [and other Directives] in a Member State does place an obligation on laboratories to carry out tests as specified in the Directive [see Article 12 of Directive 92/66/EEC].

Table 1: Results obtained by National Laboratories using the protocol in Directive 92/66/EEC

Serum	RL	A	B	D	Fi	Fr	GB	NI	Gr	Ge	Ir	It	Ne	Pt	Sp	Sw
A	<2	<2	<2	<2	4	<2	2	<2	2	<2	<2	<2	<2	2	<2	<2
B	512	256	1024	256	512	1024	256	256	256	512	256	1024	512	512	512	512
C	1024	512	1024	512	2048	1024	1024	512	256	512	512	2048	1024	1024	1024	1024
D	2	<2	<2	<2	4	8	2	<2	2	2	2	<2	<2	2	2	8
E	512	512	1024	512	1024	512	512	256	512	512	256	1024	1024	1024	1024	512
F	<2	<2	<2	<2	2	8	<2	<2	8	<2	<2	<2	<2	<2	<2	<2
G	512	128	512	512	1024	512	512	256	256	512	256	1024	256	512	512	512
H	1024	1024	1024	1024	2048	512	1024	1024	1024	1024	1024	2048	1024	1024	1024	2048
I	16	16	32	8	32	64	16	16	16	32	16	32	16	16	32	32
J	256	256	128	128	256	256	128	256	128	256	128	512	256	256	512	256
2C Ag	512	1024	640	640	4096	1024	512	2048	512	512	512	512		256	512	384

Table 2: Results obtained by National Laboratories using the protocol in Directive 92/66/EEC titres outside one dilution of mean titre

Serum	RL	A	B	D	Fi	Fr	GB	NI	Gr	Ge	Ir	It	Ne	Pt	Sp	Sw	mean
A					4												1.6
B			1024			1024						1024					450
C					2048				256			2048					824
D						8										8	2.2
E								256			256						609
F						8			8								1.8
G		128			1024							1024					431
H						512											1117
I				8		64											22
J												512			512		225
Number	0	1	1	1	3	5	0	1	2	0	1	4	0	0	1	1	

Table 3: Results obtained by National Laboratories using the protocol in Directive 92/66/EEC consensus titres and variations by laboratory.

	Haemagglutination inhibition titres											
Serum	**<2**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**
A	**12**[a]	GB,Gr,Pt	Fi									
B									**6**	**7**	B,It,Fr	
C									Gr	**5**	**8**	It, Fi
D	**6**	**7**	Fi	Fr,Sw								
E									NI, Ir	**8**	**6**	
F	**13**	Fi		Fr, Gr								
G								A	NI,Ir,Gr,Ne	**9**	It, Fi	
H											**13**	It,Fi,Sw
I				D	**8**	**6**	Fr					
J								**5**	**9**	It, Sp		

[a]Number of laboratories with this titre.

Table 4: Results obtained by National Laboratories using the protocol in Directive 92/66/EEC titres outside one dilution of consensus titre

Serum	RL	A	B	D	Fi	Fr	GB	NI	Gr	Ge	Ir	It	Ne	Pt	Sp	Sw	consensus
A					4												<2
B			1024			1024						1024					256/512
C									256								1024
D						8										8	<2/2
E																	512
F						8			8								<2
G		128															512
H																	1024
I				8		64											16
J																	256
Total	0	1	1	1	1	4	0	0	2	0	0	1	0	0	0	1	

Table 5: Results obtained by National Laboratories using the protocol in Directive 92/66/EEC comparison of variation between laboratories - number sera with titres two or more dilutions different.

	RL	A	B	D	Fi	Fr	GB	NI	Gr	Ge	Ir	It	Ne	Pt	Sp	Sw
RL	*	1			1	3			2							1
A		*	2	1	4	5	1		1	1		3		1	1	2
B			*	2	1	2	1	2	3		2	1			1	1
D				*	4	4			1	1		4			2	2
Fi					*	3		5	3	2	3	2	3			1
Fr						*	4	4	4	2	4	3	3	3	2	2
GB							*		2			2			1	1
NI								*	1			4	1	1	1	1
Gr									*	1	1	5	2	2	3	3
Ge										*		1				1
Ir											*	5	1	1	2	1
It												*	1			1
Ne													*			1
Pt														*		1
Sp															*	1
Sw																*

Table 6: Results obtained by National Laboratories using their normal protocols

Serum	RL	A	B	D	Fi	Fr	GB	NI	Gr	Ge	Ir	It	Ne	Pt	Sp	Sw
A	2	<2	<2	<2	<4	<4	<2	<8	2	<2	<8	<2	<2	<2	<2	<2
B	64	256	1024	256	512	512	256	256	256	512	256	1024	1024	256	512	1024
C	256	1024	1024	512	1024	1024	1024	512	512	1024	512	2048	2048	512	1024	2048
D	<2	<2	<2	<2	4	<4	2	<8	2	<2	<8	<2	<2	<2	2	16
E	128	1024	1024	512	1024	1024	512	512	512	1024	256	1024	2048	512	1024	1024
F	<2	<2	<2	<2	<4	<4	<2	<8	2	<2	<8	<2	<2	<2	<2	<2
G	64	128	512	256	512	512	512	256	256	512	256	1024	1024	256	512	512
H	256	1024	1024	1024	1024	1024	512	512	512	1024	512	2048	2048	1024	1024	1024
I	8	16	32	8	32	32	8	16	16	64	8	32	64	16	32	32
J	32	256	256	256	256	512	128	128	128	256	64	512	512	256	512	256
	256	1024		768	4096	512	256	1024		512	512	256			512	384

Table 7: Results obtained by National Laboratories using their normal protocols - consensus titres and variations by laboratory.

	Haemagglutination inhibition titres										
Serum	**<4**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**
A	**15**[a]										
B								**7**	Fi,Fr,Ge,Sp	B,It,Ne,Sw	
C									**5**	**7**	It,Ne,Sw
D	**13**	Fi		Sw							
E								Ir	**5**	**8**	Ne
F	**15**										
G							A	**5**	**7**	It, Ne	
H									GB,NI,Ir,Gr	**9**	It, Ne
I			D,GB,Ir	A,NI,Gr,Pt	**6**	Ge, Ne					
J						Ir	GB,NI,Gr	**7**	Fr,It,Sp,Ne		

[a]Number of laboratories with this titre.

Table 8: Results obtained by National Laboratories using their normal protocols titres outside one dilution of consensus titre

Serum	A	B	D	Fi	Fr	GB	NI	Gr	Ge	Ir	It	Ne	Pt	Sp	Sw	consensus
A																**<4**
B		**1024**									**1024**	**1024**			**1024**	**256**
C																**1024**
D															**16**	**<4**
E										**256**						**1024**
F																**<4**
G	**128**															**512**
H																**1024**
I			**8**			8				8						**32**
J										64						**256**
Total	**1**	**1**	**1**	**0**	**0**	**1**	**0**	**0**	**0**	**3**	**1**	**1**	**0**	**0**	**2**	

Table 9: Results obtained by National Laboratories using their normal protocols : comparison of variation between laboratories - number sera with titres two or more dilutions different.

	A	B	D	Fi	Fr	GB	NI	Gr	Ge	Ir	It	Ne	Pt	Sp	Sw
A	*	2		2	1	1	1		2	3	2	3		1	3
B		*	2		2	2	1	1		4			1		1
D			*	1	1				1	1	4	5		1	4
Fi				*		1	1			3	1	1	1		1
Fr					*	2	1	1		3					1
GB						*			1		4	5		2	3
NI							*		1		5	7		1	3
Gr								*	1		5	7		1	3
Ge									*	3			1		1
Ir										*	7	4	1	3	5
It											*		3		1
Ne												*	5		1
Pt													*		1
Sp														*	1
Sw															*

Table 10: Normal HI procedures used at National Laboratories - variations from EU protocol

Country	EU	Variations
Austria	✖	different antigen
Belgium	✖	different antigen
Denmark	✔	
Finland	✖	different antigen, settle room temp 20 min, other minor.
France	✖	different antigen, variable amount antigen, possibly U-bottom plates, no tilting to read, others.
Great Britain	✔	
N. Ireland	✖[✔]	initial 1/8 dilution
Greece	✖	different antigen
Germany	✖	different antigen, U-bottom plates, settle RBC at room temperature
Ireland	✖[✔]	initial 1/8 dilution
Italy	✔	
Netherlands	✖	different antigen, U-bottom plates, no incubation for 30 minutes
Portugal	✔	
Spain	✔	OIE
Sweden	✔	

✖protocol shows a significant difference from EU protocol
✔protocol shows no significant difference from EU protocol

NUCLEOTIDE SEQUENCES ENCODING THE REGION AROUND AND INCLUDING THE HAEMAGGLUTININ CLEAVAGE SITE OF H7 SUBTYPE AVIAN INFLUENZA VIRUSES : EVIDENCE FOR INVOLVEMENT OF PET AND FANCY BIRDS IN SPREAD

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Introduction

Viruses submitted to the European Union reference laboratory for avian influenza that are of H7 subtype are subject to nucleotide sequencing of the haemagglutinin gene to determine the amino acid sequence at the post translational cleavage site of the haemagglutinin (CEC, 1992) and thus determine the pathogenic potential of the virus. The cleavage site sequences of several H7 avian influenza viruses have been reported (Wood *et al*, 1993). These lengths of sequence represented only part of the information obtained since in all cases sequencing primer binding sites were somewhat removed from the cleavage site. There was therefore additional data available from which one could attempt to draw conclusions beyond those concerning pathogenic potential.

Avian influenza viruses of H7 subtype submitted to the reference laboratory between 1992 and the end of January 1996, including those from caged pet or fancy birds, were sequenced as reported (Wood *et al*, 1993) and their sequences compared with those of other H7 viruses that had either already been sequenced at the CVL or were available in the public domain. The sequences for A/turkey/England/647/77 (TkEng64777), A/starling/England-Q/983/79 (StarEng-Q903/79), A/parrot/Northern Ireland/VF-73-67/73 (ParrotNI73), A/turkey/Israel/2091/88 (TkIsrael209188), A/non-psittacine/England-Q/198589 (npsitEngQ198589), A/chicken/Victoria/1/92 (CkVic92), A/ostrich/South Africa/1062/92 (OstSA92), A/softbill/China/92 (softbillChin92), A/gull/Italy/692-2/93 (GullItaly69293), A/sun conure/England/766/6/YS/AL/94 (SconEng76694), A/parakeet/Netherlands/873/267497/94 (PkeetNeth87394), A/parrot/England/-1174/94 (ParrotEng117494), A/conure/England/1234/94 (ConEng123494), A/chicken/Pakistan/447/4/95 (CkPak44795), A/chicken/Queensland/667/6/95 (CkQueens66795), A/turkey/Ireland/1414/PV74/95 (TkIre141495), A/fairy bluebird/Singapore/F92/9/94 (BlubrdSingF9294) and A/common iora/Singapore/F89-/11/95 (IoraSingF8995) were obtained at the CVL. The sequence of A/FPV/Weybridge (Wey) was reported by Klimov *et al* (1992), that

of A/fowl/FPV/Rostock/34 (Rost34) by Garten (unpublished-accession no. M24457), that of A/turkey/Oregon/71 (TkOreg71) by Orlich *et al* (1990), that of A/seal/mass/1/80 (SealMass80) by Naeve and Webster (1983) and those of A/starling/Victoria/1/85 (StarVic85) and A/chicken/Victoria/1/85 (CkVic85) by Nestorowicz *et al* (1987).

Results and Discussion

The sequences are shown in Fig. 1. The gap in the alignment for the majority of the sequences represents the fact that these are low pathogenicity viruses that lack the additional nucleotides encoding the multibasic amino acid inserts that characterise the highly pathogenic avian influenza haemagglutinin cleavage site. Features of the figure referred to in the text are indicated by being emboldened and underlined. Among the features shown by the figure are the similarity of the caged bird sequences reported for 1994 and 1995. Their common characteristics include the AGG codon for the arginine immediately before the cleavage site (position 54, numbering as on Fig. 1) the arginine at this position in H7 viruses normally being encoded by AGA. The only previous finding of AGG in this position in an H7 virus was that in A/FPV/Egypt/45 (Wood *et al*, 1993). The other common unusual feature of the sequences of these six viruses was the substitution of C for T at position 111 (as on Fig. 1), this substitution being present only in the historic sequences A/chicken/Rostock/34 and A/FPV/Weybridge and in that of A/turkey/Oregon/71. A further finding, in the five of the six caged bird sequences for which the sequence ran far enough, was the A at position 18 (as on Fig. 1) in the third position on a lysine codon, the rest of the viruses having either an AAG lysine or an arginine. The finding of very similar sequences (and the same neuraminidase subtype - N1) in these six isolates, which were submitted from England, the Netherlands and Singapore, suggests a recent common origin and that avian influenza virus may have been transmitted around the world by the caged bird trade.

A further interesting feature from Fig. 1 is the similarity of the highly pathogenic Australian isolates from '85, '92 and '95, which share, and are differentiated from the rest of the isolates by, C at position 33, G at 36, T at 57, T at 58 and C at 84. This is a strong similarity, despite the isolates being separated by up to 10 years. The two 1985 isolates were of N7 neuraminidase subtype whereas the two recent isolates were N3, which suggests that reassortment may have taken place during the intervening years.

Obviously these observations are based on small regions of sequence, and should, therefore, be treated with caution. The length of sequences involved does not really justify formal phylogenetic analysis but the tentative findings referred to above suggest that it may well be worthwhile, in future, to attempt to obtain rather more sequence from routine isolates in order that epidemiological conclusions could be firmly drawn.

Acknowledgements
We thank Sally Franklin for her assistance.

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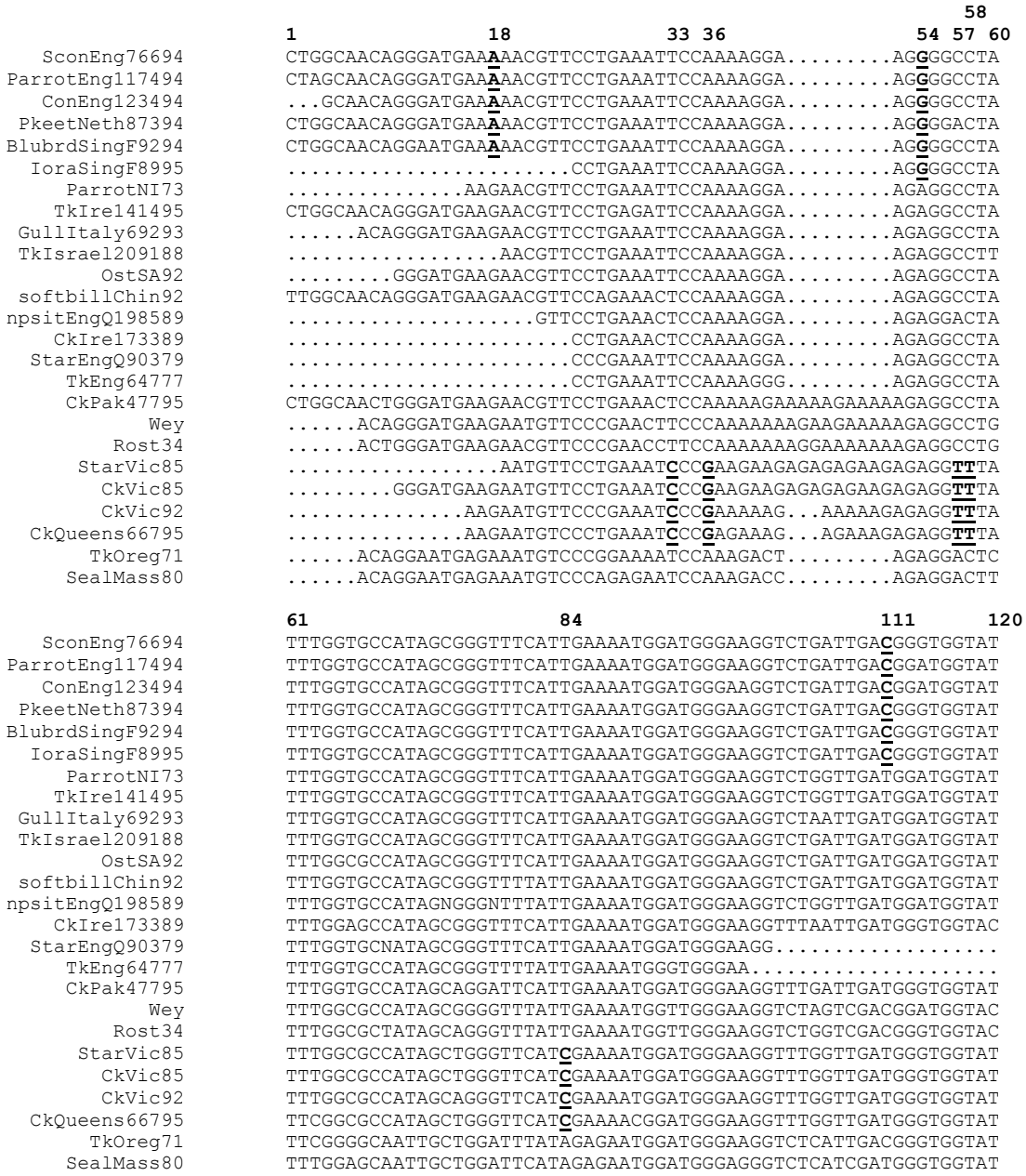
Fig 1. Nucleotide sequences around the haemagglutinin cleavage sites of avian influenza viruses of H7 subtype



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                    121                     147
   SconEng76694     GGCTTCAGGCATCAAAATGCACAAGGA
ParrotEng117494     GGCTTCAGGCATCAAAATGCACAAGG.
   ConEng123494     GGCTTCAGGCATCAAAATGCACAAGGA
 PkeetNeth87394     GGCTTCAGGCATCAAAATGCACAAGGA
BlubrdSingF9294     GGCTTCAGGCATCAAAATGCACAAGG.
  IoraSingF8995     GGCTTCAGGCATCAAAATGCACAAGG.
     ParrotNI73     GGCTTC......................
    TkIre141495     GGCTTCAGGCATCAAAATGCACAAGGA
 GullItaly69293     GGCTTCAGGCATCAAAATGCA......
 TkIsrael209188     GGCTTC......................
       OstSA92      GGCTTCAGGCATCAAAATGCACAAGGA
 softbillChin92     GGCTTCAGGCATCAAAATGCACAAGGA
npsitEngQ198589     GGCT........................
    CkIre173389     GGCTTT......................
  StarEngQ90379     ............................
     TkEng64777     ............................
     CkPak47795     GGCTTCAGGCATCAA.............
           Wey      TGTTTCAGGCATCAGAAT.........
         Rost34     GGTTTCAGGCATCAGAAT.........
      StarVic85     GGTTTCAGGCATCAAAATGCACAAGGA
        CkVic85     GGTTTCAGGCATCAAAATGCACAAGG_
        CkVic92     GGTTTCAGGCATCAAAATGCACAAGGA
  CkQueens66795     GGTTTCAGGCATCAAAAT.........
       TkOreg71     GGTTTTCGGCATCAAAATGCACAAGGA
     SealMass80     GGTTTCAGGCATCAAAATGCACAAGGA
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IMPORTANCE OF THE AEROGENIC TRANSMISSION OF VELOGENIC NEWCASTLE DISEASE VIRUS.

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Introduction

During the last epizootic of Newcastle disease in Belgium, we observed that most of the cases were diagnosed in backyards flocks. The importance of such cases as a possible source of contamination for industrial poultry flocks needs therefore to be estimated. Indeed, if the infecting strain can be spread by the air, there is no need for a direct contact to get contamination between backyard or other birds - eg: ducks, canaries,-which were found to be accidentally infected, and industrial poultry.

The purpose of our work is to evaluate the risk of aerogenic transmission of NDV from infected chickens, ducks and canaries to susceptible chickens.

Materials and methods

Virus

The viral strain used, 93/159, was isolated from canaries in 1993. A stock virus was made in SPF eggs and titrated in 6-week-old SPF chickens held in isolators. For each experimental inoculation, we used 10^6 CLD_{50}.

Haemagglutination inhibition test

The haemagglutination inhibition tests were performed using four haemagglutinating units following the EU protocol.

Experimental procedure

Experiment n°1:

Thirteen SPF chickens, 6 weeks old, were inoculated by eye drop with NDV strain 93/159 and placed in an isolator (isolator 1). The air exhaust from this isolator was connected directly to the air entry of a second isolator (isolator 2) by by-passing both the air exhaust filter of the first isolator and the air entry filter of the second.

Thirteen uninoculated 6-week-old SPF chickens were held in the second isolator and used as indirect contact birds.

All chickens were observed for ten days.

Experiment n°2:
Twenty-three 4-week-old Pekin ducks were inoculated by eye drop with NDV strain 93/159 and placed in isolator 1. Ten uninoculated 6-week-old SPF chickens were held in isolator 2 and used as indirect contact birds.
All birds were observed for 21 days.

Experiment n° 3:
Thirty canaries were inoculated by eye drop with NDV strain 93/159 and placed in isolator 1. Ten uninoculated 6-week-old SPF chickens were held in isolator 2 and used as indirect contact birds.
All birds were observed for forty days.

Results

Experiment n°1:
The first clinical signs were observed in the inoculated chickens, three days after infection. On the sixth day, all inoculated chickens were dead. On the seventh day of observation, clinical signs were observed in the uninoculated indirect contact chickens which were all dead on the tenth day of observation.

Experiment n°2:
Neither clinical signs nor mortality were observed in the infected ducks or uninoculated indirect contact chickens during the whole observation period.
Serological examination of all the birds demonstrated a seroconversion for only eight infected ducks.

Experiment n°3:
The first clinical signs were observed in the canaries 10 days after infection. The first canary died on the 12th day. On the 36th day of observation 12 canaries were dead (40 %). Neither clinical signs nor mortality were observed at the end of the observation period in the uninoculated indirect contact chickens. Serological examination of those birds demonstrated the absence of seroconversion.

Discussion
The aerogenic transmission of NDV is highly dependent on the biological properties of the infecting velogenic strain. Alexander et al. (1986) demonstrated that the pigeon virus was only transmitted by the faeces.

The results of experiment n° 1 show clearly that infected chickens shed a representative viral strain responsible of the epizootic of 1993 in the air which can therefore be a source of infection for chickens. This is not the case when

ducks or canaries are infected with the same virus (experiments n°2 and 3), as those birds do not appear to excrete virus in the air.

Isolation of velogenic NDV from ducks and ducklings is reported in the literature (Lancaster and Alexander, 1975). During the last epizootic of 1993, we also isolated a velogenic strain from ducks which experienced 30% mortality. We were however unable to reproduce clinical signs or mortality after experimental infection in ducks; nevertheless, those birds were susceptible to infection as demonstrated by the seroconversion observed in some ducks.

Infected canaries showed clinical signs and mortality. It is interesting to observe that the incubation period is longer in canaries than in chickens. The role of the canaries, and perhaps of passerines, in the spread of Newcastle disease seems to be highly uncertain as those birds do not shed the virus in the air. Moreover, we were unable to infect SPF chickens by feeding them with a mixture of meat, faeces and cage debris from infected canaries.

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SESSION V
- DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

The participants discussed the conclusions and recommendations that had been formulated at the First Meeting and modified at the Second.

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.*

The participants asked to what extent this was still a problem. The representative of the Commission stated that while most countries had legislation in place that implemented the directive there was still one or two problems to be resolved in some countries. He pointed out that this was continually under review by the Commission.

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.*

The participants again endorsed this recommendation. The representative of the Commission pointed out that there were plans to visit certain laboratories.

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.*

This was discussed, the main problem seems to be the impracticability of enforcing legislation.

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.*

The participants re-endorsed this view.

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.*

The participants endorsed this recommendation.

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.*

The participants endorsed this recommendation.

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 adopted by the Council of Ministers of all Member States. 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.*

The Meeting re-endorsed this recommendation.

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.*

The representative of the Commission confirmed that proposals for the quarantine of ratites and caged birds are in hand.

Several other points were raised and discussed:

ICPI and IVPI tests
In addition to recommendation 9 above, some of the participants felt that the requirement to test all isolates in pathogenicity index tests was unnecessary when

multiple outbreaks have occurred in a given area and that identification of a virus as the epizootic virus using currently available characterisation tests should suffice [see paper by Werner above - Ed.]. The participants expressed some sympathy with this approach and it was considered a subject that should be discussed at the next meeting unless addressed by the Commission before then.

Presenting results

There was some debate on how results, especially serology for export testing, should be presented with particular regard to the wording. The representatives for Great Britain stated that they had addressed this problem to some extent and the relevant report forms they issued contained the following statement:

> The HI titrations were done using the method described in Annex III of Council Directives 92/66/EEC - Newcastle disease and 92/40/EEC - avian influenza which state that a positive serum is one showing a titre of 2^4 or more.

Submitting viruses to the Reference Laboratory

Several laboratories questioned the need or practicality of sending all isolates made during an epizootic to the Reference Laboratory especially when the numbers were large. It was generally agreed that there must be some pragmatism applied. For example, little would be achieved by sending more than one isolate, shown to be of the same virulence, from a single flock. The representative of the Commission pointed out that the objective of submitting viruses to the Reference laboratory was to enable further characterisation and comparisons between viruses from different countries which would enable identification of epidemiological links. Clearly large numbers of viruses submitted together would stretch the resources of the Reference Laboratory, but National Laboratories could help in such circumstances by indicating which of the isolates were most likely to be of importance or interest.

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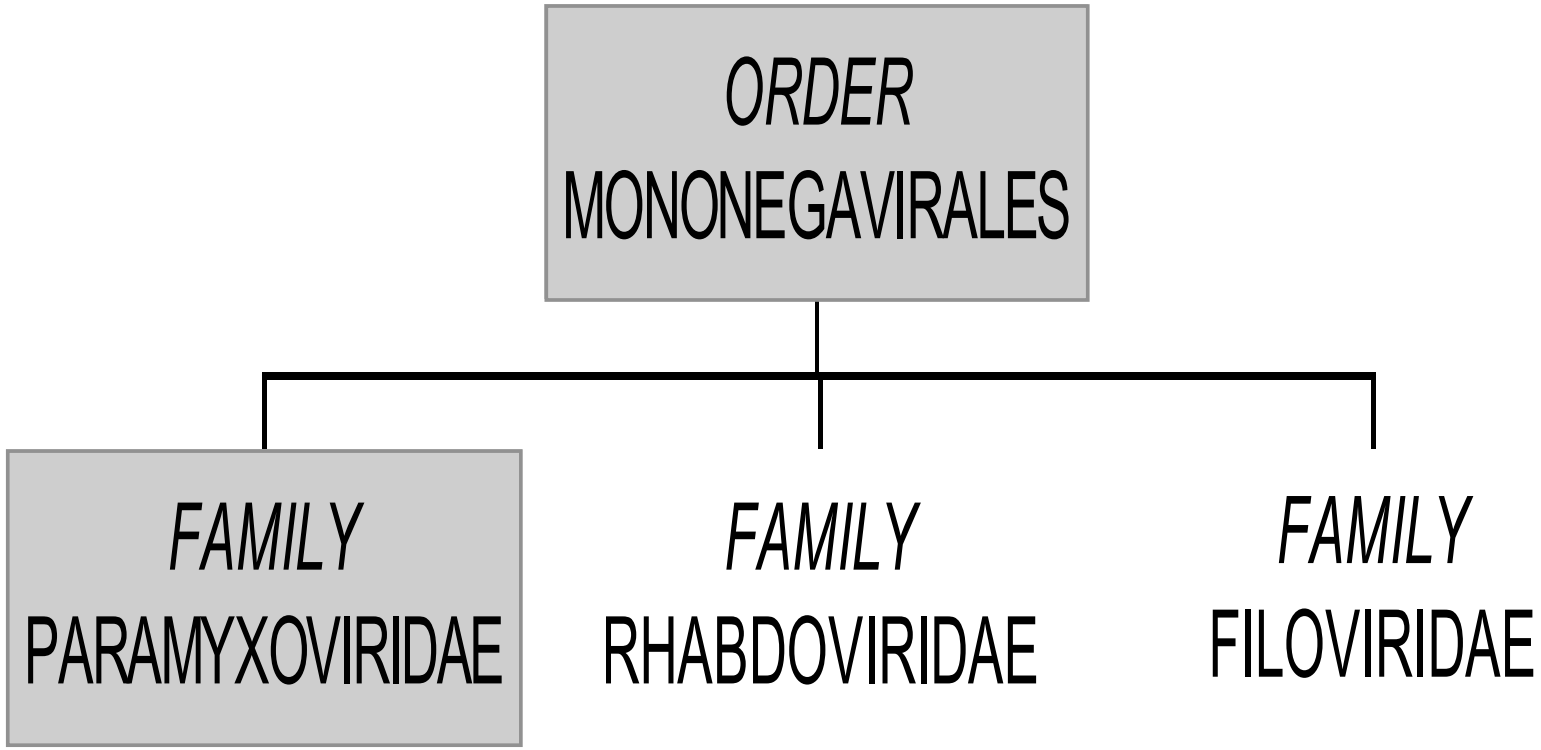
INFORMATION: - NEW TAXONOMY FOR PARAMYXOVIRUSES

Recent revisions of the taxonomy of viruses have affected Newcastle disease [ND] virus taxonomy (Rima *et al*., 1995). The three virus families Rhabdoviridae, Filoviridae and Paramyxoviridae form the order Mononegavirales; i.e. viruses with negative sense, single stranded, non-segmented, RNA genomes. The taxonomy and nomenclature of the family Paramyxoviridae has been modified recently and now has four genera forming two subfamilies. The subfamily Pneumovirinae consists of one genus *Pneumovirus* which includes respiratory syncytial virus and avian pneumovirus [responsible for turkey rhinotracheitis and swollen head syndrome].The subfamily Paramyxovirinae consists of three genera. The genus *Morbillivirus* includes measles, rinderpest and the distemper viruses; no members have been isolated from avian species. The genus *Paramyxovirus* is formed from Sendai virus and other mammalian parainfluenza viruses. The genus *Rubulavirus* is formed from mumps virus, human parainfluenza viruses 2 and 4, ND virus (APMV-1) and the other avian paramyxoviruses (APMV-2 to APMV-9). It was decided to retain the name avian paramyxoviruses rather than adopt the name "avian rubulaviruses".

Rima, B., Alexander, D.J., Billeter, M.A., Collins, P.L., Kingsbury, D.W., Lipkind, M.A., Nagai, Y., Orvell, C., Pringle, C.R. & ter Meulen, V. (1995). *Paramyxoviridae.* Virus Taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses. F.A. Murphy, C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo AND M.D. Summers (Eds), Wien, Springer-Verlag pp268-274.

TAXONOMY OF PARAMYXOVIRUSES



TAXONOMY OF PARAMYXOVIRUSES