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

HELD IN VIENNA, 9th-10th NOVEMBER 1998

Edited by Dennis J. Alexander

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JOINT ANNUAL MEETINGS OF EUROPEAN UNION NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES 1998

PROGRAMME

Monday 9th November 1998

09.15: Welcome by *Dr Peter Weber*, CVO, Austria.

09.30: Introduction and aims of meeting - *Kirsten Sander/Dennis Alexander*

COUNTRY REPORTS FOR 1998 - AVIAN INFLUENZA

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

Current situation of avian influenza in other non-EU countries [Norway, USA, South Africa, Poland, Hungary. Slovak Republic, Slovenia, Czech Republic]

General discussion of current avian influenza situation in Europe and the rest of the World

COUNTRY REPORTS - FOR 1998 - NEWCASTLE DISEASE

1. Greece
2. Spain
3. Portugal
4. Italy
5. Austria

6. France
7. Belgium/Luxembourg
8. The Netherlands
9. Germany
10. Sweden
11. Finland
12. Denmark
13. Ireland
14. United Kingdom

Current situation of Newcastle disease in other non-EU countries [Norway, USA, South Africa, Poland, Hungary. Slovak Republic, Slovenia, Czech Republic]

General Discussion of Current Situation of Newcastle disease in Europe and the rest of the World.

14.00: ORIGINAL CONTRIBUTIONS

14.00: Risk Analysis *Dirk Verwoerd*

14.30: RT-PCR assays for the detection of avian influenza virus. *E. Starick and O. Werner*

15.00: Infectious clone of Newcastle disease virus. *Guus Koch*

about 15.30: BREAK

16.00: Human infections with H5N1 influenza virus in Hong Kong. *J.C. de Jong*

17.00: INFECTIOUS BURSAL DISEASE
Brief discussion of the current situation of infectious bursal disease in EU and rest of the World.

Tuesday 10th November 1998

09.00: EU REPORTS

09.00: Report of the EU Reference Laboratory for avian influenza and Newcastle disease 1998. *Dennis Alexander*

09.30: Report from the Commission on aspects of Newcastle disease and avian influenza legislation in the European Union. *Kirsten Sander*

10.00: Results of the comparative virus identification tests in different laboratories. *Dennis Alexander*

10.45: BREAK

11.15: DISCUSSION OF THE FOLLOWING TOPICS

- proposed EU and OIE new definitions of ND
- discussion of recommendations made for influenza diagnostic testing in National Laboratories.
- use of reference sera and monoclonal antibodies in National Laboratories
- Control measures for outbreaks of ND and AI in backyard flocks and racing pigeons
- achievement of standardised tests throughout EU
- role of the Community Reference Laboratories in 1999

13.00: CLOSING REMARKS AND CLOSE

COUNTRY REPORTS FOR 1998

AVIAN INFLUENZA

Representatives of the following countries made statements of no avian influenza outbreaks during 1998:

The Netherlands
Austria
Sweden
Spain
Portugal
Greece
Belgium/Luxembourg
Finland
Denmark
Norway
South Africa
Poland
Hungary
Slovak Republic
Slovenia
Czech Republic

AVIAN INFLUENZA - SITUATION IN GERMANY 1997/1998

Ortrud Werner

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No outbreak of highly pathogenic influenza has occurred in Germany since 1979.

From the last quarter of 1995 until the beginning of 1997 influenza H9N2 infections occurred in turkey flocks in the North West of Germany.

The pathogenicity of the virus isolates was low. The course of the disease and the economic losses were age-dependent. The mortality reached 50% in some cases in flocks with young turkeys and with a high bacterial pressure. In older animals the mortality was below 20%.

Animals which recovered from influenza often became infected with *Pasteurella multocida* a few weeks before slaughtering. Body weight reduction of 1-3 kg was noticed at the slaughter age of 22 weeks.

Layer turkey hens had egg production drops from 75% to 20% for a few weeks. After infection the laying performance was only about 40%.

Antibiotic treatments were employed to reduce the *Mycoplasma* and bacterial infections.

For specific prophylaxis a flock-specific inactivated vaccine was produced by using the virus isolated during the outbreak. Turkey flocks which were vaccinated once during the rearing period were protected against clinical signs. Vaccination of nearly all young turkeys in the affected area reduced the economic losses.

No further outbreaks have been recorded since the beginning of 1997.

During 1998 a few respiratory disorders without tendency to spread to other flocks were noticed in some turkey flocks in the South of Germany. Three influenza viruses, most probably of subtype H6, were isolated. Their pathogenicity was low with IVPI values of 0.

A further influenza virus isolated from a broiler flock proved to be of subtype H9. In the flock of origin no signs were noticed. The pathogenicity of the isolate is low. The source of infection is unknown.

Table 1: Isolation of avian influenza viruses in Germany

Year	Number	Host	Subtype	Pathogenicity
1995	3	turkey	H9N2	low
	1	chicken	H9N2	low
	1	duck	H9N2	low
1996	6	turkey	H9N2	low
1997	-			
1998	3	turkey	H6	low
	1	chicken	H9	low

AVIAN INFLUENZA : SITUATION IN FRANCE DURING NOVEMBER 1997 TO SEPTEMBER 1998

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No cases of avian influenza as it is defined in the EU directive 92/40/EEC were reported during the relevant period. Although egg drop problems occurred in turkey breeder flocks, no viral diagnosis was undertaken, only serological analysis were achieved by AGP test, by diagnostic laboratories. Positive serological reactions were confirmed by the national reference laboratory using HI tests. In that way, viruses belonging to the subgroups H1, H6, and H9 were suspected to be circulating in poultry (Table 1).

Table 1 : Avian influenza serology (Confirmation of positive sera from diagnostic laboratories) November 1997 - September 1998

Country Department	Month year	Species	Case history	AGP	HI
22	Nov. 97	Turkey	Egg drop	+	H6
22	Feb. 98	Turkey	Egg drop	+	H9
22	March 98	Turkey*	Egg drop	+	H6
22	May 98	Turkey	Egg drop	+	H1

* : Same birds as November 1997

NON-PATHOGENIC AVIAN INFLUENZA IN IRELAND IN 1998

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Introduction

Reports of severe disease involving highly pathogenic avian influenza A during the past 30 years have been rare (1). In Ireland there has been only one outbreak, which occurred in turkeys in 1983 and was due to an H5N8 subtype influenza virus. However, occasionally non-pathogenic or low pathogenic strains of influenza virus of subtypes H7 and H5 are isolated. On their own such strains may or may not be the cause of disease problems e.g. low or significant mortality or decreased egg production in breeding of laying flocks.

During the Spring of 1998 29 outbreaks of non-pathogenic avian influenza occurred in Ireland. Twenty-seven of the outbreaks occurred on commercial turkey sites; one outbreak was in turkey breeders and the other occurred on a broiler breeder farm.

Aetiology

The disease outbreaks were the result of infections with avian influenza A viruses of subtype H7N7 which were isolated from cloacal and tracheal swabs taken from birds on six infected sites. The virus was readily isolated 48 hours after inoculation into 9-day-old embryonated fowls' eggs.

Isolated viruses were forwarded for typing and pathogenicity testing to the Community Reference Laboratory for Avian Influenza. All viruses tested had intravenous pathogenicity indices of 0.00. The amino acid sequence at the cleavage site of the haemagglutinin was -PEIPKGR*GLF- which is typical of virus of H7 subtype of low or no pathogenicity. In some flocks there was secondary bacterial infection with *Pasteurella multocida*.

Clinical signs and post mortem findings

In the broiler breeder flock, which was 40 weeks old, there was decreased activity, a marked drop in egg production, decreased food consumption and a small number of deaths were recorded each day.

In commercial turkeys uncomplicated infections with H7N7 virus resulted in inappetance for 24 hours, the birds appeared dull and huddled together, there was pronounced respiratory signs with coughing, sneezing and rales and a small

number of deaths each day. Clinical signs persisted for about one week and the n the birds recovered. The lesions seen in dead birds were small fibrino-necrotic lesions in the lungs.

In flocks suffering from a combined infection of H7N7 influenza virus and *P. multocida* the lesions were much more severe and extensive and associated mortality was much higher. In these cases the lesions seen were severe necrotic pneumonia and oedema of the lungs, fibrinous airsaculitis affecting both the abdominal and thoracic airsacs, fibrinous pericarditis and peritonitis.

Source and spread of the virus.
The original source of the virus was not determined. Wild water birds were considered the most likely source. The infection was spread initially from the first site to the others by catching crews.

Surveillance testing and control.
During the outbreaks 8,410 blood samples were taken from 229 farms in the area. These samples were tested for antibodies to the H7N7 virus. In addition a further 4,510 blood samples were taken from farms after the outbreaks had stopped and these too were tested for antibodies to the H7N7 virus.

All infected flocks were voluntarily slaughtered. The houses were cleaned and then, washed disinfected and fumigated. All the infected litter was brought out of the area for destruction.

Extensive further serological monitoring of new birds placed on previously infected sites took place. No breakdowns were recorded.

Discussion
This episode represents a good example of the successful eradication of an influenza A virus from a large poultry population. In total the virus had infected 320,000 birds in over 100 houses on 29 farms in a very short time. The first outbreak was reported on 18th March 1998 and the last outbreak occurred on 17th April 1998. In approximately seven weeks from the date of the first outbreak, and when all cleaning, disinfection, and fumigation of the infected houses and disposal of the infected litter was completed the disease was considered eradicated. However, serological surveillance was continued and a close watch was kept on all poultry in the area for clinical signs.

In this epizootic the severity of the disease varied considerably. In those flocks that had a combination of the H7N7 virus and *P. multocida* infections the mortality rate was very high. On one site with such dual infections mortality reached 80% over a four day period.

The results of treatment also varied with the presence or not of dual infection. While turkeys suffering from *P. multocida* alone responded reasonably well to a combination of "Baytril" and "Tribrissen" in the drinking water, these drugs had no effect when there was a combination of influenza H7N7 and *P. multocida* infections.

Reference

1. Easterday, B.C. & Hinshaw, V.S. (1991). Diseases of Poultry 9th edition p533.

AVIAN INFLUENZA: SITUATION IN GREAT BRITAIN 1998

Dennis J. Alexander and 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 remained extremely low during 1998 (Table 1).

Table 1. Avian influenza investigations in poultry during 1992-1998

	1992	1993	1994	1995	1996	1997	1998
Suspect cases investigated:	2[a]	4	1	2	1	0	0
Influenza virus isolated:	1[a]	1	1	1	1	0	1
Confirmed HPAI:	1[a]	0	0	0	0	0	0

[a]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 H6N1 virus with IVPI 0.00 obtained, in May 1998, from a 10-day-old commercial duckling showing depression and respiratory signs (Table 2). The duckling had been submitted as a result of mortalities in the flock and suspicion of duck viral hepatitis.

Influenza isolations from other birds

Although in previous years influenza viruses have been obtained from other birds in Great Britain, especially captive caged birds, none were isolated during 1998.

Table 2. Isolations of avian influenza viruses from poultry 1990-1998

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
1996				
		mallard/England/1078/96	H4N6	0.18
1997				
		no isolates		
1998				
		duck/Wales/484/98	H6N1	0.00

AVIAN INFLUENZA IN NORTHERN IRELAND: CURRENT SITUATION

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Based on the definition given in Council Directive 92/40/EEC, there were no cases of avian influenza (AI) in Northern Ireland during 1998.

However, three low pathogenicity isolates of H7N7 avian influenza virus were made in County Fermanagh during this period, as part of an outbreak extending from the Republic of Ireland. Details of these isolates, determined in conjunction with the EU Reference Laboratory are given in Table 1.

Although no action was required under Directive 92/40/EEC, these isolates still raised potential concerns in relation to the perceived public health risk (following the cases in Hong Kong) and also as a complicating factor in serological monitoring for AI.

The results of a transmission experiment with isolate VF-97-1930 suggested that the virus did not spread easily from bird to bird even within houses, and were consistent with the epidemiological findings which suggested that infection was initially introduced to the commercial sector from wild birds and subsequently spread by personnel/fomites.

The infection was eliminated by a programme of co-operation with the industry involving voluntary early site de-population, cleansing and disinfection and biosecurity measures.

Table 1. Details of avian influenza viruses isolated from commercial poultry during 1998.

Strain reference	Month	Species/type	Age	Clinical signs	Virus subtype	IVPI	Amino acid sequence at the HN0 cleavage site
VF-98-1545	March	turkey/growers	18 weeks	Respiratory, enteric, low mortality	H7N7	0.00	.....NVPEIPKGR*GLF... .
VF-98-1930	April	chicken/broiler breeder	43 weeks	Egg drop, coughing, low mortality	H7N7	0.00	.....NVPEIPKGR*GLF... .
VF-98-2023	April	turkey/breeder	?	Respiratory, low mortality	H7N7	0.00	.....NVPEIPKGR*GLF... .

THE EPIDEMIOLOGICAL SITUATION OF AVIAN INFLUENZA IN ITALY DURING 1998

A.Fioretti, M. Calabria, A. Piccirillo and L.F. Menna

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No cases of highly pathogenic avian influenza [HPAI] were recorded in Italy during 1998. However, in February 1998 a strain of influenza virus type A, subtype H5N9, IVPI 0 and with a haemagglutinin cleavage site which demonstrated its apathogenicity, was isolated in Emilia Romagna in a group of approx. 2000 40-day-old chicks.

The amino acid sequence of the cleavage site supplied by the Community Reference Laboratory for Avian Ortho and Paramyxoviruses at the CVL, Weybridge differed slightly from the sequence found by the Italian Reference Centre of the WHO for Human Influenza in the Superior Health Institute, Rome as in the latter sequence arginine substituted lysine (site CVL: PQRETRGLF; site ISS: PQKETRGLF). *[Editor's note PQKETRGLF is correct]*

This isolation followed an epidemiological investigation carried out in Veneto and Friuli Venezia Guilia, the two north-eastern Italian regions affected by the influenza epidemic in 1997.

The results of the investigation have allowed us to establish precisely the commercial flow of live poultry to and from the identified sources of infection, and in particular we have managed to identify the poultry farm which supplied the chickens showing the disease in 2 of the 7 rural farms which were identified as infected last year.

It is necessary to clarify that in this poultry farm samples were taken only in order to complete the epidemiological investigation as no poultry presented any signs which could be linked to avian influenza, nor were there abnormal mortality rates, nor had there been any reports in this respect even in 1997. The samples were then treated by the Diagnostic Section in Lugo di Romagna (RA) of the Experimental Zooprofillatic Istitute in Lombardia and Emilia Romagna and it was possible to isolate a haemagglutinating agent in an embryonated eggs at the first passage, which then proved to be the above-mentioned apathogenic influenza virus.

The interest stimulated by this strain has been notable and can be linked to the possibility of selecting this virus for vaccine use in humans, considering the antigenic correlation demonstrated towards the H5 isolated in poultry in Italy and in particular with the strain Hong Kong 1997 H5N1, which has been notoriously transmitted to man in some cases.

Finally, it has emerged from a study recently presented by the Italian study group on human and animal influenza viruses at the 1st Congress of The European Society for Emerging Infections (13-16 September 1998, Budapest, Hungary), entitled "Avian Influenza A (H5N2) outbreak in Italy: evaluation of the risk of transmission to humans" (authors: Di Trani L., Campitelli L., Puzelli S., Fioretti A., Selli L., Donatelli I.) that there is a 94.2% homology at the level of the HA gene between the Italian pathogenic strain H5N2 and the apathogenic strain H5N9, which is superior to that found for both the viruses when compared with the Hong Kong H5N1 strain (93.6% and 91.4% respectively). In brief, the results presented have established the following:

a) the H5N2 pathogenic strains isolated in the North-East of Italy during 1997 are substantially identical (homology of gene HA equal to 99.4%-99.9%).
b) the homology between the pathogenic strains H5N2 and the non pathogenic Italian strain H5N9 in the HA gene is equal to 94.1%-94.5%, indicating a phylogenetic difference between these viruses.
c) although all the H5 strains isolated in Italy are closely linked to the highly virulent avian influenza virus named A/turkey/England/50-92/91, the degree of homology is greater for the apathogenic strain H5N9 which is named A/ck/It/9097/98, namely equal to 96.9% compared to that found for the virulent strains H5N2 (95.4% - 95.6%).

This data, confirmed by phylogenetic analysis, demonstrates that, unlike the epidemiological indications collected during the epizootic, the highly pathogenic Italian avian influenza strains H5N2 are closely linked to the Hong Kong H5N1 strain of 1997, while the apathogenic H5N9 virus isolated in Emilia Romagna this year belongs to the sub-lineage of the European strain A/turkey/England/50-92/91.

As far as the possibility that pathogenic influenza viruses for birds involved in the epizootic of 1997 could be transmitted to people in contact with the infected animals is concerned, this has been evaluated in the above study by the attempted isolation from clinical samples using throat-swabs and from the results of the analysis of sera collected and measurement anti-H5 antibodies by the standardised microneutralization test from the Center for Diseases Control in Atlanta (USA) as the inhibition of haemagglutination cannot be considered a sufficiently sensitive test to show human antibodies caused by avian influenza

viruses. The transmission of avian viruses to man was not demonstrated virologically, while more controversially weak seropositivity was detected in 2 of the 32 sera tested. These serological findings on human sera will be further confirmed using the Single Radial Haemolysis test for antibody to influenza viruses.

AVIAN INFLUENZA - CURRENT SITUATION IN THE UNITED STATES OF AMERICA

S.C. Trock and D.A. Senne.
presented by Chris M. Groocock*

*American Embassy Vienna, Department of State, Washington DC, USA.

In 1998, surveillance activities continued in the live-bird markets in the north eastern states and Florida to detect the presence of avian influenza virus (AIV). A total of 2,497 specimens was tested. Isolations of AIV were made from birds and premises (environment) in the states of New York, New Jersey, Pennsylvania, and Connecticut. The predominant subtype of AIV isolated was H7N2. This was characterised as being low pathogenic by the chicken pathogenicity test and amino acid profile of the haemagglutinin cleavage peptide. Six other subtypes were isolated: H3N2, H1N1, H3N8, H6N2, H6N8, H1N2.

AIV was also isolated from premises other than the live-bird markets. In Pennsylvania, AIV subtype H7N2 was isolated from gallinaceous birds in 14 premises; one isolate was from the environment. AIV subtype H5N2 was isolated from chukar partridges on a game farm in Minnesota. Both AIV H7N2 and H5N2 were characterised as low pathogenic. Seven other subtypes were isolated: H3N2 in Delaware, Pennsylvania and New York, H3N8 in Michigan and Pennsylvania, H4N6 in New York, H4N8 in Pennsylvania, H6N2 in New York, H6N8 in Pennsylvania, and H9N2 in California.

Antibody to subtype H7 was found in chickens in Pennsylvania and North Carolina and in ostriches in Florida and Texas. H5 specific antibody was found in partridges and pheasants in Minnesota and in chickens in Pennsylvania. Waterfowl sera contained antibodies to multiple H and N subtypes of AIV. The antibodies in most turkey sera were specific for subtype H1N1.

A low pathogenic H7N2 has been circulating in commercial poultry, mainly layer flocks, since December 1996 in Pennsylvania. Twenty-two commercial flocks with 2.73 million birds were affected. Because of fears the virus may undergo a change in virulence, the state authorities initially depopulated many of the flocks; however, as time went by without change in virulence and because of mounting costs, several flocks were placed under quarantine and subsequent action based on economic concerns. The last quarantine was lifted in September of 1998.

On October 8, five pheasants from a game bird farm in New Jersey were submitted to the poultry diagnostic laboratory at the University of Pennsylvania, New Bolton Center. The laboratory reported positive antibody for H5 AIV and submitted samples to NVSL. The farm was quarantined on October 14. The NVSL reported the isolation of AIV subtype an H5N2 subtype. This virus was subsequently characterised as low pathogenic AIV. The birds on the farm are being disposed of by controlled slaughter; no live birds leave the premises.

COUNTRY REPORTS FOR 1998

NEWCASTLE DISEASE

Representatives of the following countries made simple statements of no Newcastle disease outbreaks during 1998:

Spain
The Netherlands
Ireland
Poland
Hungary
Slovak Republic
Slovenia
Czech Republic

NEWCASTLE DISEASE: SITUATION IN GREECE IN 1998

Nicholas Batianis

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Epidemiology

During the year of 1998 a total of seven suspect cases was investigated for ND, including poultry, pigeons and caged birds.

All of them were negative.

The last outbreaks of ND occurred during 1984-1985 and the source of infection was attributed to back-yard poultry.

Diagnosis and control.

The diagnosis and control methods follow EU Directive 92/66/EEC.

Vaccination

There is no compulsory vaccination for ND, but practically all flocks of broilers, layer and breeders are vaccinated with live or inactivated vaccines.

Emergency vaccination and other control measures may be imposed by the Ministry of Agriculture in case of an outbreak.

NEWCASTLE DISEASE SITUATION IN PORTUGAL 1997-1998

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During 1997 a total of 48 suspected cases of Newcastle disease was investigated in poultry. Fourteen viruses were isolated (Table 1). Nine outbreaks occurred in industry flocks and five in small flocks.

The isolates were grown in SPF-eggs, identified serologically with a standard polyclonal antiserum against ND virus and monoclonal antibodies (mAb) specific to La Sota (mAb 7D4) and pigeon-type PMV-1 (mAb 617/161). All viruses were sent to the EU Reference Laboratory - CVL, for antigenic characterisation and determination of the intracerebral pathogenicity index (ICPI) in one-day-old chicks.

Antigenic grouping of 1997 NDV isolates by immunocytochemistry revealed three groups; E, C and H. The ICPI was determined for 5 isolates with values ranging from 1.64 to 1.95. Determination of the MDT/MLD was carried out with five isolates. With the exception of isolate 5639/97, all isolates have a MDT >60h. Plaque formation on MDBK cells was done with ten isolates. All produced plaques in the absence of trypsin. Seven isolates were subjected to nucleotide sequencing to determine the amino acids at the cleavage site of the haemagglutinin. All isolates had multiple basic amino acids at the cleavage site implying high pathogenicity for poultry (Table 1).

These results suggest the existence of mixed infections. The presence of virulent viruses in the country since 1990 and the compulsory use of vaccines on the last 4 years, may well explain the presence of virulent and non-virulent viruses in the birds.

In 1998 there were 6 isolates from 32 suspected cases (Table 2). All viruses were isolated from pigeons, five from the Lisbon area and one from Estremoz-Alentejo. The latter, virus 8145/98, was isolated from a pigeon suspected of adenovirus infection. The virus was first grown in chicken embryo liver cells where it caused a light cytopathic effect. Clarified cell supernatant was negative for haemagglutination activity. The virus was then grown in eggs where it caused embryo deaths in 72h.

With the exception of isolate 8742/98 all other isolates were inhibited by Mab 617/161. Characterisation by immunocytochemistry with a panel of mAbs at the EU reference laboratory revealed that the first four isolates of 1998 belong to the PPMV-1 antigenic group.

ICPI was determined for four isolates and ranged between 0.96 and 1.375. IVPI was 0.00 on two isolates tested.

The amino acid sequence at the F0 cleavage site of four isolates showed that all have the minimum requirement for pathogenicity, motif **RQKRF**.

Table 1: Isolation of PMV-1 viruses in 1997

Isolate	Bird	Characteristics					
		MDT	PF(b)	ICPI (a)	IVPI (a)	mAb(a)	AA SEQUENCE (cleavage site)
2035/97	broilers	> 60 h	Yes	1.83		H	SGG**RR**QK**RF**I
2036/97	broilers	> 60 h	Yes	1.64		E	SGG**RR**QK**RF**I
2037/97	chicken		Yes	1.95		C	
2132/97	broilers		Yes			C	
3647/97	broilers		Yes			H	
3742/97	broilers		Yes			C	
5639/97	broilers bk	< 60 h	Yes	1.72		E	SGG**RR**QK**RF**I
5679/97	broilers					H	SGG**RR**QK**RF**I
5737/97	broilers					H	SGG**RR**QK**RF**I
5922/97	broilers bk	> 60 h				C	
6185/97	broilers bk		Yes			H	
6186/97	broilers bk					C	
6187/97	broilers bk		Yes	1.79		C	SGG**RR**QK**RF**I
8674/97	broilers	> 60 h	Yes				SGG**RR**QK**RF**I

(a) Characterisation tests were done at the EU Reference Laboratory for Newcastle Disease and Avian Influenza (CVL-UK).
(b)Plaque formation on MDBK cells in the absence of trypsin.

Table 2: Isolation of PMV-1 viruses in 1998

ISOLATE	BIRD	CHARACTERISTICS			
		ICPI(a)	IVPI(a)	mAb(a)	AA SEQUENCE (cleavage site)
4474/98	*pigeon*	0.96	0.00	P	SGG**GRQKRF**I
4638/98	*pigeon*	1.25	0.00	P	SGG**KRQKRF**I
6667/98	*pigeon*	1.34		P	SGG**KRQKRF**I
6717/98	*pigeon*	1.375		P	SGG**KRQKRF**I
8145/98	*pigeon*				SGG**RRQKRF**I
8742/98	*pigeon*				

(a)Characterisation tests were done at the EU Reference Laboratory for Newcastle Disease and Avian Influenza (CVL-UK).

NEWCASTLE DISEASE : SITUATION IN ITALY DURING 1997 TO 1998

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Investigations on Newcastle disease during 1997 and 1998:

Non virulent strains :
a total of 5 non virulent (vaccine) strains was isolated in 1997

Virulent strains

1997

Case investigated	Date	Province	Species	Flock	Virus type	ICPI
13/AV/97	09.01.1997	Firenze	ostrich	rural	PPMV1, P*	1.12
47/AV/97	18.02.1997	Verona	pigeon	rural	PPMV1, P	1.44
138/AV/97	15.05.1997	Viterbo	raptor	--	PPMV1, P	1.14
152/AV/97	29.05.1997	Cuneo	pigeon	rural	PPMV1, P	1.32
201/AV/97	03.07.1997	Roma	pigeon	rural	PPMV1, P	1.11
298/AV/97	29.09.1997	Verona	pigeon	free	PPMV1, P	1.06

*monoclonal antibody group

Non virulent strains :
a total of 2 non virulent (vaccine) strains was isolated in 1998

Virulent strains

1998

Case investigated	Date	Province	Species	Flock	Result*
116/AV/98	20.03.1998	Gorizia	chicken	rural	PMV1, C1,ICPI 1.69
192/AV/98	25.05.1998	Pisa	pigeon	free	PPMV1, P, ICPI 1.3
209/AV/98	04.06.1998	Parma	pigeon	free	PPMV1, P, ICPI 1.23
290/AV/98	10.09.1998	Parma	pigeon	free	PPMV1, P, ICPI 0.85

*identification, mAb group, intracerebral pathogenicity index

All viruses isolated were obtained on SPF eggs and were characterised according to EU directive 92/66/EEC. In our laboratory, identification, preliminary

characterisation with mAbs (supplied by CVL) and virulence tests were performed on all isolates.

Of the ten virulent ND viruses isolated during the last two years year, nine were pigeon paramyxovirus type 1 strains obtained from different birds. The examination of data collected in the past years also indicates that infection with this virus is present in the pigeon population and in rural or free-ranging birds, and this represents a potential threat for intensively reared poultry. It is the intention of the Italian Reference Laboratory for Newcastle disease to encourage vaccination in pigeons through official veterinarians and through the Ministry of Health.

NEWCASTLE DISEASE IN AUSTRIA 1996-1997

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Introduction

In the 10 years to 1998 there were eight outbreaks of ND in Austria, two in 1993 in unvaccinated broiler chickens (Kissling, 1994), four in 1996 and two in 1997 (Damoser, 1998). In 1998 between 12th March and 28th August six outbreaks were confirmed (Table 1), as with the 1996 and 1997 outbreaks these were in pigeons and hobby flocks.

Table 1 Confirmed outbreaks in Austria in 1998

Date	Province	District	Number and type of birds	ICPI
12.03.98	Vienna	District 7	17 pigeons	1.48
06.04.98	Upper Austria	Urfahr-Umgebung	14 pigeons 14 chickens	1.16
09.04.98	Upper Austria	Vocklabruck	40 pigeons	1.41
25.05.98	Lower Austria	Krems	7 pigeons	1.47
02.06.98	Upper Austria	Urfahr-Umgebung	40 pigeons 3 fowls	1.37
28.08.98	Upper Austria	Eferding	32 pigeons	1.45

The Community Reference Laboratory confirmed all viruses isolated from the 1998 outbreaks to be the pigeon panzootic variant (PPMV-1) showing monoclonal antibody [mAb] binding pattern P.

References

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NEWCASTLE DISEASE : SITUATION IN FRANCE DURING NOVEMBER 1997 TO SEPTEMBER 1998

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During the period under consideration, a total of 8 suspected cases was investigated from poultry, pigeons, ornamental birds and cage birds. Six cases were negative (Table 1). Only 2 cases were positive (Table 2). Of the PMV-1 isolates, one was pathogenic with an ICPI of 1.08 and identified as the pigeon variant NDV (PPMV-1).

Table 1 : Suspected cases investigated with negative results November 1997 to September 1998

Month Year	Species	Case history	Department
Feb. 98	Canary	Respiratory signs	91
Feb. 98	Turtledove	Mortality	67
Feb. 98	Diamants de gould	Mortality	54
Feb. 98	Diamants de gould	Mortality	54
June 98	Backyard chicken	Mortality	59
Aug. 98	Fowl	Mortality	68

Table 2 : APMV-1 Viruses isolated from November 1997 to September 1998

Date	Species	Case history	ICPI	Department
April 98	Fowl	Mortality	0,22	02
Sept. 98	Pigeon	Mortality	1,08	44

NEWCASTLE DISEASE SITUATION IN BELGIUM

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Investigations of Newcastle disease in poultry during 1998.

No outbreak of Newcastle disease was confirmed during the year 1998.

Newcastle disease in racing pigeons.

From the beginning of June, 15 cases of infection of racing pigeons with the variant PPMV-1 virus were confirmed by viral isolation.
The ICPI value of the different isolates and the sequence at the fusion protein cleavage site were determined and are given in Table 1.
All strains belong to the pathogenic PMV-1 viruses although the ICPI values are generally low and in certain cases below the 0.7 level.
Sequencing of the cleavage site of the fusion protein is probably a better measure of virulence than ICPI for PMV-1 strains isolated from birds other than poultry.

Table 1.: NDV strains isolated from pigeons in 1998

Date	Reference Number	Province	Town	ICPI	Amino acids at the F0 cleavage site
5/06/98	98/103VB	Brabant	Rhode-St-Genèse	0,75	^{113}RQKRF^{117}
22/07/98	98/169VB	Brabant	Pepingen	0,57	RQKRF
6/08/98	98/187VB	Brabant	Bever	0,73	RQKRF
11/08/98	98/190VB	Liège	Stemberg	0,79	RKKRF
11/08/98	98/196VB	Brabant	Nivelle	0,6	RQKRF
18/08/98	98/202VB	Liège	Bombaye	1,02	RQKRF
21/08/98	98/200VB	Brabant	Pepingen	0,77	RQKRF
24/08/98	98/210VB	Namur	Achêne	0,76	RQKRF
28/08/98	98/225VB	Liège	Berloz	0,55	RQKRF
3/09/98	98/217VB	Hainaut	Thullies	0,5	RQKRF
7/09/98	98/238VB	Brabant	Braine-le-Château	1,25	RQKRF
15/09/98	98/247VB	Hainaut	Chièvres	0,77	RKKRF
16/09/98	98/248VB	Hainaut	Herchies	0,32	RQKRF
25/09/98	98/253VB	Anvers	Emblem		RQKRF
29/09/98	98/273VB	Hainaut	Bray		

NEWCASTLE DISEASE - SITUATION IN GERMANY 1997/1998

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Vaccination against ND is compulsory for chickens and turkeys in Germany. Due to this policy Germany has been free of ND since April 1996.

Since all chickens and turkeys have vaccine induced antibodies serological screenings for surveillance purposes are not feasible.

All suspected cases are therefore investigated virologically.

In 1997 1087 cases were investigated in the regional diagnostic laboratories. Twenty eight viruses were isolated and submitted to the National Reference Laboratory (Table 1). The isolates were typed by HI test with monoclonal antibodies and tested for their pathogenicity.

Twenty isolates proved to be pigeon type PMV-1. All of them were isolated from pigeons. Eight isolates proved to be lentogenic NDV. They came from chickens, turkeys and one from a pigeon.

During this year 40 virus isolates were sent for characterisation (Table 2). Thirty four were identified as PMV-1. Twenty two of these isolates originated from pigeons and were pigeon type PMV-1. One velogenic NDV was obtained from a dead wild pigeon, but there was no evidence of an epizootic in further wild birds.

Ten lentogenic PMV-1 viruses were isolated from chickens and one from turkeys. All were viruses of the La Sota type since they reacted in HI tests with mAb 7D4.

During the year we received two haemagglutinating viruses isolated from parakeets. We could identify them as PMV-3.

Four other apathogenic haemagglutinating isolates from turkeys and chickens are not identified up to now. Influenza virus could be excluded by PCR, immunofluorescence test and HI test. By electron microscopical examination Paramyxovirus (PMV) was diagnosed. The serotype could not been determined.

The isolates will be sent to the EU Reference Laboratory for further characterisation.

Table 1: Biological characteristics of PMV-1 isolates

Virus	1996	1997	1998[1]
Velogenic	2	-	1
Pigeon PMV-1	23	20	22
Lentogenic	5	8	11

[1]upto 30/9/1998

Table 2: Virus isolates characterised by the National Reference Laboratory in 1998 up to 30.9.

Number	Virus	Subtype	isolated from
34	PMV-1	22 pigeon type	pigeons
		1 velogenic	pigeon
		11 lentogenic	chickens 10, turkey 1
2	PMV-3		parakeets
4	PMV	unknown	turkeys 2, chickens 2

NEWCASTLE DISEASE - COUNTRY REPORT FOR SWEDEN 1998

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Accreditation

The Swedish National Newcastle and Avian Influenza Laboratory has, during 1998, been accredited as testing laboratory according to SS-EN 45001 (Swedish Standard European Norm).

Clinical disease

No clinical outbreak of Newcastle disease (ND), in any type of bird, has occurred in Sweden from January 1998 upto the present date.

Investigated samples

In two flocks of commercial breeders (parents) tested on routine basis for NDV 8% of the samples showed low titres on HI but were negative on ELISA. Re-testing were done and samples with doubtful HI reactions were all negative when tested at VLA, Weybridge. No clinical signs were observed in the flocks

Serological reagents

Three flocks of cormorants investigated in a health monitoring projects were tested for antibodies to paramyxoviruses. Of the 75 birds eight showed serological reactions to PMV-1. No virus was isolated. The investigation will continue during 1999.

Serological surveys

All breeder flocks are tested annually with 60 serum samples taken at the point of lay. A total of 12,000 samples was tested during 1998. Apart from the earlier mentioned unspecified reactions no antibodies against NDV were detected in any flock.

Vaccination

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

COUNTRY REPORT - NEWCASTLE DISEASE IN FINLAND 1997-1998

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In September 1997 PMV-1 was isolated from a mallard duck. The ICPI was 0.17 and the strain was placed in the H mAb group. The further characterisation of the strain by RT-PCR and sequencing is in progress.

In 1998 no isolations of NDV were made.

Annual screening for ND in breeding flocks has been done with negative results (Table1.).

Table 1. Annual screening for ND 01.01.1997 - 05.11.1998.

	Number of samples	Number of holdings
1997	5016	75
1998	3842	

NEWCASTLE DISEASE - CURRENT SITUATION IN DENMARK OCT 1997 - OCT 1998.

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Definition.

No alterations

Diagnostic methods.

No alterations

Epidemiological situation.

In February 1998 ND was diagnosed in two flocks of turkeys. The birds were 3-17 weeks of age and they belonged to the same turkey meat producing company. The number of birds in the flocks was 95,000 and 45,000, respectively and they were killed on the spot and destroyed.

The ICPI of the APMV-1 isolated from the flocks was 1.75-1.84 and the amino acid sequence at the F protein cleavage site conformed with that of virulent virus strains. Immunoperoxidase tests with monoclonal antibodies at the EU Reference Laboratory at CVL on virus isolates from both flocks revealed patterns identical with the C1 group.

The source of the virus could not be identified but transmission from feral birds is considered possible.

NEWCASTLE DISEASE: SITUATION IN GREAT BRITAIN 1998

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Investigations of Newcastle disease in poultry during 1998.

During 1998 a total of 12 suspected cases of Newcastle disease (ND) was investigated in poultry. No viruses were isolated (Table 1).

Table 1. Newcastle disease investigations in poultry during 1992-1998.

	1992	1993	1994	1995	1996	1997	1998
Cases investigated	17	16	22	11	44	107	12
NDV isolated	1	4	11	2	3	23	0
Confirmed cases	0	0	0	0	1	11	0

ND in racing pigeons

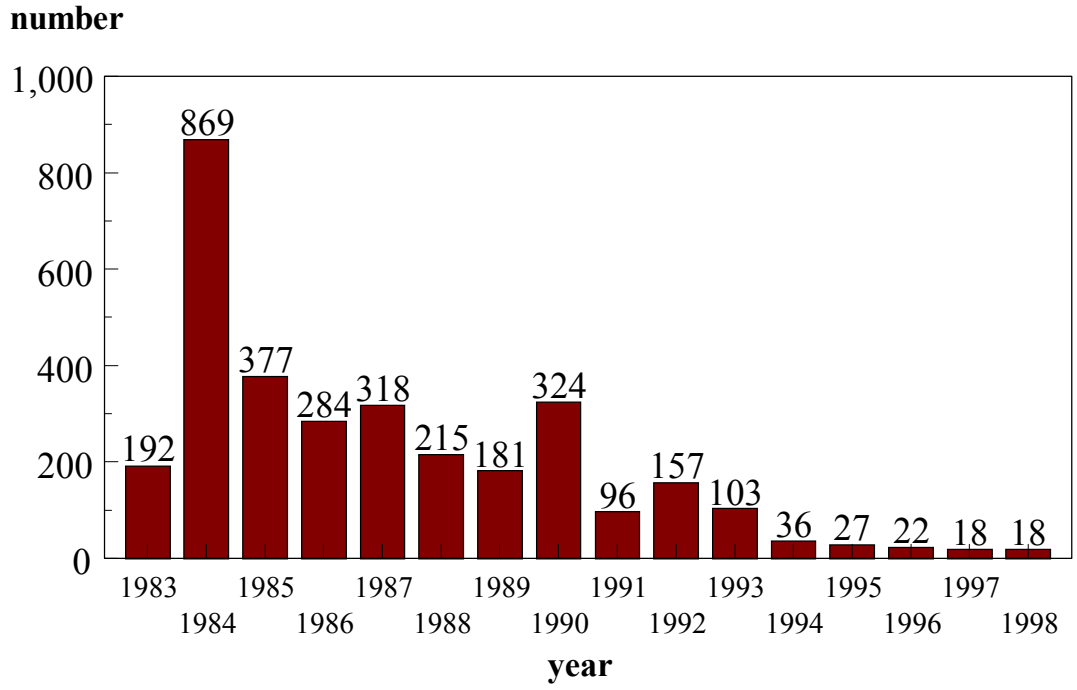
Infections of racing pigeons with the variant PPMV-1 virus may be confirmed in Great Britain by clinical signs alone, serology, virus isolation or a combination of these. The numbers of confirmed cases in Great Britain each year since the introduction of this variant virus in 1983 are shown in Figure 1. There were 18 confirmed cases in 1998, the same as 1997. This is the first year the number of confirmed outbreaks has not declined since the enforcement of the requirement to vaccinate birds taking part in races.

ND in other birds

During 1998 there were 95 virus isolation attempts on birds dying in quarantine. No ND viruses were isolated, but one APMV-3 virus was obtained from a king parakeet.

In March 1998 ND virus was isolated from two fancy pigeons found to be dying at Heathrow Airport on route from Bahrain to Oslo. The virus had an ICPI of 1.6 and was placed in monoclonal antibody group A.

Figure 1. Confirmed outbreaks of PPMV-1 infections in racing pigeons in Great Britain



NEWCASTLE DISEASE IN NORTHERN IRELAND: CURRENT SITUATION

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The last outbreak of Newcastle disease (ND) associated with the 1997 epizootic in Northern Ireland was in April 1997, with a single subsequent case confirmed in pheasants near Lough Neagh in County Antrim on 19/8/97. Since that time there have been no further cases.

Wild bird survey.

During the 1997 epizootic, four of the first five outbreaks of ND in Northern Ireland, including the index case, were located within 3km of major waterways which were being used by large numbers of waterfowl. This was consistent with the hypothesis that migratory waterfowl were responsible for introducing the infection, both in Northern Ireland and England (Alexander et al 1998).

In order to test this hypothesis, virus isolation was performed on samples from 104 waterfowl and waders, collected primarily on the shores of Lough Neagh and Strangford Lough. In addition, virus isolation was performed on samples from 126 non-aquatic wild birds to examine the possibility that these birds either introduced infection by contaminating feed at Belfast docks or were involved in the spread of infection between poultry units. Samples consisted of (i) faeces collected from feeding and nesting sites of waterfowl and waders (ii) tracheal and cloacal swabs or tissues from birds shot at the docks or in the vicinity of infected premises and (iii) tissues from waterfowl drowned in fishing nets. Full details of dates and locations of sampling, species and numbers of birds sampled, sample type and virus isolation results are given in Table 1.

Haemagglutinating agents were isolated on only 3 occasions, all from samples collected from waterfowl at Strangford Lough. Characterisation of these isolates in conjunction with the EU Reference Laboratory showed two of these to be avian influenza viruses, both of H6N1 subtype, with IVPI values of 0.15 (ex Whooper swan) and 0.00 (ex Greylag geese) respectively. The third isolate was an avian paramyxovirus type 8 (ex Brent geese).

While no evidence was found to support the involvement of wild birds in introducing or disseminating infection with APMV-1 in Northern Ireland, the isolation of other para- and ortho-myxoviruses from aquatic birds emphasises the

potential for such viruses to be introduced to commercial flocks from this source. This is highlighted by the isolation of H6N1 influenza virus from an outbreak of disease associated with high mortalities in ducklings in Wales during May 1998 (Alexander & Manvell, 1999).

References

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Table 1. Virus isolation results for wild bird survey conducted in February/March 1997.

Reference	Date	Species	Sample type	No. birds	Location	Result
VF-97-899	15/2/97	Ducks	Fa*	8	Lough Neagh (Derrycrow,	Negative
		Moorhen	"	1	Kinnego & Oxford Island)	"
		Geese	"	4		"
		Swans	"	8		"
VF-97-912	16/2/98	Pigeons	Cloacal/tracheal swabs	40	Belfast Harbour	Negative
VF-97-923	16/2/98	Wood pigeon	Fa, Br*, Tr*	1	Coleraine	Negative
VF-97-978	18/2/97	Pigeon	Fa, Br	1	Carrickfergus	Negative
VF-97-988	19/2/97	Starling	Cloacal swab	40	Belfast Harbour	Negative
VF-97-1154	24/2/97	Mallard	Fa	6	Strangford Lough	Negative
		Brent geese	"	6	(Kiltonga, Comber,	**PMV-8**
		Whooper swan	"	4	Greyabbey, Castle Espie)	**H6N1****
		Teal	"	6		Negative
		Greylag geese	"	5		**H6N1*****
		Wigeon	"	6		Negative
		Canada geese	"	5		"
		Shelduck	"	1		"
VF-97-1419	4/3/97	Sparrow	Fa, Br, Tr	9	Dungannon	Negative
		Robin	"	1		"
		Great tit	"	1		"
		Lapwing	"	1		"
VF-97-1432	5/3/97	Sparrow	Fa, Br, Tr	3	Lough Neagh	Negative
		Starling	"	2		"
VF-97-1443	5/3/97	Swan	Fa	11	Lough Neagh (Ardboe,	Negative

		Wigeon Golden eye Lapwing	" " "	2 4 3	Derrycrow)	" " "
VF-97-1485	6/3/97	Moorhen	Fa, Br, Tr	1	Dungannon	Negative
VF-97-1505	6/3/97	Swans Tufted duck Mallard	Fa " "	4 5 4	Lough Neagh (Ballyronan, Pollan Bay, Three Island)	Negative " "
VF-97-1552	6/3/97	Starling Sparrow	Fa, Br, Tr "	3 5	Lough Neagh	Negative
VF-97-1510	7/3/97	Wood pigeon House sparrow Bewick swan Whooper swan Mute swan	Fa, Br, Tr Fa, Br, Tr Fa Fa Fa	1 3 3 2 1	Lough Neagh (Ballinderry, Ardboe, Kinrush)	Negative " " " "
VF-97-1564	7/3/97	Sparrow Starling Rook Jackdaw Black headed gull	Fa, Br, Tr " " " "	2 2 2 1 1	Dungannon	Negative " " " "
VF-1997-1669	11/3/97	Ducks	Fa, Br, Tr	2	Lough Neagh (Ardboe)	Negative
VF-97-1781	14/3/97	House sparrow Starling	Fa, Br, Tr "	6 3	Magherafelt	Negative
Total				**230**		

*Fa = faeces, Br = brain, Tr = trachea, ** IVPI = 0.00, ***IVPI = 0.15

NEWCASTLE DISEASE: REPORT FOR NORWAY OCTOBER 1997 to OCTOBER 1998

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No outbreak of Newcastle disease/PMV-1-infection has been recorded in Norway during the period.

Every year at least one flock on all parent poultry farms is tested for PMV-1 antibodies as a part of a health certification and documentation programme for the breeding stock.

During the report period approximately 7400 blood samples representing 138 flocks have been tested in this connection.

24 flocks, including 6 ostrich flocks, in import quarantine have also been tested serologically.

In addition samples from 8 disease outbreaks have been examined for diagnostic purposes.

All blood samples have been negative for antibodies to PMV-1 and there has been no isolation of PMV-1.

In a large breeding plant with a history of several years with an endemic infection with an apathogenic or lentogenic PMV-1 infection, no antibodies have been detected during the report period. This improved situation should be considered in relation to an extensive sanitation programme enforced.

NEWCASTLE DISEASE REPORT: UNITED STATES OF AMERICA

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presented by Chris M. Groocock*

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In 1998, three nonlentogenic isolates of Newcastle disease virus (NDV) from domestic sources were characterised at the National Veterinary Services Laboratories (NVSL). The first isolate was characterised as a mesogenic pathotype of NDV. This virus was isolated at the National Wildlife Health Research Center, Madison, Wisconsin, from samples collected during a die-off in double crested cormorants in Oregon (Clatsop county) in July 1997. The second isolate was characterised as viscerotropic velogenic NDV. The virus was isolated in May 1998 at the California Veterinary Diagnostic Laboratory System, Fresno, California, from game chickens in a backyard flock in Fresno. The third isolate was characterised as a velogenic pathotype. This isolation was made at the NVSL in September 1998 from yellow cheek amazon parrots confiscated by the U.S. Customs and held in the Mission, Texas, quarantine centre.

The outbreak of viscerotropic velogenic ND in game chickens was in a backyard flock of fighting birds in a residential area. The single affected premise had 48 game chickens, 32 of which were sick and ten had died. The rest were destroyed and the premise depopulated by June 8. The eradication operation surveyed all 751 of the homes in the 56 square block residential area around the infected premises. Fifty-two premises had were found with a total of less than 100 birds. There was no evidence of contact with neighbouring birds, nor were there suspicious signs of illness in any of the surrounding premises. There was no evidence that the disease had moved from the index premises to any other location. There was no known contact with the commercial poultry population, the nearest of which is 17 miles away, and it is believed that there was no apparent risk to commercial poultry from this outbreak. The probable source of this Exotic Newcastle disease virus is still not known.

ORIGINAL CONTRIBUTIONS

RT-PCR ASSAYS FOR THE DETECTION OF AVIAN INFLUENZA A VIRUSES

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Introduction

Avian influenza (AI) is diagnosed by virus isolation and further characterization or by serological methods. Currently, 15 haemagglutinin (HA) and 9 neuraminidase (NA) subtypes are known. The pathogenicity of AI viruses (AIV) depends on strain and on host. High pathogenicity in chickens is mainly determined by the presence of multiple basic amino acids at the cleavage site of the HA protein. Only H5 and H7 subtypes have been characterized as highly pathogenic in chicken until now (Wood et al., 1996). Therefore, a detailed characterization of these subtypes is particularly important. Especially if the intravenous pathogenicity index is not greater than 1.2, nucleotide sequencing of the HA gene should be carried out to determine whether multiple basic amino acids are present at the cleavage site of the HA protein or not (CEC, 1992).

RT-PCR technique was used prior to sequencing of the haemagglutinin cleavage sites of H7 and H5 AI viruses (Wood et al., 1993; Senne et al., 1996; Banks et al., 1998). Because of the high variability of HA genes, several primer pairs have been applied in these investigations.

In the present work, RT-PCR assays for AIV of H5 and H7 subtypes including the HA cleavage sites have been developed to allow subsequent sequencing. Efforts particularly focused on the design of each uniform primer pair feasible for all strain variants.

Materials and methods

Viruses

Reference virus strains originated from the virus repository of the National Reference Laboratory for Avian Influenza. H5 and H7 subtype isolates were kindly provided by the Institute for Health Protection of Consumers and Veterinary Medicine, Berlin, Germany. The material was propagated in SPF chicken eggs (Lohmann, Cuxhaven, Germany) and the allantoic fluids were used for further analysis.

RNA preparation

The preparation of RNA was performed following the acid guanidinium-thiocyanate method (Chomczynski and Sacchi, 1987; Starick, 1999). Alternatively, two RNA isolation kits (QIAamp Viral RNA Kit, QIAGEN; High Pure Viral Nucleic Acid Kit, BOEHRINGER Mannheim) were used.

Primer design

The design of oligonucleotides was based on the sequence data submitted to the EMBL/GenBank/DDBJ. Tab. 1 summarizes the primers used in this study. Two of it, H7HA 1/1 and H7HA 1/5, were adopted (Senne et al., 1996; Banks et al., 1998) but altered by further degeneration. The primers were custom-ordered from GIBCO BRL.

RT-PCR

Essential program parameters for reverse transcription and PCR are shown in Tab. 2. RT was carried out with 5.3 µl RNA sample, 0.5 µl primer 1 (20 pmol), 0.1 µl RNasin (4U) and 0.35 µl AMV reverse transcriptase (3.5U) (PROMEGA) in a 15 µl reaction volume. The RT reaction product was supplemented with 35 µl PCR buffer including 0.5 µl primer 2 (20 pmol) and 0.25 µl Taq polymerase (1.25U) (PROMEGA). After amplification, ten µl of the reaction product were analyzed by electrophoresis on a 1.5 % agarose gel stained with ethidium bromide.

Results and discussion

After optimal conditions for the RT-PCR assays had been set up the specificity of the selected primers was evaluated by amplifying the genome of ten H7 and five H5 virus strains and isolates. All H5 viruses yielded positive results by RT-PCR with the primer pair H5HA 1/H5HA 2. Likewise, AIV-RNA isolated from the ten H7 viruses were specifically detected using each of the RT-PCR assays with the three different H7 primer pairs (Table 1). To confirm the specificity of the assays, controls were set up using paramyxoviruses serotypes 1 - 4 and 6 - 9. When RNA preparations of these viruses were tested, no false positive results were obtained.

A type specific amplification of influenza A virus RNA following reverse transcriptase was possible using primers selected from a strongly conserved region, for instance of the matrix protein coding gene. For this, a primer pair has been designed which was able to detect influenza A virus RNA independently of the subtype and the host species (data not shown). Because of the high variability of the HA gene, it was, however, difficult to develop uniform primers for the region covering the HA cleavage site of any AI subtype. Horimoto et al. (1995) developed a RT-PCR for the amplification of the HA cleavage site of H5 viruses prior to sequencing. However, the comparison with sequences available to date has indicated that there might be up to four mismatches between the gene and

primer sequences. No data are currently available on H7 HA cleavage site amplification using only one primer pair. In the present work, we made use of the advantages of wobble primers to overcome the difficulties caused by limited homology of the target sequences. For the RT-PCR of the H5HA cleavage site one primer pair has been developed. Three different primers for the reverse transcription of H7-AIV RNA were designed for the time being and evaluated on their suitability to detect virus RNA from different isolates. So far, the same results have been achieved with all primers. In case of confirmation of these results using additional AIV isolates (especially concerning the H5 viruses) the data presented here provides a fast, sensitive and reliable method for the detection, subtype characterization and subsequent sequencing of H5 and H7 avian influenza viruses.

Table 1: RT-PCR primers for detection of H5 and H7 AI viruses

primer	localisation[a]	sequence[b]
H5HA 1	677 - 699	5`GGAACATCAACACTRAAYCAGAG`3
H5HA 2	1091 - 1110	5`TCTACCATTCCYTGCCATCC`3
H7HA 1/1	1 - 20	5`AGCAAAAGCAGGGGWTACAA`3
H7HA 1/2	316 - 332	5`MGRGAAGGRAAYGATRT`3
H7HA 1/5	616 - 635	5`GARCAGACCAARYTMTATGG`3
H7HA 2	1149 - 1129	5`AGTTCCYTCYCCYTGTGCATT`3

[a] primer positions refer to the sequences for A/ck/Scotland/59 (H5N1) and A/FPV/Rostock/34 (H7N1)
[b] abbreviations for wobble primers: R: A+G; Y: C+T; W: A+T; M: C+A

Table 2: Programme steps for reverse transcription and PCR

RT-Primer	Annealing	Reverse transcription	PCR
H5HA 1			A
H7HA 1/1	70 °C, 10 min.	42 °C, $1^1/_4$ h	A
H7HA 1/2			B
H7HA 1/5			B

A: 90°C, 5 min.; 30 x (95°C, 1 min.; 53°C, 1 min., 72°C, 1 min.); 72°C, 7 min.
B: 95°C, 5 min.; 30 x (95°C, 1 min.; 50°C, 1 min., 72°C, 1 min.); 72°C, 7 min.

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RESCUE OF NEWCASTLE DISEASE VIRUS FROM CLONED CDNA: EVIDENCE THAT CLEAVABILITY OF THE FUSION PROTEIN IS A MAJOR DETERMINANT FOR VIRULENCE.

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Introduction

Newcastle disease is a serious avian disease with world wide distribution that can cause severe economic losses in the poultry industry (2). The causative agent of the disease is Newcastle disease virus (NDV) or avian paramyxovirus type 1 (18). Similar to other Paramyxoviridae, NDV contains a nonsegmented single-stranded RNA genome of negative polarity (11). The RNA is 15,186 nt in size (10, 12, 22) and contains 6 genes which encode the nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin-neuraminidase (HN), and large polymerase protein (L) (17).

NDV strains can be classified into highly virulent (velogenic) strains, intermediate (mesogenic) strains, or non-virulent (lentogenic) strains on the basis of their pathogenicity for chickens (5). The molecular basis for pathogenicity of NDV is mainly determined by the amino acid sequence of the protease cleavage site of the F protein and by the ability of cellular proteases to cleave the F protein of different pathotypes (19, 20). Cleavage of the precursor glycoprotein F0 to F1 and F2 by host cell proteases is required for progeny virus to become infective (5, 19, 23). As a result, lentogenic viruses can only replicate in areas with trypsin-like enzymes such as the respiratory and intestinal tract, whereas virulent viruses can replicate in a range of tissues and organs resulting in fatal systemic infection (19, 20). Lentogenic NDV strains such as the Hitchner B1 (9) and La Sota (6) strains are widely used as live vaccines against Newcastle disease. However, despite their widespread use, NDV live vaccines may still cause disease signs, depending upon environmental conditions and the presence of complicating infections.

To improve the efficacy and safety of current NDV live vaccines, we set out to develop a system that would allow the genetic modification of NDV. Genetic modification of non-segmented negative-strand RNA viruses has recently become possible (reviewed in 4, 21). This process, which is often referred to as "reverse genetics", involves the in vivo transcription, by means of T7 RNA polymerase, of cDNA-encoded antigenomic RNA and the simultaneous

expression, from co-transfected plasmids, of those viral proteins that are required for replication and transcription of the viral RNA.

Here we report the generation of infectious NDV entirely from cloned cDNA and we demonstrate that this procedure can be used to generate genetically modified NDV strains. We show that the virulence of NDV can be changed dramatically by modifying the protease cleavage site of the F0 protein. By using cDNA-derived NDV strains that differ only in the amino acid sequence of the protease cleavage site of the F0 protein we deliver the definite proof that cleavability of the F0 protein is a key determinant for virulence of NDV.

Results

A full-length cDNA clone of Newcastle disease virus (NDV) vaccine strain La Sota was assembled from subgenomic overlapping cDNA fragments and cloned in a transcription plasmid between the T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme. The resulting plasmid was designated pNDFL. Transfection of this plasmid into cells that were infected with a recombinant fowl pox virus that expressed T7 RNA polymerase, resulted in the synthesis of antigenomic NDV RNA. This RNA was replicated and transcribed by the viral NP, P and L proteins, which were expressed from cotransfected plasmids. To recover infectious virus, the supernatant of transfected monolayers was injected into the allantoic cavity of 9- to 11-day-old embryonated SPF eggs.

Four days later the allantoic fluid was harvested, analysed in a haemagglutination assay, and passaged further in eggs. The results showed that only allantoic fluid from eggs inoculated with the supernatant of cells transfected with a combination of pNDFL and the NP, P and L support plasmids yielded a positive reaction in the haemagglutination assay (data not shown). The allantoic fluid was subsequently analysed in a haemagglutination-inhibition (HI) test by using NDV HN specific mAbs 7B7, 5A1, 7D4, and 4D6 (13, 16). The results showed that the NDV strain that was recovered from the inoculated eggs showed the same reaction pattern as the original La Sota strain (Table 1). The virus that was recovered from the inoculated eggs was designated NDFL to distinguish it from the original La Sota strain.

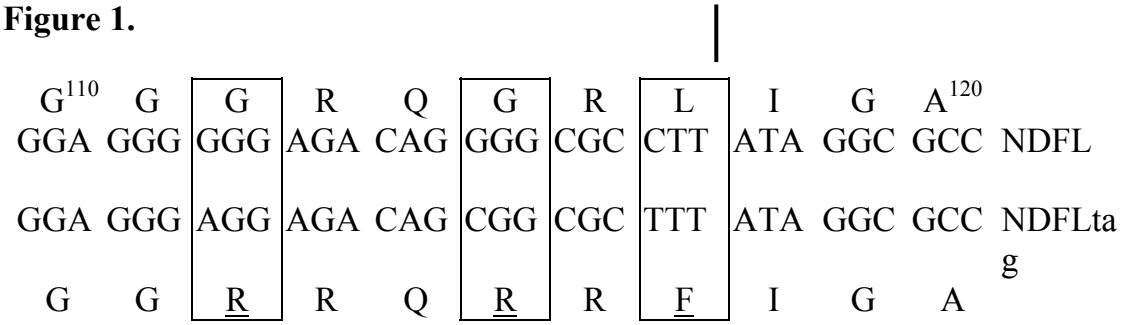
To show unambiguously that the cotransfection system could be used to recover infectious virus from cloned full-length NDV cDNA, a genetic tag was introduced in plasmid pNDFL. To this end, the amino acid sequence of the protease cleavage site in the Fo protein was changed from that of the LaSota strain (GGRQGR↓L) to the consensus sequence of virulent NDV strains (GRRQRR↓F) (23) by means of PCR mutagenesis (Fig. 1).

Table 1. Haemagglutination-inhibition titres[1] of antisera and mAbs.

Strain	NDV serum	control serum	APMV-3 serum	mAb 7B7	mAb 7D4	mAb 5A1	mAb 4D6	ICPI[2]	Pathotype
La Sota E13-1	256	-	-	320	5120	40	10240	0.30	lentogenic
NDFL	128	-	-	2560	10240	1280	10240	0.00	lentogenic
NDFLtag	256	-	-	640	10240	20	10240	1.27	mesogenic
Hitchner B1	256	-	-	80	-	320	10240	0.25	lentogenic
Herts	128	-	-	2560	-	-	-	1.88	velogenic
Texas GB	128	-	-	10240	-	-	10240	1.75	velogenic

[1] Titres are expressed as the reciprocal of the highest serum dilution that caused inhibition of hemagglutination.
[2] The intracerebral pathogenicity index was determined as described by Alexander (1).
[3] Lentogenic strains: ICPI<0.7; mesogenic strains: ICPI 0.7-1.4; velogenic strains: ICPI > 1.4.

Figure 1.



The resulting plasmid was designated pNDFLtag. By changing the amino acid sequence of the proteolytic cleavage site of the F gene, we aimed at introducing both a genetic tag as well as a phenotypic tag. Plasmid pNDFLtag was used to generate virus by using the cotransfection system described above. Infectious viruses, designated NDFLtag, was recovered from the allantoic fluid of embryonated eggs. In an HI test, all mAbs including 7D4, which is specific for the La Sota strain (13, 16), showed the same reaction pattern with the newly generated virus as with the original La Sota strain (Table 1).

Pathogenicity tests in day-old chickens showed that strain NDFL, which was derived from the unmodified cDNA, was completely non-virulent (intracerebral pathogenicity index [ICPI] = 0.00). However, strain NDFLtag which was derived from the cDNA in which the protease cleavage site was modified, showed a dramatic increase in virulence (ICPI = 1.28 out of a possible maximum of 2.0). Pulse-chase labelling of cells infected with the different strains followed by radio-immunoprecipitation of the F protein showed that the efficiency of cleavage of the F0 protein was greatly enhanced by the amino acid replacements (data not shown). These results demonstrate that genetically modified NDV can be recovered from cloned cDNA and confirm the supposition that cleavage of the F0 protein is a key determinant in virulence of NDV.

Discussion

That genetic modification is a powerful tool to study the biological functions of viral gene products is demonstrated by our observation that modifying the protease cleavage site of the F0 protein can dramatically change the virulence of NDV. Amino acid sequencing of the F0 precursor proteins of many NDV strains has shown that lentogenic viruses have a single arginine (R) that links the F2 and F1 chains, whereas mesogenic or velogenic strains possess additional basic amino acids forming two pairs at the site of cleavage. Furthermore, the F2 chain of virulent strains generally starts with a phenylalanine (F) residue whereas that of nonvirulent strains generally starts with a leucine (L) (23). When the protease cleavage site of the Fo protein of strain NDFL (GGRQGR↓L) was converted into the consensus protease cleavage site of virulent NDV strains (GRRQRR↓F), this

modification resulted in a dramatic increase in virulence from ICPI = 0.00 for NDFL to ICPI = 1.28 (out of a possible maximum of 2.0) for NDFLtag (Table 1). Thus, the virulence of NDFLtag is similar to that of a mesogenic strain whereas the antigenic profile is similar to that of the lentogenic parent strain LaSota (Table 1). These results deliver the definite proof that the efficiency of cleavage of the F0 protein is the key determinant for virulence of NDV. By using the same approach, the cleavage site of the Fo protein may be modified, at will, to any other amino acid sequence. This may lead to the generation of a series of NDV strains that may display a spectrum of virulence levels in vivo.

Our results indicate that the efficiency of cleavage of the Fo protein is not the only determinant that is responsible for virulence of NDV. Velogenic NDV strains may exhibit an ICPI of as high as the maximum possible value of 2.0 (2). This indicates that, apart from the cleavage site of the F0 protein, additional nucleotide sequences in the genomic RNA of NDV contribute to virulence. For instance, differences in transcription and translation may modulate growth and cell-to-cell spread of the virus and/or cytopathogenicity (14, 15). The availability of an infectious cDNA of NDV allows for the systematic analysis of sequences that are involved in transcription and replication. This may lead to the design of new NDV vaccines that combine optimal immunogenicity with complete safety.
One of the largest drawbacks of currently used live vaccines and inactivated vaccines is the fact that vaccinated animals cannot be distinguished from infected animals with currently used screening techniques such as haemagglutination-inhibition or virus neutralisation tests. Virulent field strains may still spread in vaccinated flocks since disease symptoms are masked by vaccination. Since virus isolation and characterisation of virulence by in vivo techniques is not feasible on a large scale, there is great need for new and effective attenuated live vaccines which can be serologically discriminated from field strains. Such vaccines, called marker vaccines, are not yet available. However, now that genetic modification of NDV is possible, it should also be possible to generate NDV marker vaccines. By using such marker vaccines, the prevalence of NDV field strains can be monitored and the appropriate measures can be taken to prevent infection and spread of virulent field strains in commercial flocks. This may significantly reduce both morbidity and mortality as well as economical losses caused by NDV infections.

Several properties make NDV an attractive vaccine vector for vaccination of poultry against respiratory or intestinal diseases. 1) NDV can be easily cultured to very high titres in embryonated eggs. 2) NDV vaccines are relatively stable and can be simply administered by mass application methods such as addition to drinking water or by spraying or aerosol formation. 3) The natural route of infection of NDV is by the respiratory and/or intestinal tract, which are also the major natural routes of infection of many other poultry pathogens. Since other paramyxoviruses have successfully been used for the incorporation and

expression of foreign genes (7, 8, 24) we expect that NDV can also be used as a vaccine vector for the delivery of foreign antigens to the immune system.

Acknowledgement

We thank Francis Balk for the inoculation of eggs and for performing haemagglutination and haemagglutination inhibition tests.

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EU REPORTS

REPORT OF THE EUROPEAN UNION REFERENCE LABORATORIES FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE 1998

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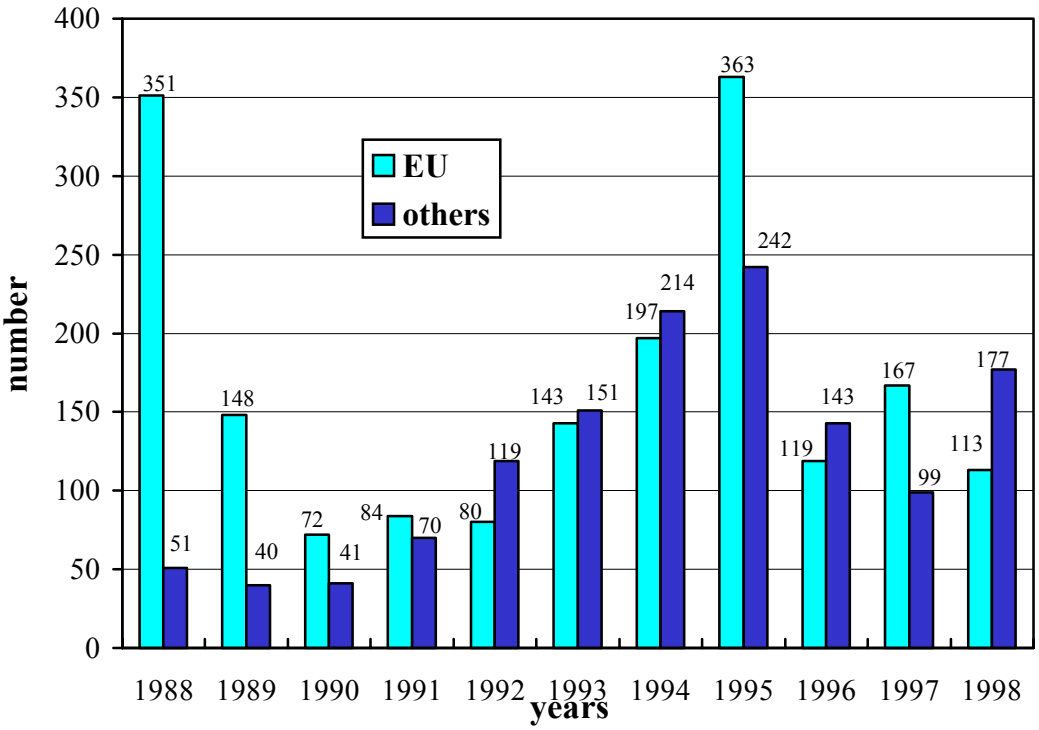
Viruses received by International Reference Laboratory

The numbers of viruses submitted by all countries to the reference laboratory for each of the years 1988 to 1998 are 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 reference laboratory is often asked to characterise isolates retrospectively.

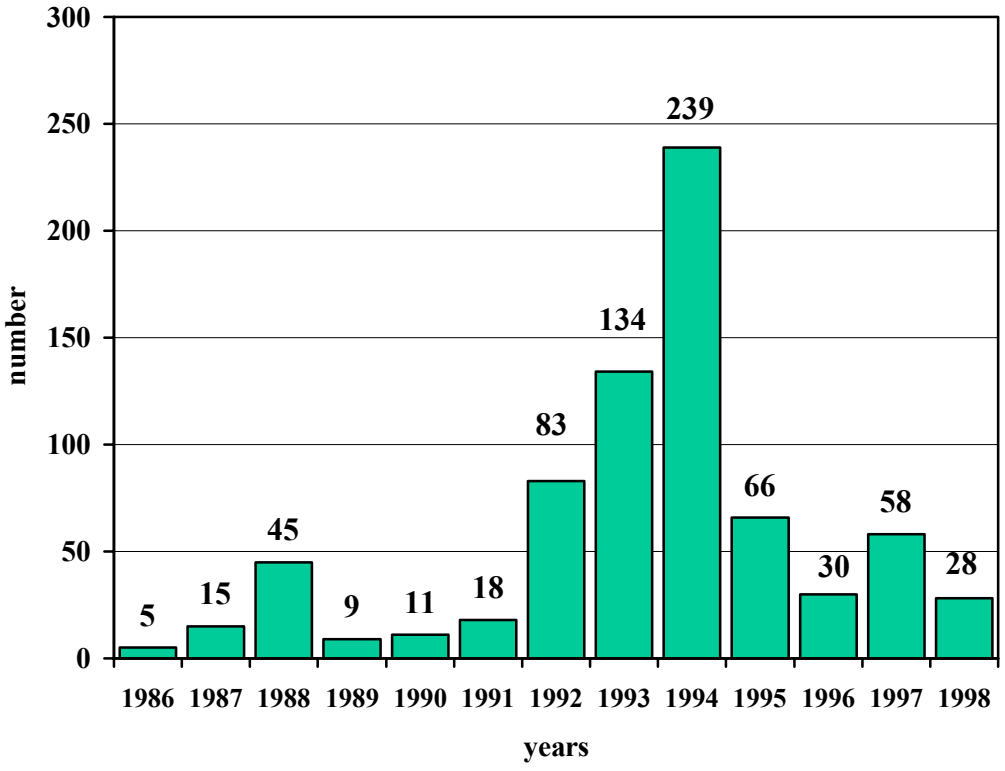
Figure 1. Viruses submitted to the International Reference Laboratory VLA Weybridge during 1988 to 1998



The number of submissions received in 1998, 290, is slightly higher than 1996 and 1997, when 262 and 266 were received respectively. These numbers are

relatively low compared to some years, but are consistent with the number of outbreaks of Newcastle disease occurring in the EU in 1998 (Figure 2).

Figure 2. Reported outbreaks of Newcastle disease in EU countries 1986-1998.



Influenza viruses submitted during 1998

Influenza virus submissions are listed in Table 1. There were 38 received from non-EU countries and 22 from EU member states.

The 15 H9N2 viruses submitted from Iran reflected the widespread disease problems in poultry in that country as a result of infections with this virus of low pathogenicity.

Viruses received from EU countries included the last of the 8 outbreaks of HPAI of H5N2 occurring in poultry in the North East of Italy during October 1997-January 1998. The H7N7 viruses of low virulence from Ireland and N. Ireland were representatives from the outbreaks occurring on the island of Ireland in 1998 [see country reports].

Surveillance of feral water fowl in Northern Ireland resulted in the isolation of two H6N1 viruses. Virus of the same subtype was isolated from domestic ducklings in Great Britain.

Nucleotide sequencing H5 and H7 viruses.
Following RT-PCR and nucleotide sequencing the amino acid sequence at the cleavage site of the haemagglutinin precursor protein of each H5 and H7 virus submitted was deduced. In keeping with the *in vivo* virulence tests the only virus that had multiple basic amino acids at the cleavage site of the HA0 protein was the H5N2 virus from the outbreak in Italy (Table 2).

Table 1: Influenza A viruses submitted to the International Reference Laboratory during 1998*.

Country	Number	Host	Subtypes	IVPI
		non-EU countries		
South Africa	4	ostriches	H6N8	0.00
South Africa	8	waterfowl	H10N9	0.00
Singapore-Q	2	ducks	H4N6	0.00
Singapore-Q	1	ducks	H9N2	
Singapore-Q	1	ducks	H5N2	0.00
Saudi Arabia	2	chickens	H9N2	
Iran	15	chickens	H9N2	0.00
New Zealand	2	feral ducks	H5N2	0.00
New Zealand	1	feral duck	H4N6	
Canada	2	turkeys	H6N1	
Total	**38**			
		EU countries		
Italy	1	chicken	H5N2	2.81
Italy	1	chicken	H5N9	0.00
Germany[a]	3	chickens	H9N2	
Germany	3	turkeys	H6N5	
Ireland	5	turkeys	H7N7	0.00
Ireland	1	chickens	H7N7	0.00
N. Ireland	2	turkeys	H7N7	0.00
N. Ireland	1	chickens	H7N7	0.00
N. Ireland	1	swan	H6N1	0.15
N. Ireland	1	greylag goose	H6N1	0.00
Great Britain	1	dom. ducks	H6N1	0.21
Denmark	2	finches	H4N6	
Total	**22**			

**Not all viruses received had been isolated within this period.*
[a]*Two of these did not originate in Germany*

Table 2. Deduced amino acid sequences at the HA0 cleavage site of H5 and H7 viruses submitted to the International Reference Laboratory.

Virus	IVPI	amino acid sequence
poultry/Italy/98 (H5N2)	**2.81**	**-PQRRRKKR↓GLF-**
chicken/Italy/98 (H5N9)	0.00	-PQKETR↓GLF-
poultry/Ireland/98 (H7N7) x 6	0.00	-PEIPKGR↓GLF-
poultry/N.Ireland/98 (H7N7) x 3	0.00	-PEIPKGR↓GLF-
duck/Singapore-Q/98 (H5N2)	0.00	-PQRETR↓GLF-
ducks/N.Zealand/97 (H5N2)	0.00	-PQRETR↓GLF-

Paramyxoviruses received during 1998

The viruses received from EU countries in 1998 (Table 3) were consistent with the disease situations reported with the exception of the large number of viruses from Denmark which were related to an investigation into poultry virus vaccines contaminated with NDV.

Table 3: European Union countries submitting APMV viruses during 1998.

Country	APMV-1	PPMV-1	APMV-2	APMV-3	APMV-8
Great Britain	1	6		1	
N. Ireland					1
Germany	2		5		
France	3				
Austria		9			
Portugal	1	7			
Belgium		14			
Italy	2	3	1		
Denmark	31				
Total	**40**	**28**	**6**	**1**	**1**

**Not all viruses received had been isolated in 1998 and numbers may include viruses isolated in countries other than the submitting country.*

In 1998 totals of 109 viruses typed as APMV-1 and one virus type as APMV-3 (Table 4), were received from 11 countries from outside the EU representing a wide cross section from different continents and geographical areas.

Table 4: Other countries submitting APMV viruses during 1998.

Country	APMV-1	APMV-3	not viable
South Africa	2		
Saudi Arabia	5		14
UAE	62		
Botswana	12		1
Mexico			12
Israel		1	
Brazil	12		
New Zealand	2		
Sri Lanka	3		
Bulgaria	8		
Australia	3		
Total	**109**	**1**	**27**

**Not all viruses received had been isolated in 1998.*

Antigenic grouping of Newcastle disease virus [APMV-1] isolates.
All APMV-1 viruses received during 1998 were characterised by their ability to cause binding of a panel of 28 monoclonal antibodies [mAbs] to cell cultures infected with the viruses. For convenience and the need to summarise, the results are shown in mAb groups (Alexander et al, 1997) in Tables 5-6. 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 5-6 on the basis of virulence for chickens.

The results suggest that the variant pigeon PMV-1 virus which is placed in mAb binding group P remains present in the EU, primarily in pigeons.

Acknowledgements
The nucleotide sequencing of the influenza viruses was done by Jill Banks and Emma Speidel.

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Table 5. Antigenic grouping of APMV-1 [Newcastle disease virus] isolates from EU countries submitted during 1998

Country	Number of viruses in monoclonal antibody binding group				
	velogenic				*lentogenic*
	P	A	C1	?[a]	E
Gt Britain	6	1			
Germany				2	
France	1			2	
Austria	9				
Portugal	7		1		
Belgium	14				
Italy	3		1		1
Denmark			2		25

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

Table 6. Antigenic grouping of APMV-1 [Newcastle disease virus] isolates from non-EU countries submitted during 1998.

Country	Number of isolates in monoclonal antibody binding group									
	velogenic						*lentogenic*			
	P	B	C1	A	?[a]	D	F	E	H	?[a]
South Africa						1		1		
Saudi Arabia	4							1		
UAE*	6	9	19	1	4		3			
Botswana			12							
Brazil					12					
New Zealand									2	
Sri Lanka						3				
Bulgaria		6	2							
Australia					2					1

[a]*not grouped - viruses placed in this category did not necessarily show the same mAb pattern. *5 reoviruses and 5 herpesviruses also isolated*

AVIAN INFLUENZA AND NEWCASTLE DISEASE IN THE EUROPEAN COMMUNITY: SANITARY SITUATION AND LEGISLATIVE DEVELOPMENTS IN 1998 TO DATE

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DISEASE SITUATION

The outbreaks of avian influenza and Newcastle disease reported by the Member States in the years 1994 – 1998 are shown in the following tables 1 and 2.

Avian influenza

Table 1: Number of outbreaks of avian influenza reported by the Member States*

COUNTRY	1994	1995	1996	1997	1998
Austria	0	0	0	0	0
Belgium	1	0	0	0	0
Denmark	0	0	0	0	0
Finland	0	0	0	0	0
France	0	0	0	0	0
Germany	0	0	0	0	0
Greece	0	0	0	0	0
Ireland	0	0	0	0	0
Italy	0	0	0	7	1
Luxembourg	0	0	0	0	0
Netherlands	0	0	0	0	0
Portugal	0	0	0	0	0
Spain	0	0	0	0	0
Sweden	0	0	0	0	0
United Kingdom	0	0	0	0	0
TOTAL	1	0	0	7	1

* reports according to the ADNS system

Just one outbreak was reported in 1998 by Italy.

Newcastle disease

Table 2: Number of outbreaks of Newcastle disease reported by the Member States*

COUNTRY	1994	1995	1996	1997	1998
Austria	0	0	4	2	6
Belgium	1	11	7	2	14
Denmark	0	14	4	0	2
Finland	0	0	2	0	0
France	0	0	0	1	1
Germany	173	28	2	0	0
Greece	0	0	0	0	0
Ireland	0	0	0	1	0
Italy	42	2	4	2	2
Luxembourg	0	2	0	0	0
Netherlands	8	5	2	1	0
Portugal	12	2	3	10	0
Spain	13	0	0	0	0
Sweden	0	1	0	1	0
United Kingdom	0	0	2	38	0
TOTAL	236	65	30	58	25

* reports according to the ADNS system

Compared to recent years the number of reported outbreaks has reduced considerably. This reflects the success of the specific Community legislation, in some Member States combined with additional legislative measures. The latter includes compulsory vaccination in flocks independent of their size. However, the implementation of stricter rules should be considered in particular in racing pigeons as most of the Newcastle disease outbreaks reported in 1998 occurred in such birds.

The two most serious outbreaks occurred in Denmark, where about 140.000 turkeys had to be slaughtered due to the infections diagnosed in two flocks.

LEGISLATIVE DEVELOPMENTS

Community Reference Laboratory

A decision was adopted to provide for financial aid for the Community Reference Laboratories for Newcastle disease and for avian influenza: Decision 98/587/EC. It provides that the Community Reference Laboratory for both diseases would be the Central Veterinary Laboratory (Weybridge), Addlestone, United Kingdom. We anticipate renewing this contract for 1999.

Avian influenza in Hong Kong

In late 1997 and early 1998 an H5N1 influenza A virus epidemic created an alarming situation in Hong Kong. Human deaths were epidemiologically linked to the presence of AI virus in chicken. The Community Reference Laboratory for avian influenza was able to keep the Commission and the Member States up to date on the developments in Hong Kong. Due to the evolution of the epidemic the Commission adopted a Decision suspending imports of birds other than poultry coming from or originating in Hong Kong and China (Decision 98/85/EC). Imports of poultry were not possible as neither Hong Kong nor China is included in Decision 95/233/EC, listing countries from which Member States authorize imports of live poultry and hatching eggs. As the disease situation improved in Hong Kong, Decision 98/85/EC was repealed on 29 May 1998 (Decision 98/396/EC).

Import requirements for fresh ratite meat

Ratites are part of the bird family and include inter alia ostriches and emus. Trade with ratite meat is an important economic factor for some third countries, in particular countries of southern Africa.

The requirements for imports of fresh ratite meat are not yet harmonized. According to Commission Decision 97/467/EC Member States may import such meat on the basis of bilateral agreements until 1 October 1999.

During 1998 a draft Decision laying down a harmonized certificate was discussed with the Member States and with some third countries particularly interested in the subject. The draft certificate was agreed technically by the Member States and shall be transmitted to the WTO for comments soon.

Main elements of the draft certificate are the following:

- The certificate lays down animal and public health requirements.

- As regards animal health, special conditions are foreseen for avian influenza, Newcastle disease and Crimean Congo Haemorrhagic Fever.

- The animal health conditions are similar to those required in the certificate for poultry other than ratites. These conditions include in particular the basic rules of Article 10 of Council Directive 91/494/EEC: The meat must come from a third country either free from avian influenza and Newcastle disease or applying measures to control them which are at least equivalent to those foreseen within the Community.

COMPARATIVE TESTS FOR ANTIGEN IDENTIFICATION IN DIFFERENT EU NATIONAL LABORATORIES

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Introduction

One of the functions and duties of the Community Reference Laboratories for Newcastle Disease and Avian Influenza is to organise "periodical comparative tests in diagnostic procedures at Community level". To fulfil this duty a simple test of the ability of the National Laboratories to identify Newcastle disease and influenza was organised.

There were three basic objectives:

1. To test the ability of National Laboratories to determine the presence of notifiable viruses.
2. To test the ability of National Laboratories not to confuse other viruses
3. To identify areas where improvements can be made

Materials and methods

Each National Laboratory was sent 7 unknown antigens with the following instructions:

"*Please find enclosed 7 ampoules of inactivated antigens labelled A to G. One bijou contains wet stock of virus and the other 6 ampoules should be re-constituted in 1.0 ml of distilled water.*

Please try and carry out identification of these antigens by HA and HI tests. If possible send the results that you obtain, and a copy of the protocol normally used in your laboratory to reach us by the end of October 1998 to enable a report to be prepared for the 5th Annual Meeting."

Antigens supplied were formalin or betapropiolactone inactivated whole viruses. Laboratories are expected to be at least able to identify H5 and H7 influenza viruses and PMV-1 [Newcastle disease] virus. However implicit in this expectancy is that they will not erroneously identify other viruses as these. The antigens supplied were therefore selected to test these points. It was not

necessarily expected that every National Laboratory would fully identify all the antigens. The antigens supplied and the minimum expected results were:-

antigen	virus	expected result
A	A/African starling/England-Q/79 (H7N1)	H7
B	A/ostrich/Denmark/72420/96 (H5N2)	H5
C	PMV-1 Ulster 2C	PMV-1
D	PMV-3 turkey/England/1087/82	PMV-3
E	A/duck/Germany/1215/73 (H2N3)	?
F	PMV-7 dove/Tennessee/4/75	?
G	PMV-1 pigeon/England/617/83	[P]PMV-1

It was expected that antigens A-C would pose no problem in identification.

Antigen D was a PMV-3 virus, these often show high levels of reaction with PMV-1 antisera, but it is important that National Laboratories are able to distinguish these from PMV-1 viruses as they are often present in turkeys and caged birds and may show ICPI values >0.7.

Antigens E and F were included as an influenza virus and a paramyxovirus which were unrelated to the viruses which cause notifiable diseases, although PMV-7 may show some low level reaction with PMV-1 antisera.

Antigen G was a representative of the variant PMV-1 virus responsible for the continuing panzootic in pigeons. National Laboratories should be able to at least identify this as a PMV-1 virus. However, a monoclonal antibody, 161/617 is available which is specific in haemagglutination inhibition tests for the variant pigeon virus [PPMV-1] and the Community Reference Laboratory has agreed to make small amounts of this mAb available on request, there is, therefore, no reason why this antigen should not have been identified as PPMV-1.

Results

The results obtained and submitted to the Community Reference Laboratory are presented in Table 1. In this table the Laboratories are coded 1-18, based on the order in which results arrived at the Reference Laboratory these consist of all the National Laboratories plus Norway and the Reference Laboratory [which is coded 1]. The total is 18 because Belgium also represents Luxembourg, the United Kingdom is represented by two Laboratories, Great Britain and Northern Ireland and in Italy the influenza and Newcastle disease Laboratories are different and results were received from each Laboratory. All Laboratories submitted their results in time for the meeting in Vienna with the exception of Laboratory 18. The results for Laboratory 18 were received after the meeting at which the other results had been presented.

The results for each antigen and possible explanations for any discrepancies will be considered separately.

ANTIGEN A - H7
All 18 Laboratories correctly identified this antigen as an H7 subtype influenza virus.

ANTIGEN B - H5
Seventeen of the Laboratories identified this antigen correctly as H5. The other Laboratory, 8, identified this antigen as H6. Clearly this is a serious problem that could result, at best, in delays in detecting the presence of potentially highly pathogenic virus.

Fortunately Laboratory 8 supplied a detailed account of the procedures used and it was clear that this error was due to the low titre obtained with the H5 antiserum used [1/64 compared to the homologous titre of 1/1024 with a/chicken/Scotland/59 (H5N1)] and high level cross reaction due to the N2 neuraminidase of the H5N2 virus used for antigen B and the H6N2 virus [A/turkey/Massachusetts/3740/65 (H6N2)] used to prepare H6 antiserum resulting in an HI titre of 1/256 compared to the homologous HI titre of 1/1024.

ANTIGEN C - PMV-1
All 18 Laboratories correctly identified this antigen as a PMV-1 virus.

ANTIGEN D - PMV-3
Eleven Laboratories identified this antigen correctly as PMV-3. Two laboratories, 5 and 12, considered this antigen to be a paramyxovirus other than PMV-1, but did not confirm the antigen as PMV-3. Five Laboratories identified antigen D incorrectly as PMV-1. Four of these had not used PMV-3 antiserum to test the antigens which would almost certainly have indicated the true identity of the antigen. In the other Laboratory PMV-3 antiserum had been used, but subsequent testing had revealed that due to a storage problem it had lost its HI titre.

ANTIGEN E - H2
Although the H2 caused problems for a number of the Laboratories 15/18 produced the expected result, 6 confirming the antigen as H2 and nine, who did not have the full range of antisera, recording an unknown or uncertain result. Three Laboratories identified E as H5 and a further Laboratory tentatively suggested it could be H5. None of these Laboratories had used antisera to H2 and while the sera used were not specified in all four cases, it seems likely that the erroneous results were the result of HI titres obtained as a result of cross reaction

between the N3 neuraminidase of antigen E and the neuraminidase of virus used to produce H5 antiserum [the H5 reference strain is A/tern/S. Africa/59 (H5N3)].

ANTIGEN F - PMV-7
Again the desired result with this PMV-7 antigen was not necessarily that it was identified correctly but that it was not identified as a PMV-1 virus. Eight Laboratories correctly identified antigen F as PMV-7; a further 5 concluded F was a paramyxovirus but not PMV-1 and two others were unable to identify the antigen but were convinced it was not H5, H7 or PMV-1, all these results are adequate. Three laboratories concluded antigen F was PMV-1, presumably as a result of detecting low cross reactions with PMV-1.

ANTIGEN G - PPMV-1
All Laboratories identified antigen G as a PMV-1 virus. Eight laboratories offered further identification confirming that the virus was PPMV-1 by using either mAb 161/617 or their own in house antisera. Surprisingly, one Laboratory, 7, had mAb 161/617 available and in routine use, but for some reason did not employ it in this exercise.

OTHER RESULTS
It was assumed that Laboratories would restrict their testing to HI tests using the antigens, but one enterprising Laboratory, 15, used the antigens in agar gel precipitation tests to confirm that antigens A, B and E were influenza A viruses.

GENERAL
Excluding the Reference Laboratory, 1, only two Laboratories, 9 and 15, fully and correctly identified the seven antigens. Two Laboratories, 7 and 17, correctly identified all the antigens, but restricted identification of antigen G to PMV-1. A further seven Laboratories produced adequate results in that they obtained at least the minimum expected result and did not incorrectly identify any antigen. Only one Laboratory failed to correctly identify all the notifiable antigens, Laboratory 8 identifying antigen B as H6 instead of H5; but five other Laboratories incorrectly identified at least one of the three non-notifiable antigens, D, E and F as PMV-1 or H5 and two Laboratories, 4 and 11, did this for all three antigens.

Discussion

The functions of the National Laboratories are not specified in great detail in the Directives on control of Newcastle disease and avian influenza. However, Directive 92/66/EEC does state in Article 14: *"1. Member States shall insure that in each Member State there is designated: (a) a national laboratory at which facilities and expert personnel shall be maintained to permit full antigenic and biological typing of Newcastle disease virus....."* and Directive 40/92/EEC states in Article 14: *"1. Member States shall insure that in each Member State*

there is designated: (a) a national laboratory at which facilities and expert personnel shall be maintained to permit assessment of pathogenicity of influenza isolates ..... and identification of influenza A viruses of H5 or H7 subtypes;". It could be argued, therefore, that with the exception of the single laboratory failing to identify the H5 antigen the Laboratories had fulfilled this function. However, implicit in the minimum requirement is a requirement that National Laboratories do not identify other viruses as those which require notification or further attention and in this aspect five laboratories failed with one [laboratory 18], two [14, 16] or three [4, 11] antigens [Table 1]. In addition nine Laboratories, while reporting essentially correct results with all antigens, could have improved their identification with the inclusion of additional antisera in their tests.

Recommendations

1. All laboratories should hold *at least* two antisera to H5 influenza A subtype prepared against viruses with different neuraminidase subtypes and *at least* two antisera to H7 influenza A subtype prepared against viruses with different neuraminidase subtypes.

2. All laboratories should hold PMV-3 antiserum.

3. Laboratories, especially those with incorrect results, should re-consider the panel of antisera maintained for the identification of notifiable avian viruses.

4. Another comparative antigen identification test should be done in 1999.

TABLE 1. Results of comparative tests for antigen identification in different EU National Laboratories

Laboratory code	A	B	C	D	E	F	G
1	H7	H5	PMV-1	PMV-3	H2	PMV-7	PPMV-1
2	H7	H5	PMV-1	PMV-3	FLU?	PMV-7	PPMV-1
3	H7	H5	PMV-1	PMV-3	?	PMV-?	PPMV-1
4	H7	H5	PMV-1	***PMV-1***	***H5***	***PMV-1***	PPMV-1
5	H7	H5	PMV-1	PMV-?	FLU	PMV-?	PMV-1
6	H7	H5	PMV-1	PMV-3	FLU?	PMV-7	PPMV-1
7	H7	H5	PMV-1	PMV-3	H2	PMV-7	PMV-1
8	H7	***H6***	PMV-1	PMV-3	FLU?	PMV-7	PMV-1
9	H7	H5	PMV-1	PMV-3	H2	PMV-7	PPMV-1
10	H7	H5	PMV-1	PMV-3	?	PMV-?	PMV-1
11	H7	H5	PMV-1	***PMV-1***	***H5***	***PMV-1***	PMV-1
12	H7	H5	PMV-1	PMV-?	H5?	PMV-?	PMV-1
13	H7	H5	PMV-1	PMV-3	FLU?	PMV-?	PPMV-1
14	H7	H5	PMV-1	***PMV-1***	H2	***PMV-1***	PMV-1
15	H7	H5	PMV-1	PMV-3	H2	PMV-7	PPMV-1
16	H7	H5	PMV-1	***PMV-1***	***H5***	?	PMV-1
17	H7	H5	PMV-1	PMV-3	H2	PMV-7	PMV-1
18	H7	H5	PMV-1	***PMV-1***	?	?	PMV-1

DISCUSSIONS, CONCLUSIONS, RECOMMENDATIONS

DISCUSSION OF THE FOLLOWING TOPICS

- ***proposed EU and OIE new definitions of ND***

The Meeting considered two proposed new definitions of Newcastle disease for the purposes of implementing control measures.

In "*The Definition of Newcastle Disease - Report of the Scientific Committee on Animal Health and Animal Welfare Adopted 24 March 1998*" the SCAHAW makes the following recommendations:-

1. For the purposes of Newcastle Disease notification, it is imperative to define "poultry". The following definition is recommended:

- Poultry are all birds which are reared or kept in captivity for breeding, the production of meat or eggs for consumption, the production of other commercial products or for restocking supplies of game.

2. The Committee recommended the definition of Newcastle disease should be as follows:

- "Newcastle disease" is defined as an infection of poultry caused by a virus of avian paramyxovirus serotype 1 (APMV-1) which has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater.

- As an alternative to the ICPI test, the presence of "Newcastle disease" virus can also be confirmed by the demonstration (either directly or by deduction) of multiple basic amino acids [at least three arginine or lysine residues between residues 113 and 116*] at the C-terminus of the F2 protein and phenylalanine [F] at residue 117, which is the N-terminus of the F1 protein. Failure to demonstrate the presence of multiple basic amino acids or F at 117 would require characterisation of the isolated virus in an ICPI test.

* numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113-116 corresponds to residues -4 to -1 from the cleavage site.

3. The Committee recommends that the derogation for vaccine viruses specified in paragraph 3 Article 5 of Directive 92/66/EEC should be removed.

In contrast the following definition for discussion had been drafted by the OIE Standards Committee in "Appendix to Report of OIE Standards Commission, September 1998.":

Proposed OIE definition of Newcastle disease

The Standards Commission proposes to amend the *Manual of Standards for Diagnostic Tests and Vaccines* chapter 2.1.15 on Newcastle disease with the addition of the following definition:

Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) which has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 1.2 or greater, or which has both an ICPI of 0.7 or greater and multiple basic amino acids at the cleavage site of the F protein as defined below.

Newcastle disease can also be confirmed by the demonstration in the virus (either directly or by deduction) of multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues –4 to –1 from the cleavage site."

The merits of the two definitions were discussed. The meeting welcomed the continued use of infection rather than disease in the EU definition and the move to infection from disease in the OIE proposed definition, in view of the well known infection and excretion of virulent NDV strains by some bird species or poultry with antibodies without evidence of clinical signs. The general consensus was that the addition of an option of using a nucleotide sequencing alternative to *in vivo* tests was applauded. However, the Meeting indicated its preference for the SCAHAW definition. Those present either saw no reason to increase the ICPI value from 0.7 [which had been the definition in Directive 92/66/EEC] to 1.2 or felt that this was of relatively little consequence in view of the very few naturally occurring viruses with ICPI values between 0.7 and 1.2. The majority of those present also considered it preferable to keep "poultry" in the definition rather than extend it to all birds.

EDITOR'S NOTE

At the 67th General Session of the OIE held in Paris in May 1999 Resolution No. XIII was to amend the Manual of Standards for Diagnostic Tests and Vaccines, Chapter 2.1.15 on Newcastle disease so that the definition of ND was:

> ***Newcastle disease is defined is an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:***
>
> ***a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater.***
>
> ***or***
>
> ***b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test.***
>
> ***In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues –4 to –1 from the cleavage site."***

This resolution was adopted with 12 votes against [Argentina, Australia, Brazil, Canada, Chile, Colombia, Costa Rica, El Salvador, Guatemala, Saudi Arabia, South Africa and the United Arab Emirates] and three abstentions [Cuba, France and the Philippines].

- ***discussion of recommendations made for influenza diagnostic testing in National Laboratories [see 4th Proceedings p112].***

These specific points and more a general discussion was undertaken. The representative of the National laboratories agreed to review the antigens and antisera used for influenza diagnosis.

- ***use of reference sera and monoclonal antibodies in National Laboratories***

This discussion mainly covered the relatively poor showing of some laboratories in the comparative tests for virus identification [see these proceedings]. It was re-emphasised that each National Laboratory should have the capacity to fully

identify influenza subtypes and it was also considered reasonable that laboratories should be able to identify at least APMV-2 and APMV-3 and distinguish the pigeon variant APMV-1 from other APMV-1 viruses. Reference sera and monoclonal antibodies to help in this were available from the Community Reference Laboratory.

- ***control measures for outbreaks of ND and AI in backyard flocks and racing pigeons***

Although not strictly the business of the National Laboratories the current situation whereby large sectors of the poultry industry in any country may be badly affected and imports into third countries banned as a result of ND or AI in just one or two backyard chickens or pigeons was discussed. Several of those present felt that lesser restrictions than those in the Directives should be applied to backyard chickens and there should be a lesser level of notification. There was a consensus that this required further debate and that perhaps the Directives needed re-considering. In view of the proposed OIE review of this topic, at least for ND, in 1999 it was felt that perhaps any review of Directive should occur after this OIE review.

- ***achievement of standardised tests for Newcastle disease and avian influenza throughout the EU***

The representatives of the Commission stated that standardisation should already exist. The methods to be used are define in directive 92/40/EEC [for avian influenza] and 92/66/EEC [for Newcastle disease] these are legally binding.

- ***role of the Community Reference Laboratories in 1999***

The work of the Community Reference Laboratory was discussed the general consensus was that the representatives were happy with the level of help and support given in the past. The 1999 proposed work programme of the CRL was presented to the meeting and approved with one or two minor modification. The agreed work programmes for the two CRLs are annexed to these proceedings.

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WORK PROGRAMME FOR THE COMMUNITY REFERENCE LABORATORY FOR NEWCASTLE DISEASE, 1999

CONTRACTUAL DUTIES

The functions and duties are specified in Annex V of Council Directive 92/66/EEC (Official Journal of the European Communities No L 260 of 5.9.1992).

OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 1999

Collecting and editing of material for a report covering the annual meeting of National Newcastle Disease Laboratories held in Vienna, November 1998.

Characterising viruses submitted to the Laboratory by Member States and third countries listed in Commission Decision 95/233/EC (Official Journal of the European Communities N° L 156, p. 76) as amended by Decision 96/619/EC (OJ N° L 276, p. 18). This will, at the request of the European Commission or the submitting National Laboratory or at the discretion of the Reference Laboratory, include:

2.2.1 Determining the intracerebral pathogenicity index (ICPI)

2.2.2 Determining the basic amino acid composition adjacent to the cleavage site of the F0 protein in the virus and phylogenetic analysis

2.2.3 antigenic grouping of viruses

Maintain a virus repository and stocks of reagents necessary for virus characterisation. Distribute viruses held in the repository and limited amounts of reagents to national laboratories on request.

Prepare and distribute antisera, antigens and reagents for the inter-laboratory comparison tests.

Analysis of results submitted by National Laboratories for the inter-laboratory comparison test.

Conduct work to evaluate reported problem areas in diagnosis.

Prepare programme and working documents for the Annual Meeting of National Newcastle Disease Laboratories to be held in 1999.

Preparation and publications of articles and reports associated with above work.

WORK PROGRAMME FOR THE COMMUNITY REFERENCE LABORATORY FOR AVIAN INFLUENZA, 1999

1. CONTRACTUAL DUTIES

The functions and duties are specified in Annex V of Council Directive 92/40/EC (Official Journal of the European Communities N° L 167 of 22.6.1992).

2. OBJECTIVES FOR THE PERIOD JANUARY - DECEMBER 1999

2.1 Collecting and editing of material for a report covering the annual meeting of National Avian Influenza Laboratories held in Vienna, November 1998.

2.2 Characterising viruses submitted to the Laboratory by Member States and third countries listed in Commission Decision 95/233/EC (Official Journal of the European Communities N° L 156, p. 76) as amended by Decision 96/619/EC (OJ N° L 276, p. 18). This will include:

2.2.1. determining the intravenous pathogenicity index (IVPI)

2.2.2. antigenic typing of viruses and both haemagglutinin and neuraminidase subtypes

2.2.3. determining the amino acid sequence at the haemagglutinin cleavage site of H5 and H7 subtype viruses

2.2.4. limited phylogenetic analysis to assist in epidemiological investigations.

2.3 Maintain a virus repository and stocks of reagents necessary for virus characterisation. Distribute viruses held in the repository and limited amounts of reagents to national laboratories on request

2.4 Prepare and distribute antisera, antigens and reagents for the inter-laboratory comparison tests.

2.5 Analysis of results submitted by National Laboratories for the inter-laboratory comparison tests.

2.6 Conduct work to evaluate reported problem areas in diagnosis.

2.7 Prepare programme and working documents for the Annual Meeting of National Avian Influenza Laboratories to be held in 1999.

2.8 Preparation and publications of articles and reports associated with above work.