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

HELD IN BRUSSELS, 18-19TH OCTOBER 1994

Edited by Dennis J. Alexander

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JOINT ANNUAL MEETINGS OF EUROPEAN UNION NATIONAL NEWCASTLE DISEASE AND AVIAN INFLUENZA LABORATORIES 1994

PROGRAMME

Tuesday 18th October 1994

10.00: Welcome and introduction.

10.15: SESSION I **COUNTRY REPORTS - AVIAN INFLUENZA**

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

SESSION II **COUNTRY REPORTS - NEWCASTLE DISEASE**

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

12.45: LUNCH

14.00: SESSION II continued

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

15.15: SHORT BREAK

15.30: SESSION III **EC REPORTS**

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

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

16.30: General Discussion Of Current Situation In European Union Countries

Wednesday 19th October 1994

10.00: SESSION IV - **DISCUSSION OF PROBLEMS FACING NATIONAL LABORATORIES.**

This session will consist of an open discussion of the facilities, including equipment and reagents, available, currently and ideally, to the National Laboratories and the effect these have on their ability to carry out diagnosis of the two diseases effectively and safely within the demands of the Directives.

10.45: SESSION V - ORIGINAL CONTRIBUTIONS

10.45: *Jan Cees van den Wijngaard.* Some epidemiological aspects of Newcastle disease.

11.15: SHORT BREAK

11.30: *Gy. Czifra, B. Engström, D. Alexander and R. Manvell* Detection of PMV-1 specific antibodies with a monoclonal *antibody* blocking enzyme-linked immunosorbent assay.

12.00: *J.P. Picault, Michèle Guittet, Josiane Lamande, H. Le Coq, Isabelle Pierre and Chantal Allee.* Quality control comparisons in French laboratories on the Newcastle disease HI test and the influenza type A AGP test

12.30: LUNCH

13.45: *Angela Oberdörfer and Ortrud Werner:* Characterisation of the cleavage site of NDV fusion protein of some strains isolated from chickens and pigeons in 1993 and 1994- method and preliminary results.

14.15: *Michèle Guittet.* Safety and efficacy of Newcastle disease vaccines in game birds

14.45 SESSION VI - CONCLUSIONS AND RECOMMENDATIONS

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

15.30: CLOSING REMARKS

15.45: CLOSE

SESSION I - COUNTRY REPORTS: AVIAN INFLUENZA

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

Belgium/Luxembourg
Denmark
Ireland
Germany
Sweden
Finland
Austria
Norway

There were no participants from **Greece** or **Portugal** but representatives of the National Laboratories had reported the absence of influenza outbreaks in these countries.

AVIAN INFLUENZA - CURRENT SITUATION IN SPAIN

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Definition

The definition of avian influenza [AI] in Spain follows that presented in EC Directive 92/40/EEC for introducing Community measures for controlling this disease. The Spanish Government has adopted the Directive into the National Legislation through a Royal Decree of 25.08.93 [Offical State Bulletin 07.10.93]. this Act is a reflection of the EC Directive and can be considered its implementation at a National level in Spain.

Diagnostic methods

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

Epidemiological situation

AI has never been diagnosed in Spain and no suspect outbreaks have been reported in the last six years. A paper published in 1994 [1] based on a 1988 serological survey by the University of Cordoba on AI in wild waterfowl on the Doñana National Reserve, Andalucia (southern Spain), reported that of 712 birds examined 44 (6.2%) had antibodies to AI. An indirect ELISA using a peroxidase-labelled anti-chicken conjugate was used to detect antibodies. Positive birds were found in 10 of the 13 species sampled. This study suggests that AI may circulate in the wildfowl population of the Doñana National Reserve.

Vaccination

Vaccination against influenza is prohibited in Spain and no vaccine is licensed.

Laboratories

Confirmatory serological identification of virus isolates and pathogenicity index tests are done at the National Reference Laboratory.

Other laboratories are located in several Autonomous Communities. These laboratories investigate disease outbreaks in flocks and send specimens for virological examination to the National Reference Laboratory. The initial diagnosis

should be made at the Regional Diagnostic Laboratory based on clinical and pathological signs, virus isolation in eggs and preliminary differentiation using HI tests. Subsequent diagnosis will include isolation of a haemagglutination by inoculation of embryonated egs, HI tests for H5 and H7 viruses and immunodouble diffusion tests for type A influenza.

The National Laboratory requires training of one or two workers to improve and standardise the methods employed in the laboratory. This training could be done at the Community Reference Laboratory.

Facilities

A number of precautions are taken to maintain the laboratories free from extraneous micro-organisms. The buildings are air tight and all incoming air is passed through an absolute filter. Employees are required to shower before entering the clean area.

Analyses of samples are done under conditions ranging from conventional to germfree isolators. There is one building for multipurpose animal experiments in which different species can be housed. It consists of five boxes and two rooms for a total of two isolators. This building is contected to the Virology Department through a SAS. All waste materials and sewage are sterilized.

The Virology Department has strict regulations aimed at preventing the escape of infectious agents. The isolation area is cut off from the outside by means of a closed structure which ensures internal negative pressure and all air leaving the building passes through an absolute filter. The National Reference Laboratory is particularly well equiped for dealing with AI isolates and samples. The facilities for handling such infectious micro-organisms were specially built with laminated epoxy resin surfaces, particularly in animal stalls, and special gas-tight constructions for windows and door. All concrete surfaces are resistant to chemicals and easy to clean and disinfect. Gas tight doors are made tight by means of lever-operated compressible seals and are constructed from stainless steel.

Reference

1. Astora et al (1994) Avian Pathology 24, 339-344.

REPORT OF DISEASE INCIDENCE OF AVIAN INFLUENZA IN THE NETHERLANDS IN 1994.

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Avian influenza has never occurred in the Netherlands, and in 1994 Dutch poultry continued to remain free of this disease. Although two influenza viruses were isolated, both were of low (potential) virulence as concluded from laboratory tests and therefore did not meet the criteria stipulated in the EU directive 92/40/EEC.

Influenza virus isolated from emus and cassowaries

In April an incident occurred with emus and cassowaries that were exported from the Netherlands to the United States in February. In the United States, an influenza virus was recovered from routine cloacal swabbing that was performed during the quarantine period. We later learned that the isolated influenza virus was typed as a low-pathogenic H5N9 subtype at the National Service Laboraories in Ames, Iowa. The animals were sent back to the Netherlands at the end of February, transported to the farm of origin and kept in quarantine at that farm. Blood samples were drawn from all animals. Antibodies directed against H5 and H7 subtypes were detected in sera of several animals that were re-exported form the Unites States and in 8/138 animals that had remained at the farm. Swabs were taken independently on the same day by the Regional Health Service and by the State Veterinary Services. The latter swabs were sent to the Institute of Animal Health (ID-DLO). No virus was isolated from these swabs, but an influenza H5 virus was isolated from swabs of one animal after 3 passages in embryonated eggs at the regional Health Service for Poultry. This virus isolate was sent to the Institute for Animal Health to determine the IVPI of the virus. The virus was passed in embryonated eggs and the subtype of the virus was confirmed; 8 HAU of virus were completely inhibited by H5-specific antiserum. Ten 6-week-old SPF chickens were injected intravenously with more than 8 HAU of virus and observed daily for 10 days. Three days after inoculation, all chickens were dead or very sick; the IVPI value was 2.07. Although two emus had died in the middle of April, one with a pneumonia and the other with gastritis and intestinal disorders, none of the other emus showed clinical signs. Nevertheless the animals were immediately killed on obtaining the IVPI test result. As a routine control, virus isolation from brain, trachea, lung and spleen tissue of the injected SPF-chickens was performed. A velogenic NDV and no influenza virus was recovered from these tissues. The isolated NDV virus had an ICPI value of 1.86. We next passaged the sample that was used for the inoculation of the chickens, once again on embryonated eggs. The HA activity of

the allantoic fluid from these eggs was completely inhibited with anti-H5 serum. NDV could be isolated from this sample only after neutralising the influenza virus before inoculation of embryonated eggs.

The influenza virus isolated from emus was immediately send to the European Reference Laboratory, Weybridge after confirmation of its identity by the ID-DLO. The Reference Laboratory confirmed the H5 subtype and they also found the IVPI to be 2.03. During the IVPI test at the Reference Laboratory some of the chickens showed nervous signs which is atypical for avian influenza. The amino acid sequence around the cleavage site of the haemagglutinin was typical for an avirulent H5 virus; no basic arginine residue pairs were detected. At this point the Reference Labotory was warned by us of the contamination with NDV. Virus was then re-isolated from the chickens and identified as a NDV with an IVPI value of 2.7.

Since the sample containing the influenza virus was sent in the same box as samples containing NDV, the velogenic NDV is most likely a laboratory contamination that occurred during transport or directly thereafter. Remarkably, the low level of NDV contamination of the influenza virus remained undetected in the HAI test with specific sera even after 3 passages in embryonated eggs, whereas, in contrast, after intravenous injection of chickens NDV and not influenza virus was recovered. Thus virus isolation should be routinely performed on organ suspensions of chickens that died in IVPI tests to make sure that chickens became sick or died because of the virus detected in the inoculum and not because of undetected contaminants.

Influenza virus isolated from parakeets.

In July an influenza virus was isolated from parakeets that originated from an aviary with high mortality. The influenza virus was of the H7N1 subtype. The IVPI which was determined in 6-week-old chickens was 0.0. The amino acid sequence around the haemagglutinin cleavage site, which was determined at the EU Reference Laboratory, did not contain pairs of basic amino acids; the sequence was -PEIPKGR*GLF-. The cause of death of the parakeets in the aviary was not elucidated.

AVIAN INFLUENZA : SITUATION IN FRANCE FROM OCTOBER 1993 TO SEPTEMBER 1994

Michèle Guittet

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No suspected outbreaks were reported from October 1993 to September 1994.

In January 1994 antibodies against subtype H5 were demonstrated in sera from a breeder flock of turkeys at the end of the production. The clincal signs were a significant drop in egg production. For economical reasons, the flock was slaughtered before the exact cause was determined. Later, in sera which had been collected earlier for the detection of mycoplasma antibodies, some Influenza AGP positive reactions were observed and confirmed by haemagglutination inhibition tests. Consequently, a serological survey was carried out in the neighbourhood of this turkey breeder flock which has not revealed any positive reactions.

THE EPIDEMIOLOGICAL SITUATION OF AVIAN INFLUENZA IN ITALY IN 1994

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The National Reference Centre for the study of Avian Influenza Viruses set up by the Ministry of Health at the Bird and Rabbit Experimental Centre in Varcaturo, associated to the Avian Pathology Section of the Department of Pathology, Disease Prevention and Foodstuff Inspection at the University of Naples "Federico II", has developed and conducted its own control activity through the epidemiological surveillance of laying hens intensively reared in the Campania region. In particular attempted isolations of avian influenza viruses were carried out on the farms where there were egg production problems.

We have conducted a study on three farms obtaining as samples, cloacal swabs from laying hens in two poultry houses per farm. We sampled 40 laying hens per poultry house in every farm for a surveillance period of six months (Table 1). In the fourth months of control we isolated a strain of avian influenza A subtype H9N2 from only one farm, but in both poultry houses sampled. Intravenous pathogenicity index (IVPI) tests showed that the virus was slightly pathogenic for chickens (Table 2), results of serotyping and determination of pathogenicity were confirmed by European Communities Reference Laboratories for Avian Influenza and Newcastle Disease, Weybridge (U.K.). The sera taken at the 19th week of age were assayed in haemagglutination inhibition tests with the enzootic influenza virus strains (H6N2, H5N2 and H9N2) but showed no immunological response.

In association with the influenza virus (H9N2), we also isolated two PMV-1 viruses which belonged to the C1 group with high IVPIs as assessed by the European Communities Reference Laboratories, Weybridge. The only clinical problem seen in the infected hens was a slight egg drop throughout the laying period.

In Italy we have to report, also, the isolation of influenza virus from a meat turkey flock in the Veneto region. The virus was isolated by "Ististuto Zooprofilattico di Brescia, Sezione di Forlì", but we do not know the subtype or the pathogenicity of the isolate. We know that the outbreak was marked by high mortality, severe respiratory signs and typical anatomorphatological findings of avian influenza. Unfortuantely, the virus has not been sent to the National Reference Laboratory and no further information is available.

The virus isolation methods used in SPF embryonated eggs were those suggested in the Report and Recommendations of the Scientific Group on Contagious Diseases of Poultry (Avian Orthomyxoviruses) published by the EEC in 1986, as were the techniques for the haemagglutination and haemagglutination inhibition tests and the intravenous and intracerebral pathogenicity index tests.
In Italy the use of inactived vaccine for Avian Influenza is not officially allowed, but a lot of turkey farms are vaccinating the breeder flocks with an inactivated vaccine containing the enzootic H6N2 and H9N2 type A influenza viruses.
No influenza viruses were isolated during the survey of imported exotic birds in quarantine in Campania region of Italy, but the number of these birds admitted was very low.

References

1. Fioretti A., Menna L.F., Calabria M., Conzo G., Papparella V.: Isolamento di Ortomyxo- e Paramyxovirus aviari da galline ovaiole con problemi di calo d'ovodeposizione. XXXIII Convegno Nazionale Società Italiana Patologia Aviare, Forlì, Ottobre 1994, in press.
2. Massi P.: Attualità nelle malattie respiratorie del pollame. XXXIII Congresso Nazionale Società Italiana Patologia Aviare, Forlì, Ottobre 1994, in press.
3. Massi P.: Current situation of avian pathology in Emilia Romagna Region in Italy during 1994. Personal comunication XXXIII Congresso Nazionale Società Italiana Patologia Aviare, Forlì, Ottobre 1994, in press.

AVIAN INFLUENZA: SITUATION IN GREAT BRITAIN OCTOBER 1993 TO SEPTEMBER 1994

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Avian influenza investigations

The incidence of avian influenza (AI) virus infections of turkeys and chickens in Great Britain remains extremely low and no infections of these birds was investigated during the relevant period. The only investigation of domestic poultry was of commercial ducks in the county of Norfolk (Table 1).

Table 1. Avian influenza: Influenza investigations during 1992-1994

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

[a] to 30th Septemberr 1994

[b]relates to outbreak occurring at the end of December 1991

Isolations of avian influenza viruses from domestic poultry

The single investigation of commercial ducks in 1994 resulted in the isolation of a virus of H4N2 subtype with an intavenous pathogenicity index in six-week-old chickens of 0.00. This is only the second isolate from commercial poultry in Great Britain since the highly pathogenic virus associated with the outbreak in turkeys in 1991 and only the sixth isolate from domestic poultry in the 5 year period since 1990 (Table 2). It is worth noting that 5 of these 6 outbreaks occurred in birds reared in the county of Norfolk which, in addition to including within its boundaries the major area for rearing commercial ducks, covers an area representing an important "stop over" on a major migratory waterfowl route.

Table 2. Isolations of avian influenza viruses from domestic poultry 1990-1994

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

Influenza isolations from other birds

During the year two influenza isolates were obtained from birds other than domestic poultry.

The first was obtained from a sun conure (*Aratinga soltitialis*) during an investigation of mortalities amongst psittacine species at a caged bird suppliers. The virus proved to have an IVPI of 0.00, but since it was of H7N1 subtype nucleotide sequencing was done to determine the deduced amino acid sequence at the cleavage site of the haemagglutinin molecule. The sequence obtained, **...PEIPKGR*GLF...**, meant the virus did not fall within the EU definition of highly pathogenic avian influenza [1].

The second virus was obtained from a duck found dead at a wildfowl trust sanctuary in Norfolk and was of H6N2 subtype.

The two virus isolates during the year are typical of the incidence of isolations of influenza viruses from captive exotic birds and feral waterfowl in Great Britain as a result of routine diagnosis. Over the past 5 years only 5 viruses have been obtained from this source (Table 3).

Table 3. Influenza isolations from other birds 1990-1994.

Date	County	Virus	Subtype	IVPI
1991				
05/91	Yorkshire	pheasant/England/939/91	H2N9	0.00
10/91	Shropshire	turaco/England/2004/91	H5N2	0.00
1992				
03/92	Hampshire	teal/England/413/92	H11N9	0.00
1994				
07/94	Staffordshire	sun conure/England/766/94	H7N1	0.00
08/94	Norfolk	duck/England/935/94	H4N6	0.42

Isolations of influenza viruses from birds in quarantine.

The number of isolations of influenza viruses from birds in quarantine has fluctuated enormously since 1976 when quarantine was first imposed [2]. No influenza viruses were obtained from this source during the period under report.

References

1. Council Directive 92/40/EEC introducing Community measures for the control of avian influenza. Official Journal of the European Communities No. L 167 1-16.
2. Alexander, D.J. (1994). Avian influenza: Current situation in Great Britain. Proceedings of the Joint First Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of the European Communities. Brussels, 1993. Commission E.C.:Brussels. pp 22-25.

SESSION II - COUNTRY REPORTS: NEWCASTLE DISEASE

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

Ireland
Austria
Finland

There was no participant from **Greece** but a representative of the National Laboratory had reported the absence of any ND outbreak.

NEWCASTLE DISEASE: SITUATION IN GREAT BRITAIN DURING 1994

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Investigations of Newcastle disease in poultry during 1994.

During 1994 a total of 22 suspected cases of Newcastle disease (ND) was investigated in poultry. Twenty-one of these cases were in chickens and the other in pheasants. A large majority of the investigations in broiler chickens was as a result of respiratory disease with increased mortality towards the end of the rearing period. Viruses were isolated from the investigation of the pheasants and from 10 of the broiler investigations (Table 1)

Table 1. Newcastle disease investigations in poultry during 1992-1994.

	1992	1993	1994
Cases investigated	17	16	22
NDV isolated	1	4	11
Confirmed cases	0	0	0

All viruses isolated in 1994 were subjected to intracerebral pathogenicity index (ICPI) tests and antigenic characterisation using a panel of 28 monoclonal antibodies (mAbs) [1], the results obtained are shown in Table 2. The 10 viruses from chickens had ICPI values over the range 0.00 to 0.21, showed a "group E" mAb binding pattern and were not inhibited in haemagglutination inhibition tests by mAb 7D4. These results are consistent with ND vaccine strain Hitchner B1. Most of the broiler flocks investigated had not been vaccinated and it was assumed that spread of Hitchner B1 vaccine to such birds had exacerbated an underlying condition such as infectious bronchitis or avian pneumovirus infection. Possibly the more widespread presence of these other viruses, rather than greater spread of the vaccinal virus, accounts for the increase in isolations during 1994

The isolate obtained from pheasants had an ICPI of 0.20 and showed a "group G" mAb binding pattern. This is typical of viruses, similar to Ulster 2C, which have been associated with infections of feral birds and it is assumed that these are the source of the virus.

Table 2. Characterisation of ND viruses isolated from poultry in Great Britain during 1994

Reference	Type of bird	mAb binding	ICPI	Conclusion
28/94	broilers [48 d.o.]	**E** 7D4 -ve	0.10	B1 vaccine
33/94	broilers	**E** 7D4 -ve	0.21	B1 vaccine
119/94	broilers	**E** 7D4 -ve	0.10	B1 vaccine
290/94	broilers [35 d.o.]	**E** 7D4 -ve	0.06	B1 vaccine
484/94	broilers	**E**.7D4 -ve	0.00	B1 vaccine
520/94	broilers [52 d.o.]	**E** 7D4 -ve	0.00	B1 vaccine
542/94	broilers	**E** 7D4 -ve	0.06	B1 vaccine
706/94	broilers [44 d.o.]	**E** 7D4 -ve	0.16	B1 vaccine
875/94	broilers	**E** 7D4 -ve	0.21	B1 vaccine
954/94	chickens	**G**	0.20	Ulster 2C-like
1096/94	broilers	**E** 7D4 -ve	0.17	B1 vaccine

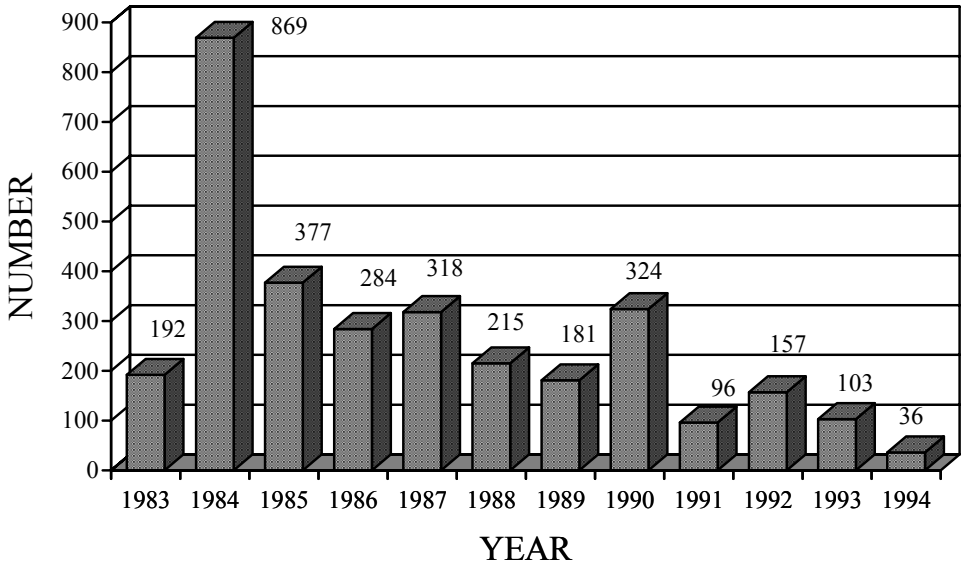
ND in racing pigeons

Infections of racing pigeons with the variant PPMV-1 virus may be confirmed in Great Britain by clinical signs alone, serology, virus isolation or a combination of these. The numbers of confirmed cases each year since the disease arrived in Great Britain in 1983 are shown in Figure 1. The total of 36 confirmed outbreaks recorded in 1994 represents the lowest in the 12 year history of the disease. In past years the majority of outbreaks have been usually confirmed in the last quarter of the year, i.e. after the end of the racing season in October [2, 3], but in 1994 only 4 of the 36 outbreaks were confirmed during October to December. It is hoped that this apparent decline in the disease is the result of enforcement of certificated vaccination of pigeons taking part in races or shows.

ND in other birds

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

Figure 1: Confirmed cases of PPMV-1 in racing pigeons 1983-1994



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2. Alexander, D.J., Parsons, G., Manvell, R.J. & Sayers, A.R. (1994). Characterisation of avian paramyxovirus type 1 infections of racing pigeons in Great Britain during 1983 to 1990. Proceedings of the European Commission meeting on Virus Diseases of Poultry - New and Evolving Pathogens, Brussels, 1992 pp 65-75.
3. Alexander, D.J. (1994). Newcastle disease: Current situation in Great Britain. Proceedings of the Joint First Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of the European Communities, Brussels 1993. pp 27-29.

NEWCASTLE DISEASE - CURRENT SITUATION IN DENMARK, 1993/94.

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Definition.

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

Diagnostic methods.

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

Epidemiological situation.

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

A total of 111 samples from poultry, submitted for routine diagnostic investigation, were inoculated in the allantoic cavity of embryonated SPF eggs in 1993. The corresponding figure for the first 9 months of 1994 is 92 samples.

In 1993 pigeon paramyxovirus (PPMV-1) was isolated from 6 non-commercial pigeon flocks with suspicion of PPMV-1 infection. The identity of the 2 of the isolates was confirmed by the EU Reference Laboratory. The remaining 4 isolates have been identified in the national reference laboratory by use of monoclonal antibodies (U86 and 161/617) supplied by the EU Reference Laboratory. No PPMV-1 has been isolated since October 1993.

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

Besides the inoculation of specimens into embryonated eggs, the national surveillance for ND in domestic fowl is based on serological monitoring of grand parent stock.

In 1993 a total of 5831 sera from fowl were tested with negative result. During the first 9 months of 1994 approximately 4700 serum samples from fowl were investigated. All routine samples were negative, while 105 samples from a flock of illegally imported pullets were positive. Investigations revealed, that the flock had been vaccinated in the country of origin. No PMV could be isolated from the flock. A report on this matter has been presented to the Commission.

REPORT OF THE NATIONAL NEWCASTLE DISEASE SITUATION IN SPAIN

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ND outbreaks

In 1993 two outbreaks of Newcastle disease (ND) were reported:

1. The first, communicated on 24th November 1993 occurred in the Vasc Country at Bergara (Gipuzcoa) in a flock of 7,100 chickens. At the time of reporting 1,100 had died and 3,800 were sick. The 6,000 surviving birds were slaughtered in accordance with the stamping out policy.

2. The second outbreak reported on 29th November 1993 was in Aragòn (Ejea de los Caballeros - Zaragoza). In this case 125,000 birds were slaughtered.

Two isolates were sent to the EU Reference Laboratory, U.K. on 28th April 1994:

Isolate 103 from outbreak 1; and Isolate 98 from Aragòn. In May 1994 the EU Reference Laboratory reported that using monoclonal antibodies 103 was indistinguishable from the viruses causing outbreaks in Northern EU countries and had an IVPI of 2.61 and an ICPI of 1.88. While 98 appeared to be a mixture of vaccine virus and a virulent virus with IVPI 2.75 and ICPI 1.89.

ND surveillance

In 1994 two papers were published on serogical surveillance of feral birds in southern Spain (Andalucia, Coto Doñana) [1,2]. Positive serology for ND virus was detected in some waterfowl and PMV-2 and PMV-3 antibodies were also detected.

References

1. Astora et al (1994) Avian Pathology 24, 339-344.
2. Maldonado et al (1994) Avian Pathology 23, 145-152.

NEWCASTLE DISEASE IN ITALY: 1994 SITUATION

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The previous epidemiological situation, vaccination strategies and official regulations concerning Newcastle disease (ND), have been reported [1, 2].

In 1994 outbreaks of disease caused by velogenic PMV-1 strains and by pigeon PMV-1 (PPMV-1) strains were diagnosed.

Several isolates of PMV-1 virus from different regions of Italy were submitted to the Department of Virology of Istituto Zooprofilattico Sperimentale delle Venezie, which acts unofficially as a national reference laboratory, for diagnosis or confirmation. The final typing and pathogenicity indices of these viruses were determined by the European Community Reference Laboratory at Weybridge.

In Table 1 data concerning outbreaks caused by velogenic and pigeon PMV-1 strains are summarised.

In Figure 1 the geographical distribution of velogenic and pigeon PMV-1 outbreaks is reported.

Commercial flocks were not affected, perhaps because of voluntary prophylactic vaccination.

The origin of the 1994 epidemic was not determined, its spread was caused by movements of animals.

The Veterinary Services of Ministry of Health adopted all the measures provided for by the Veterinary Police Regulation to eradicate Newcastle disease: establishment of protection zone and surveillance zone, prohibition of fairs, slaughter of all the animals in infected holdings and disposal off of the carcasses and litter, disinfection, reinforced vaccinations etc.

We hope that as soon as possible the Italian Ministry of Health will officially acknowledge the 92/66/EEC Newcastle Disease control directive.

References

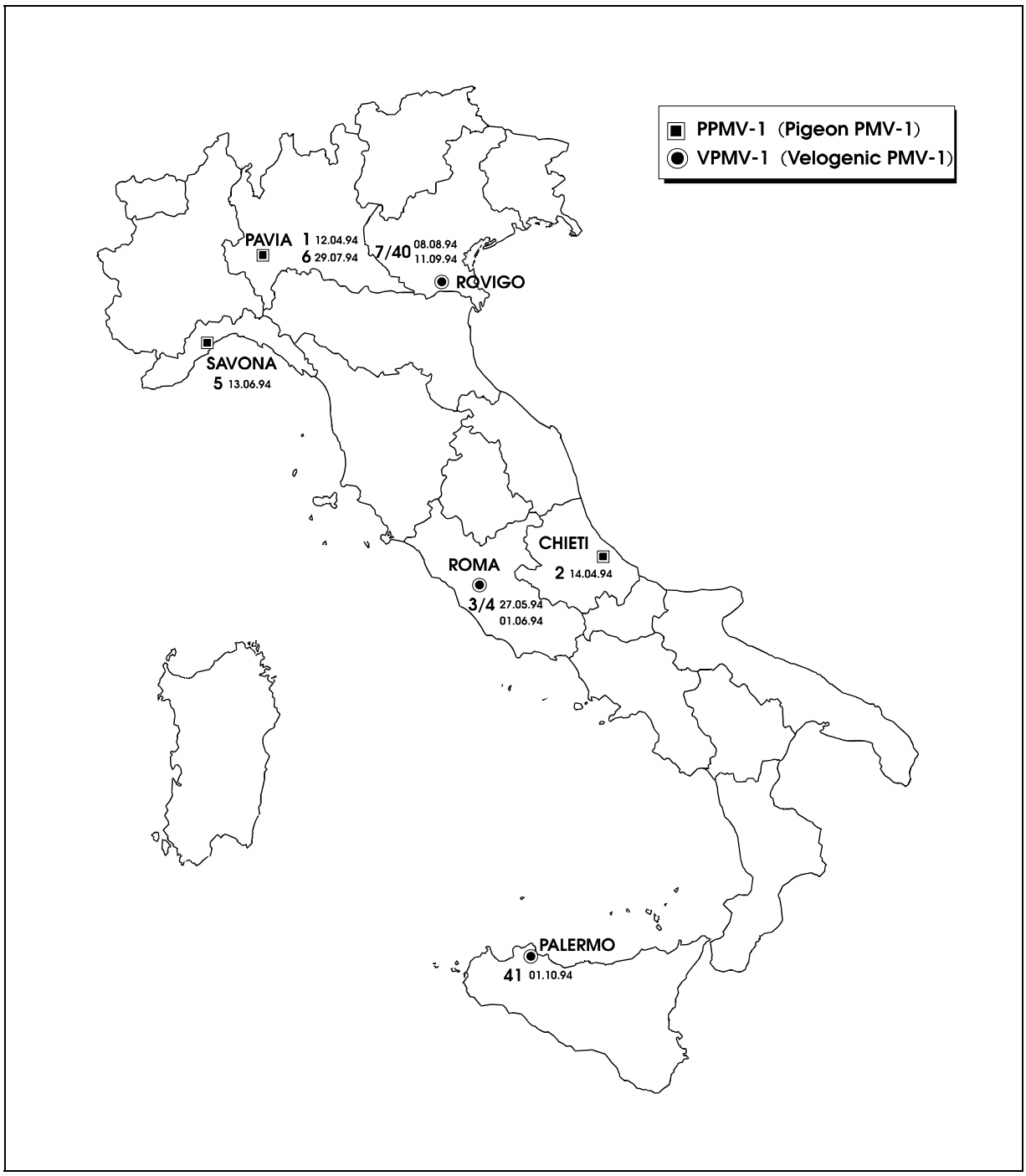
1. D'Aprile P.N. (1992) *In:* Proceedings of the Workshop on Avian Paramyxoviruses, Rauischholzhausen, Germany, July 1992 pp 35-37.
2. D'Aprile P.N. (1993) *In:* Proceedings of the Joint First Annual Meetings of the National Newcastle Disease and Avian Influenza Laboratories of the European Communities, Brussels, October 1993 pp 39-40.

Table 1 - Viruses isolated January - August 1994

Case No.	Date	Region, Province	Type of flock	Virus isolated	Species	No. of Birds	ICPI✦	IVPI✦	mAb Group
1	12.04.94	Lombardy Pavia (N)	backyard	2 x PPMV-1*	Pigeons, chickens	68	1.33 1.33	0.26 0.34	P P
2	14.04.94	Abruzzo Chieti (C)	backyard	PPMV-1	Pheasants	3	1.35	2.54	P
3	27.05.94	Latium Roma (C)	backyard	Velogenic PMV-1	Chickens, ducks, mallards, stock-doves, turkeys, psittacines, guinea fowl	37	1.89	ND	C1
4	01.06.94	Latium, Roma (C)	backyard	Velogenic PMV-1	Chickens, pigeons, ducks, quails	73	1.99	ND	C1
5	13.06.94	Liguria, Savona (NW)	hobby	PPMV-1	Pigeons , turtle-doves, chickens	19	1.14	0.00	P
6	29.07.94	Lombardy Pavia (N)	backyard	PPMV-1	Pigeons, chickens	180	1.21	□0.00	P
7	08.08.94	Veneto Rovigo (NE)	backyard	Velogenic PMV-1	Chickens, pheasants	75	1.75	2.06	C1
8 40	08.08.94 01.09.94	Veneto Rovigo (NE)	backyard	Velogenic PMV-1	Chickens	1148 in total	Secondary outbreaks originated from animals sold in a local market		
41	01.10.94	Sicily Palermo (S)	backyard	Velogenic PMV-1	Chickens	30	To be determined		

* pigeon PMV-1 ⅄intracerebral pathogenicity index ✦intravenous pathogenicity index
N = North; NW = North West; NE = North East; C = Centre; S = South

Figure 1 - Geographical distribution of PPMV-1 and VPMV-1 strains in 1994.



CURRENT NEWCASTLE DISEASE SITUATION IN SWEDEN

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Newcastle disease

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

Serological surveys

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

Vaccination

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

Results from the quarantine control

All imported birds are still kept in quarantine. During the last year we have detected antibodies against NDV on two occasions.

Ostriches are today frequently imported to Sweden from Belgium and the Netherlands. In quarantine all are sampled individually for serological examination by HI-test for both ND and avian influenza. With the HI test we usually detected low NDV-antibody titres in ostrich sera last year. In November 1993, 50 emus were imported from Belgium. After heat inactivation and haemabsorbtion, samples from 10 of the birds were still positive with titres of $>2^3$ with 4 HAU. With our monoclonal blocking ELISA all birds were positive. The birds were re-exported as the owner did not want to continue with further investigations.

After this occasion 6 batches of ostriches have been tested in quarantine. Not a single bird has had antibodies against NDV $>2^3$. Ostriches have been tested more thoroughly in the exporting countries before departure to Sweden this year.

Every year about 10 groups of day-old grand parents (GP) are imported into Sweden. In December 1993 a quarantine with GP of a European layer hybrid was tested at 8 weeks of age; 23 out of 60 samples were positive in the HI test, but after heat inactivation only one sample was still positive with a titre of 2^8.

When retested in January, at 13 weeks of age, 92 out of 100 samples were positive before and 13 out of 30 after heat inactivation. An increasing number of birds were also positive for IB. We did not manage to isolate NDV only IBV from the birds, but all chickens were killed in the quarantine.

According to the exporting company, vaccines were not used the same day at the hatchery, but NDV and IBV-vaccines are, of course, used in birds destined for most other countries. Some chicks may have picked up the virus from the hatchery facilities. The antibody response in the flock was probably delayed by the maternal antibodies derived from the dams.

Survey of migrating wild birds

Samples for virus isolation were taken from migrating birds at a research station in south of Sweden in Spring 1994., Cloacal swabs from 430 birds were collected and attempts to isolate virus were performed in embryonated hens egg. So far only one haemagglutinating agent has been isolated, from pooled samples. This year mostly passerine birds were sampled. Next Spring we will continue with water living wild birds.

NEWCASTLE DISEASE : CURRENT SITUATION IN FRANCE FROM OCTOBER 1993 TO SEPTEMBER 1994

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The epizootic from November 1992 to January 1993 in ornamental birds following the IXth Avian International Exhibition in Mulhouse, stopped without spreading to commercial flocks.

Nevertheless, the threat of the Newcastle disease to the poultry industry has led to the strengthening of vaccination regimens especially in long life birds (breeders, layers, range free broilers); although vaccination is not compulsory in France.

From October 1993 to September 1994, a total of 56 suspect cases was studied in different diagnostic laboratories in France. Ornamental fowl or cage birds were involved, and it was difficult to obtain the case history and the exact signs. Only 3 virus were isolated : one PMV-3, one PMV-4 and one PMV-1. The latter was not pathogenic, its ICPI was 0.16 (Table 1).

Table 1: Suspect cases of ND in birds in France from October 1993 to September 1994

Diagnostic Laboratory	N° of suspect cases	Species	Result
LDA22	14	Pigeon	Negative
	11	Fowl	PMV1 (1 case)
	3	Turledove	Negative
	1	Duck	PMV4
	1	Quail	Negative
	2	Ostrich	"
	1	Budgerigar	"
	1	Turkey	"
	1	Canary	"
LAV 01	2	Fowl	Negative
	2	Budgerigar	"
	1	Duck	"
	1	Pigeon	"
LAV 40	1	Partridge	Negative
	1	Parrot	"
	1	Canary	"
	5	Pigeon	"
	4	Fowl	"
	2	Lovebird	PMV3 (1 case)
	1	Budgerigar	Negative

CURRENT NEWCASTLE DISEASE SITUATION IN GERMANY

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In Germany Newcastle disease (ND) vaccination has been compulsory for chicken flocks with more than 200 birds for many years. In case of an outbreak the stamping out policy is applied.

Until 1992 only a few sporadic outbreaks of ND appeared. In 1993 the number of reported cases rose to 181. A map with the occurrence per district (Figure 1) shows that almost all Bundesländers are affected. Particularly often, although temporarily limited and therefore with epizootic features, ND occurred in the middle East of Germany (Sachsen-Anhalt, Brandenburg, Sachsen und Thüringen) as well as in the South (Baden-Württemberg and Bayern). The outbreaks occurred primarily in small and hobby flocks with less than 100 birds and only 3 outbreaks affected flocks with more than 10,000 animals.

So far in 1994, the epizootic features have been similar to those last year, although most cases were concentrated on the north-east of Germany in Schleswig-Holstein, Mecklenburg-Vorpommern, Brandenburg and Sachsen (Figure 2). To the end of August, 133 outbreaks had been reported. Of these 128 affected small flocks with less than 500 birds (Figure 3). Most of these are backyard poultry and hobby flocks and have only 10 to 50 animals. In these flocks chickens are often reared together with other species such as ornamental fowls, waterfowl, turkeys or pigeons, but in most cases the chickens are mainly affected. These flocks are not vaccinated, because, according to the existing legislation, vaccination is not compulsory for them due to their small size. They are, therefore, in constant danger.

Epidemiological investigations have shown, that the source of the disease in these backyard or hobby flocks was mostly the purchase of young hens or chicks. The disease is spread chiefly by poultry tradesmen. In Germany these tradesmen go from village to village and sell young poultry every year between March and October. Most of the outbreaks are related to this period. Although animals sold in this way are vaccinated in accordance with their certificates, they transmit the virus to the small flocks of the purchaser, infecting the non-vaccinated older birds in the flock.

Even if only in very small flocks are affected the owners demand a veterinary investigation and diagnosis for the following reasons:

1. The owner wants to claim compensation from the tradesman and/or
2. In case of occurrence of an epizootic the losses will be compensated by insurance.

For these reasons all outbreaks, without exception, are diagnosed and recorded, even if only very few birds are infected.

Unfortunately, the real origin of the infection has not yet been found. Investigation by the competent veterinary authorities of the parent flocks of the poultry tradesmen, which are mostly located in Nordrhein-Westfalen, showed that those flocks had all been vaccinated sufficiently. Neither clinical signs were found nor any other factor or illegality.

Just 5 of the 133 reported outbreaks in 1994 affected flocks with more than 500 birds (Table 1), but these were not industrial flocks. Two cases occurred in dealer flocks. These dealers buy large numbers of young chicken and sell them in small numbers in the surrounding area. These flocks are especially endangered, because of the numerous movements of birds in and out and contact with animals differing in origin, age, species and immunity.

In all cases, in large as well as in small flocks, a stamping out policy was applied by the Veterinary Services. In areas in which the disease became epizootic, compulsory vaccination of all poultry flocks, regardless of the number of birds, was imposed. The control of the disease has been supported by intensive public relations exercises, including information in leaflets, newspapers and journals. In general the vaccination of small flocks is recommended and sometimes demanded by the competent authority.

No outbreaks occurred in industrial poultry flocks. They are controlled by strict hygienic management and intensive compulsory vaccination policy. Increasingly oral vaccination via drinking water is being replaced by spray vaccination.

In the National Reference Laboratory all virus isolates from the epizootic outbreaks were characterised (Table 2). Of the 97 viruses received from chickens 90 had an ICPI of more than 1.5. Although the pathogenicity is highly homogeneous, the isolates are probably not identical. They have two different reaction patterns with monoclonal antibodies, which were developed in our laboratory. Not only are further investigations necessary but also a comparison with the results of the EU Reference Laboratory in Weybridge.

Twenty-two virus isolates from pigeons had an ICPI between 0.7 and 1.5. They had an homogeneous reaction pattern with our mAbs and are probably pigeon type viruses. In contrast 7 other pigeon virus isolates were velogenic and showed a reaction pattern typical for velogenic chicken isolates. It is possible, that pigeons were infected with chicken virus when outbreaks occurred in small mixed flocks. These birds are a special danger in the spread of the virus.

In addition, a further series of interesting isolates were obtained in Germany. As a side result of the practical work of a PhD candidate, 12 NDV were isolated from deep frozen ducks which were imported from Thailand in 1991. All these isolates had an ICPI of more than 1.5. The comparison with the field virus is still in progress.

Table 1. Outbreaks in flocks with more than 500 birds from January to August 1994

Ref. No.	Location	Number of birds	Type of bird	Type of flock	Origin of infection	ICPI of isolate
1	Glauchau (Sachsen)	3975	layer hens and ornamental fowls	dealer and hatchery	purchase of young hens	1.79
2	Nordfriesland (Schleswig-Holstein)	529	layer hens		purchase of young hens	1.89
3	Lübeck (Schleswig-Holstein)	5000	broilers		unknown	1.88
4	Werdau (Sachsen)	1656	layer hens	dealer	purchase of young hens	1.88
5	Cloppenburg (Niedersachsen)	14500	broilers		purchase of day-old chicks	1.88

Table 2. NDV isolates received by the National Reference Laboratory from January to August 1994

Number	Type of bird	Number of viruses with ICPI values		
		<0.7	**0.7 - 1.5**	**>1.5**
97	chicken	4	3	90
29	pigeon	-	22	7
3	turkey	1	-	
2	pheasant	-	-	2
1	duck	-	-	1
12	deep frozen duck	-		12

REPORT ON THE CURRENT NEWCASTLE DISEASE SITUATION IN THE NETHERLANDS

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1. Outbreaks of Newcastle disease (ND) in the Netherlands between October 1993 and October 1994.

Month/Year	Backyard poultry	Poultry industry
10/1993 to 02/1994	0	0
03/1994	2	0
04/1994	0	2
05/1994	2*	0
06/1994 to 10/1994	0	0

*One of these outbreaks was caused by virulent NDV imported with pigeons from Indonesia. The mAb pattern of this virus proved to be different from the virus that had been circulating in the Netherlands. This finding stresses the need for a sound vaccination policy as long as it is possible to import infected pet birds and backyard poultry.

2. Answers to the ND problem

- Continuous improvement of hygiene management, especially on layer, layer pullet and broiler farms.
- Increased level of immunity in broilers by improvement of the immunisation procedures. Veterinarians of the Animal Health Services visited many broiler farms to control vaccination procedures.
- Improvements in the monitoring of ND immunity in all categories of poultry.

3. Vaccination schedules.

No changes since June 1993 [see 1993 report].

4. Application of ND vaccines

Aerosol application of live vaccines is obligatory in layers, breeders and turkeys, except for the vaccination of one-day-old birds at the hatcheries. It has also been

demonstrated that broilers may be vaccinated by aerosol without serious post-vaccination reactions.

5. Conclusions

* The ND control management (hygiene management, vaccination policy including HI monitoring) is still successful.
* The improved vaccination policy with regard to layer pullets and layers [4 live vaccine and one inactivated vaccine applications before 18 weeks of age] has resulted in an impressive increase in HI titres.
* It has proved difficult to implement a uniform vaccination policy on broiler farms.
* The paucity of knowledge concerning the presence of poultry pathogens in backyard poultry means these birds may continue to represent an economical threat to the poultry industry.
* As long as it is possible to import pet birds and backyard poultry, the poultry industry will be obligated to a vaccination policy.
* From the point of view of hygiene special attention should be paid to the specialised poultry personnel which may be responsible for vaccination, debeaking and dead bird removal, interim removal of broils and egg trays.
* Good management practices with regard to the prevention of ND on poultry farms depends always and everywhere on strict application of GOOD HYGIENE AND VACCINATION.

6. Recommendations

- Prohibit the importation of pet/hobby birds unless they have fulfilled certain conditions e.g. quarantine.
- Poultry farmers should be required to obtain certification that the hygiene management on their farm is up to standard.
- Special attention is required to the possibility of spreading ND by means of slaughter house crates.
- ND vaccination should be done on each farm using spray or aerosol equipment that remains on the farm. It should be forbidden to use this apparatus on other farms.
- To amend the vaccination policy so that it is possible for controls and sanctions to be established by law for all categories of poultry, including backyard poultry and racing pigeons. It should be made compulsory that each farmer submits his flocks to regular monitoring with regard to the level of immunity to ND.
- It is necessary to promote and improve diagnostic work for backyard flocks and pet/hobby birds.
- At all times a ND control team/task force must be prepared and equipped with the necessary mandate for enforcing national measures

CURRENT SITUATION OF NDV IN INDUSTRIAL POULTRY FLOCKS IN BELGIUM.

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The first case of velogenic NDV in chickens was diagnosed in February 1992. At that time, NDV vaccination was not compulsory. As the disease became rapidly epizootic, compulsory vaccination of all poultry was imposed from the beginning of May by the Belgian Veterinary Services. All industrial poultry flocks were vaccinated at day-old but revaccination of broilers during the growing period was not imposed.

From the end of June until November 1992, this policy seemed to be very effective as no new outbreaks were noticed. However, two new cases were diagnosed in November 1992 in industrial broiler flocks located in two different provinces: Oost and West Vlaanderen (Table 1). A stamping-out policy was applied in both cases by our Veterinary Services and at the same time blood samples were taken in order to evaluate the vaccination status of the affected chickens. The results of the haemagglutination inhibition tests showed clearly that broilers which were only vaccinated once at the hatchery, using NDW vaccine, had insufficient antibody titres at the end of the growing period. Consequently, a modification of the existing legislation was introduced. A second vaccination of broilers using the La Sota strain given in the drinking water between 10 and 18 days of age was made compulsory from the end of January 1993. Since the application of this new vaccination scheme, only 5 new cases were diagnosed in broilers, the last being observed in November 1993.

In conclusion, it appears clearly that the vaccination measures taken together with the stamping-out policy allowed correct control of the epizootic. In total, approximately 450,000 broilers and 8,000 layers were slaughtered.

Table 1: Outbreaks of NDV in industrial poultry

Case number	Type of production	Vaccination scheme
92/01 (05/92)	Broilers: 152,798	Unvaccinated
92/02 (05/92)	Broilers: 7,775	Unvaccinated
92/03 (06/92)	Broilers: 44,000	Day 1: NDW Spray
92/04 (11/92)	Broilers: 20,000	Day 1: NDW Spray
92/05 (11/92)	Broilers: 12,300	Day 1: Hitchner Spray
93/01 (01/93)	Broilers: 46,200	Day 1: NDW Spray
93/02 (01/93)	Laying chickens: 7,927	Unvaccinated
93/03 (01/93)	Broilers: 18,200	Day 1: NDW Spray 1/2 dose
93/04 (O2/93)	Broilers: 19,500	Day 1: NDW Spray
93/05 (O2/93)	Broilers: 18,500	Day 1: NDW Spray
93/06 (03/93)	Broilers: 40,151	Day 1: NDW Spray. Day 12 La Sota Clone 30
93/07 (04/93)	Laying chickens: 730	Unvaccinated
93/08 (04/93)	Broilers: 10,400	Day 1: NDW Spray. Day 12 La Sota Clone 30
93/09 (06/93)	Broilers: 30,000	Day 1: NDW Spray. Day 11 La Sota Clone 30
93/10 (07/93)	Broilers: 9,208	Day 1: NDW Spray. Day 18 La Sota Clone 30
93/11 (10/93)	Broilers: 4,100	Day 1: NDW Spray. Day 14 La Sota

NEWCASTLE DISEASE IN PORTUGAL

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The situation of Newcastle disease (ND) in Portugal is significantly different from that observed in 1993. The number of outbreaks recorded in the poultry industry dropped from 34 in 1993 to 17 in 1994 and the disease is apparently under control in this sector. In contrast, the number of ND cases recorded in backyard flocks and hobby birds during 1994 was almost three times that registered in 1993, 32 and 12 cases respectively (Table 1). The presence of ND virus in these birds is a serious threat for the poultry industry because the means to control trade and movement of such birds are far from being satisfactory.

In 1994, 122 samples were submitted for ND virus investigation and of these 83 were positive.

Assessment of the virulence of NDV isolates for chickens using standard ICPI and IVPI tests is impossible in our laboratory. As an alternative to those tests we have used the plaque formation test and the mean death time of the minimum lethal dose (MDT) to assess virus virulence. All 1994 isolates tested so far, produced plaques in the MDBK cell line in the absence of trypsin and showed a MDT < 60 hours, thus suggesting potential virulence for poultry (Table 2). These isolates caused severe clinical signs and high mortality. Tracheitis, enteritis and haemorrhages were the most common lesions observed.

The biological and antigenic characterisations of these viruses have been done at the European Community Reference Laboratory, Central Veterinary Laboratory, U.K. All isolates except 2912/94 produced IIP binding patterns identical to the isolates of 1991, 1992, and 1993 (Table 3). Virulence tests (IVPI) were performed on several isolates giving indices between 2.56 and 2.67 indicating a high virulence for poultry. Isolate 2912/94 that gave an IVPI of 2.64 produced an unusual IIP binding pattern.

In a retrospective study we found that isolates 6134/91 and 6334/93 gave a mixed population of trypsin-dependent and trypsin-independent viruses. These isolates have been placed in group E (La Sota) and the ICPI found for 6334 was 0.125. Further studies are necessary for a better characterisation of these two isolates.

Efforts have been made to improve the laboratory structure and we hope in a near future to be capable of doing nucleotide sequencing of the F gene of several Portuguese isolates of NDV.

Table 1. Outbreaks of ND in Portugal during 1994 by month.

	Ja	Fe	Ma	Ap	Ma	Ju	Ju	Au	Se	Oc	No	De	total
Industry	4	2	1	1	2	1				5			17
Backyard & hobby	3	3	2	5	9	2	3		2	3			32

Table 2. Biological properties of NDV isolates from industrial and backyard birds

Virus	BIRD	IVPI[(a)]	PF[(b)]	MDT
112/94	broilers	2.57	yes	< 60 h
339/94	broilers	2.56	yes	nt
983/94	pheasants	2.67	yes	nt
1131/94	chicken	2.63	nt[(c)]	< 60 h
2127/94	chicken	nt	yes	nt
2139/94	broilers	nt	yes	nt
2785/94	chicken	nt	nt	nt
2912/94	chicken	2.64	yes	< 60 h
3020/94	chicken	2.62	yes	< 60 h
3183/94	pigeon	nt	yes	nt
3221/94	canary	nt	yes	nt
3303/94	pigeon	nt	yes	nt
5802/94	partridge	nt	nt	nt
6532/94	broilers	nt	yes	nt
6684/94	broilers	nt	yes	nt
6693/94	broilers	nt	nt	nt
6922/94	chicken	nt	nt	nt

[(a)] Determined at the Community Reference Laboratory- CVL.
[(b)] Plaque formation on MDBK cells in the absence of trypsin.
[(c)] Not tested.

SESSION III - EUROPEAN UNION COMMISSION AND REFERENCE LABORATORY REPORTS

AVIAN INFLUENZA AND NEWCASTLE DISEASE IN THE EUROPEAN COMMUNITY

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SANITARY SITUATION

1. Avian Influenza

No outbreaks of avian influenza were confirmed in the Community in the past year. One flock of Emus, Cassowaries, Crowncranes and Nandus were slaughtered in the Netherlands in April. The influenza virus (H5N9) which was isolated was later shown not to meet the definition of the disease.

2. Newcastle Disease

The situation as regards Newcastle disease was not satisfactory in 1994 with disease so far being diagnosed in 5 member states. The position has been as follows:

Country	Number of outbreaks
Germany	137
Italy	40
Portugal	8
Netherlands	7
Belgium	1

As last year, the majority of the outbreaks occurred in Germany and were almost all in small non commercial poultry flocks located in the former East Germany. A high proportion (95%) of these have been attributed to purchase of hens from a small number of dealers. Of the 137 farms infected so far this year only 3 could be described as commercial operations.

In an effort to counteract the situation, the German authorities have recently introduced new provisions by which the Landers may make vaccination compulsory in all flocks. At present this only applies to flocks over 200 birds. In addition poultry dealers are being registered and their own flocks subjected to checks. It is anticipated that these measures, if intensively and rigorously applied,

should lead to a reduction in the disease levels. However it should also be borne in mind that because the trade this type of poultry in Germany is seasonal a drop in infection levels occurred in the autumn of previous years.

Though a large number of flocks were affected in Italy, the picture was not as bad as first seemed. The majority of cases were in fact one event in Veneto where a poultry dealer and 34 flocks supplied by him were affected.

The situation in Portugal is different in two respects. Firstly, commercial flocks have been affected and secondly, the virus there is different to that in the rest of the Community. Ring vaccination campaigns were instituted around the affected premises.

There has been an improvement from last year in the situation in the Benelux countries. This has followed measures instituted to control the disease ie improved vaccination intensity, controls on markets and sales of "non commercial" birds.

One point worth noting is that though the number of outbreaks of Newcastle disease has increased, the number of birds involved has shown a sharp decline from 1993. In the first eight months of 1994 a total of 165.000 birds were affected by Newcastle disease compared to over one million in the same period of 1993. This drop relates to the fact that relatively more outbreaks are now being confirmed in the smaller non commercial farms and fewer in the larger commercial flocks.

It is also worth noting that no outbreaks have occurred in the Non Vaccinating part of the Community ie Denmark, Ireland and Northern Ireland. France, Spain and Greece also reported no cases so far in 1994.

LEGISLATIVE DEVELOPMENTS

Considerable progress as been made in European legislation on Poultry diseases in the past 12 months. Legislation has been enacted dealing with disease control, trade and non vaccination areas. In addition other topics are under discussion with legislation planned for later this year or early 1995.

1. Disease Control

- Two decisions were adopted to provide for the funding of the community reference laboratories for Newcastle disease and Avian Influenza for 1994 - decisions 93/686/EC and 93/689/EC respectively. These provided that the Community reference laboratory for both diseases would be the central Veterinary Laboratory, Addlestone (Weybridge), United Kingdom. We also anticipate renewing this contract for 1995.

- Two decisions concerning the funding of measures resulting from the confirmation of newcastle disease in 1992 were adopted. Decision 94/329/EC involved payment of 50% of the costs of slaughter and cleaning up following a single outbreak of the disease in Ireland in August 1992. Decision 94/455/EC involved payment of 50% of the costs of slaughter and cleaning up following outbreaks of the disease in the Netherlands in the same year.

- Another important development is that decision 93/152/EC comes into force on 1 January 1995. This decision sets out the criteria that must be met by vaccines used for routine vaccination in the community. The decision effectively provides that vaccines based on virulent strains of Newcastle disease virus are not used in the Community.

2. Trade in Live Poultry and Poultry Meat

- Council Directive 93/120/EC amending directive 90/539/EEC on animal health conditions governing intra-community trade in, and imports from third countries of, poultry and hatching eggs was adopted by Council in December 1993 and comes into force on 1 January 1995. This amendment;

  a. introduced amendments consequent on the adoption of Council directives 92/66/EEC and 92/40/EEC which set out Community measures for controlling Newcastle disease and Avian Influenza respectively.

  b. allowed the policy of regionalisation to be applied to imports of poultry from third countries in respect of Newcastle disease and Avian influenza and included provision for classifying third countries in respect of these diseases

  c. corrected and clarified some textual and drafting matters in the original Directive

  d. amended the time limit for the inspection of hatching eggs and poultry prior to movement in the light of experience. The major change was altering the requirement for inspection of the flock of origin in the case of hatching eggs entering intracommunity trade. A health check may now be carried out 72 hours prior to movement instead of 24 hours. As an alternative a monthly health check combined with a check of the flock records within 72 hours prior to movement will also suffice.

  e. clarified the position as regards game restocking

- Council Directive 93/121/EC amending directive 91/494/EEC on animal health conditions governing intra-community trade in, and imports from third countries of fresh poultrymeat was adopted by Council in December 1993 and also comes into force on 1 January 1995. This amendment essentially deals with points a,b, and c above for poultrymeat. In addition the amendment provides that international definitions of Newcastle disease

and Avian influenza be used for the purposes of importations of poultrymeat from third countries.

- Decision 94/438/EC on the criteria for classifying third countries as regards newcastle disease and avian influenza was adopted in June 1994. The decision set out criteria for accepting third countries or parts thereof as being free of the diseases for the purposes of importing fresh poultrymeat. The decision also modified decision 93/342/EC (classifying third countries for imports of live poultry).

- Two decisions, 94/85/EC and 94/298/EC, were adopted setting out a third country list for the importation of poultry meat. This list is a list "in principle" only and any importations would also have to meet additional animal and public health requirements.

3. Non Vaccinating Areas

- Directive 93/120/EC, mentioned above, also introduced the concept of non vaccinating status for newcastle disease. This would currently apply to Ireland, Denmark, and Northern Ireland. The candidate countries, Sweden Norway and Finland would probably also qualify. The decision states that this status is available to a member state or part of a member state which does not permit vaccination (except of racing pigeons), whose flocks do not contain vaccinated birds and where the breeding flocks are monitored once a year for Newcastle disease.

- Commission decision 94/327/EC sets out the criteria for annual testing of breeding flocks in the non vaccinating area. Essentially 60 blood samples must be taken annually on a random basis from each breeding flock.

4. Legislation Under Discussion

- A meeting has taken place to draw up conditions by which poultry meat originating in a farm in a surveillance zone for newcastle disease but outside the protection zone may receive the EC health mark. Clinical and epidemiological examination of the farm and virological examination of the birds will be required. It is anticipated that the decision will be taken in early 1995.

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

5. Forthcoming Legislation

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

- decisions on the Newcastle disease status of the new member states
- approval of the plans for establishments in the new member states
- review of the trade guarantees for trade into non vaccinating parts of the community in the light of the harmonisation of the standards of vaccines for newcastle disease in the Community - proposal to Council.

REPORT OF THE EUROPEAN UNION REFERENCE LABORATORIES FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE, 1994

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Introduction

The Central Veterinary Laboratory, Weybridge was designated the European Community Reference Laboratory for Avian Influenza in Directive 92/40/EEC [1] and the Reference Laboratory for Newcastle disease in Directive 92/66/EEC [2] to come into force on 01.01.93 and 01.10.93, respectively. The Directives also specify the functions of the Laboratory and the role that it will play in the diagnosis and control the diseases in the Community. From 01.01.94 this role came under a contractual agreement [3, 4].

CVL, Weybridge is also recognised as an International Reference Laboratory for Avian Influenza and Newcastle disease by both the Food and Agriculture Organisation of the United Nations [FAO] and Office Internationale des Epizooties [OIE].

Viruses received by International Reference Laboratory

The number of viruses submitted by all countries to the reference laboratory during the years 1987 to 01.10.1994 is shown in Figure 1. The number of viruses submitted in a particular year does not necessarily mean that the viruses were isolated that year and the Laboratory is often asked to undertake retrospective characterisation of past isolates.

The viruses received during 1987-1989 were largely those of the so-called pigeon variant PMV-1 virus submitted by laboratories wishing to have confirmation that this virus was present in their country. Since 1990, when 113 isolates were submitted, there has been a gradual increase in viruses received each year, to 294 in 1993 and 314 for the first nine months of 1994. Because of the drammatic rise in ND outbreaks occurring in countries of the European Union during this period (Figure 2) an increase in submissions would be expected. However, submissions from other countries have slightly exceeded those from EU states in the last three years (Figure 3) reflecting the increased activity of Newcastle disease throughout the world in recent years.

Figure 1: Viruses submitted to the International Reference Laboratory.

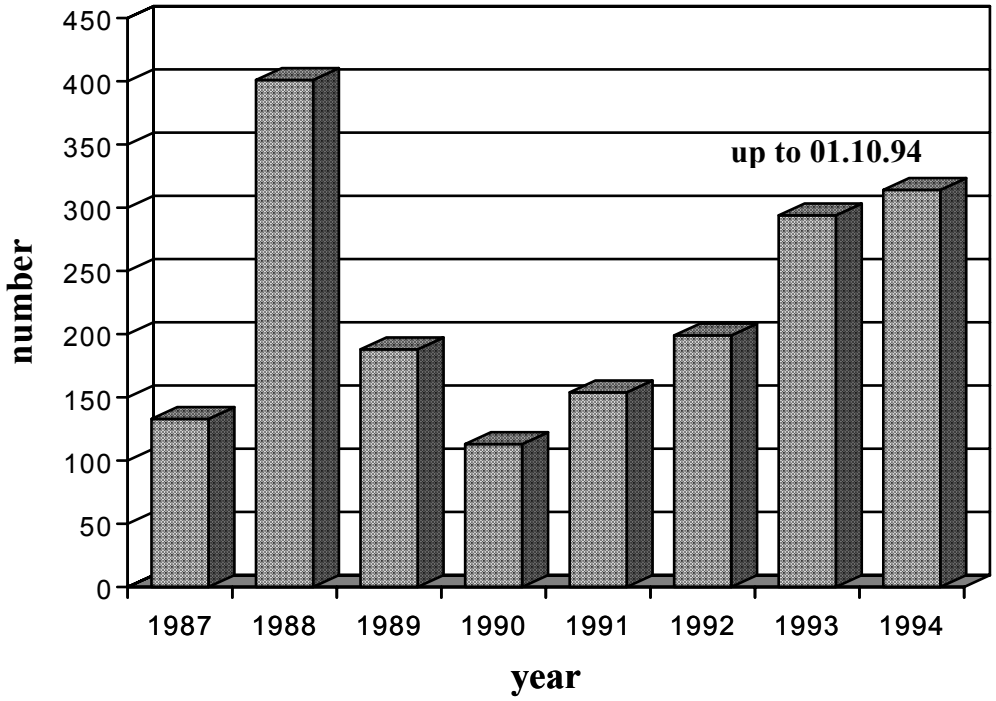


Figure 2: Reported outbreaks of Newcastle disease in European Union countries.

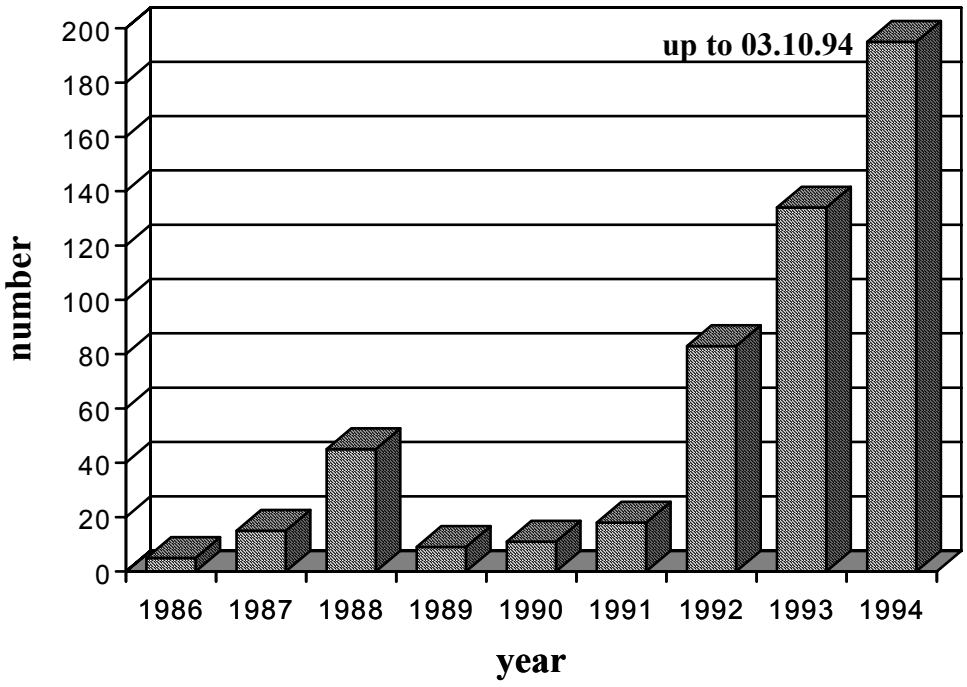
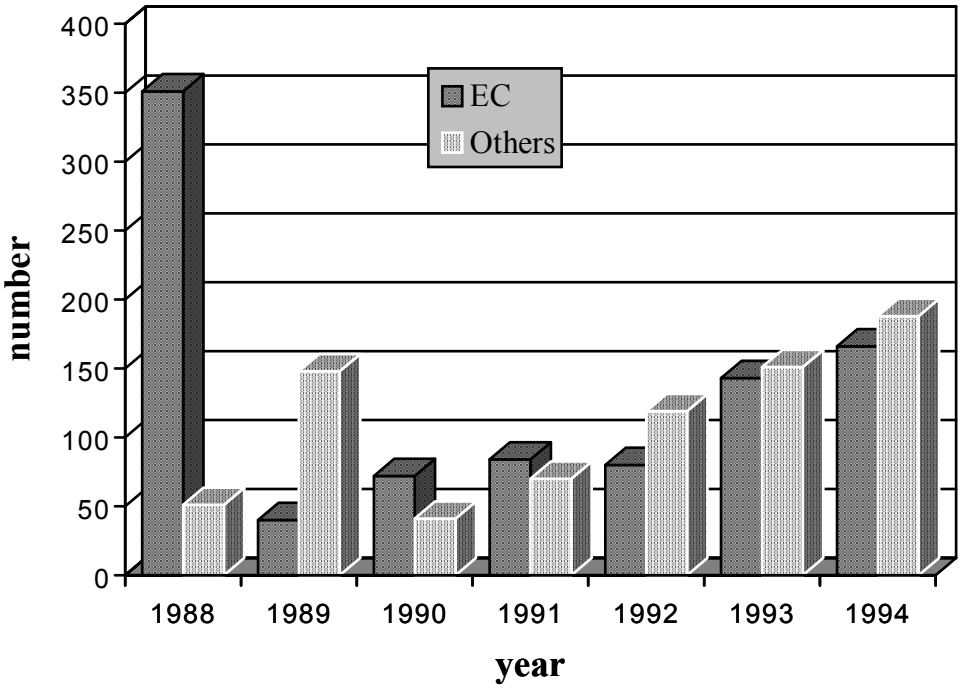


Figure 3: Viruses submitted to the International Reference Laboratory from European Union and other countries 1988 to 30.09.94.



Influenza viruses submitted during 01.10.1993 to 30.09.94

During the 12 month period from October 1993 to September 1994 a total of 15 influenza viruses was received at CVL, Weybridge from 5 different countries. The countries, number of viruses and subtype characterisation are shown in Table 1.

The total represents a decline from the 26 viruses received from 8 countries in the corresponding period in 1992-1993.

All 15 viruses had low intravenous pathogenicity indices in six-week-old chickens. Four viruses were of H5N9 subtype, three were from South Africa, two from chickens and one from ostriches, and the fourth from emus and casowaries in The Netherlands. Two viruses, both isolated from caged birds in The Netherlands and Great Britain were of H7N1 subtype. All viruses of H5 or H7 subtypes were subjected to nucleotide sequencing to determine the amino acids at the cleavage site of the haemagglutinin as required in Directive 92/40/EEC [1].

Table 1: Influenza A viruses submitted to the International Reference Laboratory during 01.10.93 to 30.09.94.

Country	Number	Host	Subtypes	IVPI
The Netherlands	1	ratites	**H5N9**	0.00
	1	parakeet	**H7N1**	0.00
United Kingdom	1	conure	**H7N1**	0.00
Italy	3	chickens	H9N2	3 x 0.00
Singapore	3	caged birds	H3N8	3 x 0.00
	3	caged birds	H4N6	1 x 0.47
				2 x 0.00
South Africa	2	chickens	**H5N9**	2 x 0.00
	1	ostrich	**H5N9**	0.00

not all viruses received had been isolated within this period.

As shown in Table 2 none of the viruses had basic amino acids at the cleavage site and do not fall within the definition of avian influenza for which the EC control policies will be implemented.

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

Virus	Cleavage site sequence
A/emu/Netherlands/94 (H5N9)	P-Q-**R**-E-T-**R***G-L-F
A/parakeet/Netherlands/94 (H7N1)	P-E-I-P-**K**-G-**R***G-L-F
A/conure/England/94 (H7N1)	P-E-I P-**K**-G-**R***G-L-F
A/ostrich/S.Africa/94 (H5N9)	P-Q-**R**-E-T-**R***G-L-F

Paramyxoviruses received during 01.10.1993 to 30.09.94.

During the 12 month period from October 1993 to September 1994 a total of 328 paramyxoviruses was received at CVL, Weybridge. Of these 170 were received from 8 countries in the European Union of which 158 were PMV-1 isolates (Table 3). The other 12 viruses being 4 PMV-2 and 8 PMV-3 viruses obtained from captive caged birds held in quarantine in Great Britian.

Table 3: European Union countries submitting PMV-1 viruses during 01.10.93 to 30.09.94.

Country	PMV-1
Belgium	1
France	1
Germany	97
Ireland	2
Italy	24
Portugal	21
Spain	2
United Kingdom	10
TOTAL	**158**

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

A total of 188 viruses was received from 13 countries from outside the EU. One hundred and forty-five were PMV-1 viruses (Table 4), 11 were PMV-2 viruses from caged birds in Singapore and 3 were PMV-4 viruses isolated in Israel.

Table 4: Other countries submitting PMV-1 viruses during 01.10.93 to 30.09.94.

Country	PMV-1
Norway	1
Switzerland	3
South Africa	3
Botswana	16
Malawi	2
Zimbabwe	17
Thailand	5
Sri Lanka	1
India	9
Indonesia	81?
Turkey	**7**
TOTAL	**145**

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

Characterisation of PMV-1 viruses using monoclonal antibodies.

All PMV-1 viruses received during September 1993 to October 1994 were characterised by their ability to cause binding of a panel of 28 monoclonal antibodies [mAb] to cell cultures infected with the viruses. For convenience and the need to summarise, the results are shown in mAb groups in Tables 5 and 6. These groups are based on those described by Russell and Alexander [5, 6] using only 9 mAbs. Generally, viruses in the same group show identical binding patterns for all 28 mAbs used. However, some isolates in the same group may vary by their ability to react with one or two mAbs.

All viruses placed in the same group tended to share the same biological properties and for convenience the groups have been separated in Tables 5 and 6 on the basis of virulence for chickens.

Table 5: Antigenic grouping of Newcastle disease virus isolates from EU and EFTA countries submitted during 01.10.93 to 30.09.94

Country	Number of isolates in monoclonal antibody binding group									
	velogenic					*lentogenic*				not done
	B	C1	P	NE	Port	C2	E	F	G	
Belgium		1								
France							1			
Germany			4	26			1			57
Ireland			2							
Italy	1	3	6				9*	5		
Portugal					21					
Spain				1			1*			
U.K.							9		1	
Norway						1				

** the virus showing pattern E from Spain and one of those from Italy were velogenic when tested in chickens - probably due to mixed cultures of live vaccine and field virus.*

The 21 isolates received from Portugal related to the outbreaks occurring during the period, these viruses showed the unique mAb binding pattern seen only with viruses isolated in Portugal since 1991.

To date 31 of the 88 viruses received from Germany have been characterised. These relate to outbreaks occurring in 1993 and 1994. Twenty-six of these viruses were placed in group NE [for North European Community] since they showed the

unique binding patterns of viruses associated with the outbreaks that occurred during 1991-1993 in The Netherlands, Belgium, Luxembourg and France.

The single isolate received from Belgium had been obtained from an ostrich, this virus showed a different binding pattern from the viruses responsible for outbreaks in Belgium in 1992 to 1993 and was placed in mAb group C1.

Table 6. Antigenic grouping of Newcastle disease virus isolates from other countries submitted during 01.10.93 to 30.09.94

Country	Number in monoclonal antibody binding group								
	velogenic						*lentogenic*		**not done**
	B	**C1**	**P**	**D**	**NE**	**J?**	**E**	**F**	
S. Africa	1	2							
Botswana						16			
Thailand							5		
Sri Lanka							1		
Switzerland			2		1				
Malawi		2							
India	1	4		3				3	
Turkey		7							
Indonesia		6							75
Zimbabwe		15					2		

Production of antigens and antisera.

The reference laboratory undertook, as part of its agreed functions, to produce antigens and antisera to assist in the diagnosis of ND and avian influenza. Initially, it was decided to produce monospecific polyclonal chicken sera to NDV [PMV-1], PMV-3, H5 influenza and H7 influenza and the corresponding inactivated antigens for use in haemagglutination inhibition [HI] tests. This has largely been completed and the stocks currently held as 1ml freeze-dried ampoules are shown in Table 7. Some considerable problems were experienced in obtaining inactivated H5 antigen which, on freeze-drying, had an acceptable shelf life and the current batch shown in Table 7 is still under test.

It was also planned to produce stocks of mAbs which can be used in HI tests and are useful in diagnosis. Problems were encountered due to the restrictions on the number of mice that can be used for ascites production under UK legislation [and the desire not to use mice for this purpose]. Attempts at production in concentrated tissue culture fluid was time consuming and unproductive. However, use of

commercially available equipment designed to produce high titred mAbs from hybridomas grown *in vitro* [Technomouse] has proven largely successful. At present about 35 ml of mAb 617/161, which is useful for identifying the pigeon variant PMV-1 viruses, has been produced in 0.5ml freeze-dried ampoules. Currently, production of mAbs 7D4, which is specific for La Sota, and U85, which reacts with most classical PMV-1 viruses, is in progress.

Table 7. Stocks of polyclonal chicken sera and virus antigens for HI tests held at the Reference Laboratory.

Type	Serum		Antigen	
	Quantity[a]	HI titre[b]	Quantity[a]	HA titre[b]
SPF	320	<2		
PMV-1	600	8	300[c]	9
PMV-3	300[d]	7	200[d]	9
H5	360	6	(70)	not done
H7	280	8	140	7

[a] *Number of freeze-dried ampoules containing 1 ml of serum or antigen at the indicated titre.*
[b] *HI and HA titres are expressed as* $\log_2$*. The SPF serum had an HI titre of <2 to each of the antigens*
[c] *Ulster 2C*
[d] *parakeet/Netherlands/449/75*

Acknowledgements

We thank Karen Frost and Sally Franklin for their assistance. The nucleotide sequencing of the influenza viruses was done by Geoff Wood and Jill Banks.

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SESSION IV: DISCUSSION OF THE PROBLEMS FACING NATIONAL LABORATORIES

Objective

The objective of this session was to give those present the opportunity to discuss the facilities, including equipment and reagents, available, currently and ideally, to the National Laboratories and the effect these have on their ability to carry out diagnosis of the two diseases effectively and safely within the demands of the Directives.

Notes of the discussion

The representatives of the different countries stated the problems particularly affecting their work. This ranged from minor irritations such as the implementation of stricter controls on laboratories aimed at preventing the release of virus, to major problems resulting in difficulty in carrying out diagnosis of disease as specified in the relevant Directives.

In vitro tests for virulence

Several of the representatives expressed the concern in their countries about the use of animals in laboratory tests and the pressure to produce an *in vitro* test for virus virulence. The chairman pointed out that a recommendation to develop and implement alternative tests had been made at the First Meeting and, although some lines of work were showing promise, at present there was no practicable alternative. [see below Recommendation 9 - Ed]

Standardisation

Several National Laboratories were still not using the precise methodology recommended in Directives 92/40/EEC and 92/66/EEC, mainly as a result of an unwillingness to change well-tried, long-standing methods currently in use. Laboratories were particularly reluctant to move from the antigen used in Newcastle disease (ND) haemagglutination inhibition (HI) tests [often La Sota strain] to strain Ulster 2C which is recommended as the standard antigen in Directive 92/66/EEC. The general reason for this reluctance was that different titres are obtained with different antigens, but it was pointed out that this was precisely the reason that standardisation throughout the National Laboratories was needed.

Training

Several representatives felt there was a need for training of members of their staff in the diagnosis of ND and avian influenza [AI], preferably at the International Reference Laboratory. It was pointed out by the Commission

Representative and agreed by the Reference Laboratory Representative that this was one of the functions of the EU Reference Laboratory. Training visits for short periods could be arranged at any time, but would be at the Member States' expense.

SPF eggs

Several countries reported difficulty in obtaining specific-pathogen-free (SPF) eggs for use in diagnosis. This was largely countries where there was no commercial flock available for supply and it was necessary to import SPF eggs.

Funding and Facilities

Funding of the laboratories was discussed, although it was made clear from the outset that funding levels were the responsibility of the Member States who by adopting the relevant directives agreed to maintain a National Laboratory for each disease. It was clear that the funds available and the methods of financing the laboratories varied enormously from country to country and, generally, although not always, poor financial support in terms of running costs paralleled poor facilities and staffing.

Some countries reported excellent laboratory and animal facilities while others felt that one (usually animal containment units) or both needed improvement.

Regional laboratories

Some countries did not have regional diagnostic laboratories and all diagnostic testing was done at the National Laboratory. In countries where there were regional laboratories there were often considerable problems associated with their rôle. These problems varied from the expense to the National Laboratory of running accreditation exercises with the regional laboratories, to perceived problems because of the geographical location of the regional laboratories in dense poultry-rearing areas. The biggest concerns for the countries reporting problems with the regional laboratories was the lack of facilities available to these laboratories and the lack of standardisation of isolation and identification techniques.

Comments by the Commission Representative

The Commission Representative indicated the Commission's concern in two areas. Firstly, the apparent lack in one or two Member States of implementation of the relevant Directive in terms of officially recognised and funded National Laboratories. Secondly, the reported problems between central and regional laboratories. It was pointed out, however, that the National Laboratory was responsible for organising standardisation of methods and techniques in regional laboratories.

SESSION V - ORIGINAL CONTRIBUTIONS

SOME EPIDEMIOLOGICAL ASPECTS OF NEWCASTLE DISEASE.

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MANUSCRIPT NOT RECEIVED.

DETECTION OF PMV-1 SPECIFIC ANTIBODIES WITH A MONOCLONAL ANTIBODY BLOCKING ENZYME-LINKED IMMUNOSORBENT ASSAY

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Introduction

Since they were first described, monoclonal antibodies (mAbs) have been widely used in microbiology. They also proved to be very useful immunochemical tools for the identification of viruses, and, for example, to classify paramyxovirus (PMV) strains and serotypes, or to monitor the spread of a distinctive epizootic Newcastle disease virus (NDV) (1). Any specific mAb can be utilised for such a purpose, irrespective of the roll of the target epitope in the host-virus interaction.

In addition the haemagglutinating (HA) epitopes of the paramyxoviruses have been localised and grouped with mAbs (2, 3).

It is important from a serodiagnostic point of view that PMV-1 and PMV-3 share some common HA epitopes. This phenomenon gives an explanation for the cross reactions in the NDV haemagglutination inhibition test (HI), and in some circumstances limiting the diagnostic value of this widely used test (4).

In the present paper we report on the evaluation of a new serological test, a monoclonal antibody blocking ELISA, which detects only PMV-1 specific antibodies. The ELISA was compared with the haemagglutination inhibition test (HI), performed according to the Council/Directive 92/66/EEC.

Materials, Methods and Results

Specificity of the MAbs

The monoclonal antibodies were produced as described by Köhler and Milstein (5).

The screening of the supernatants was done first with an indirect ELISA, then with a competitive ELISA. The mAbs, competing with NDV-specific chicken antibodies to bind to NDV-specific epitopes, were selected for further examinations.

The specificity of the selected mAbs was tested with an indirect immunoperoxidase test performed on MDBK cells, as described (6). The results are summarised in Table 1., "mAb b" (IgG_1) and "mAb f" reacted with all the

strains representing the different groups of the PMV-1, but did not react with any other PMV serotype. "mAb b" was selected and utilised in the ELISA.

Table 1. Specificity of the monoclonal antibodies

PMV	group [a]	virus strain	Monoclonal antibodies a	b	f	g
1	A	Essex 70	+[b]	+	+	+
	B	North.72/ Herts.33	+ / -	+ / +	+ / +	+ / -
	C	Kuwait / MB20	+ / +	+ / +	+ / +	+ / +
	D	GB Texas	+	+	+	+
	E	B1	+	+	+	+
	F	"F"	-	+	+	+
	G	Ulster2C / Qld V4	+ / +	+ / +	+ / +	+ / +
	H	MC 110	+	+	+	-
	L	Loon	+	+	+	+
	P	561/83	+	+	+	-
2		Yucaipa/56	-	-	-	-
3		England/MPH/81	-	-	-	-
4		Hong Kong/D3/75	-	-	-	-
6		Hong Kong/199/75	-	-	-	-
7		Tennessee/4/75	-	-	-	-
8		Delaware/1053/76	-	-	-	-
9		New York/22/78	-	-	-	-

[a]group defined as binding pattern obtained with a panel of mAbs [1].
[b]ability of virus to cause binding of mAb to infected cells in indirect immunoperoxidase tests.

The monoclonal antibody blocking ELISA

NDV-coated ELISA plates were first incubated with different dilutions of test sera for half an hour. After washing, the NDV-specific horseradish peroxidase-labelled mAb was added to the wells, and further incubated for another half an hour. The binding of the conjugated mAb was visualized after subsequent washing

with a substrate solution containing tetramethybenzidine hydrochloride (TMB) and H_2O_2. The test was performed at room temperature, wells were washed 3 times between the incubation steps with PBS containing 0.05% Tween-20. Absorbance values were determined with a microtitre plate spectrophotometer, compared with that of the NDV-negative reference serum and percent inhibition (PI) was calculated. Higher than 50% PI indicates positive result, between 40 and 50% doubtful result and lower than 40% indicates a negative result. The ELISA titre was expressed as the last dilution of the test serum sample showing PI > 50%. For routine use the working dilution of the test sera was 1:2.

Correlation between the HI and the ELISA

Experiment 1

Ten six weeks old NDV-free chickens kept in isolators, were individually marked and infected with the Ulster 2C strain of NDV. Blood samples were collected 9 times during a period of six weeks. Serum samples in twofold dilutions were titrated in HI and blocking ELISA tests.

When HI and ELISA titres were subjected to regression analysis there was a positive correlation (r = 0.89) between the two serological methods (Fig. 1). The ELISA was also more sensitive than the HI test ($P < 0.001$).

Experiment 2

When 158 serum samples from young birds were titrated with both methods the same close correlation occurred (Fig. 2). These birds, progeny of immunized parent flocks, were vaccinated at the hatchery (aerosol) or via the drinking water at the age of 3 weeks, with different doses of live NDV vaccine. Sera were collected at the age of 6-9 weeks. If an HI titre of $<2^4$ is considered as negative, only 7.5 % of the birds were positive, whereas 50.6 % of them had $\geq 2^1$ ELISA titres with our blocking ELISA.

Testing routine samples from Hungary and Sweden

Serum samples from vaccinated large-scale flocks and backyard poultry from Hungary, unvaccinated broilers and layers from Sweden and some turkeys with and without vaccination were tested with both HI test and our ELISA.

A total of 893 samples was examined at 1:2 dilution in the ELISA test and titrated in HI tests. The results are shown in the Table 2. Only 68.7% of the vaccinated birds had HI titres >3 ($\log_2$), whereas 98 % were positive with the ELISA. Twelve HI-positive birds (2%) were negative in ELISA. These sera were not saved, so we could not repeat the tests. The unvaccinated Swedish birds were all negative with both methods.

Discussion

Two selected mAb was specific for all serogroups of PMV-1, and were directed against an NDV epitope which is also recognized by the immunocompetent hosts. One mAb was utilized in the development of a monoclonal antibody blocking ELISA which was found to be specific and sensitive.

The mAb blocking ELISA has some advantages compared with the HI test in that it is PMV-1 specific and significantly more sensitive. Egg-yolk can be also tested with the blocking ELISA.

There are also advantages with the blocking ELISA compared with the traditional indirect ELISA: Serum samples from any species can be tested in our ELISA, and the possibility of using low working dilutions makes our test more easy to perform.

The local immunity induced by spray or eye drop immunization is usually followed by a poor serological response. Young birds immunized via the drinking water also usually have low HI titres. Preliminary results indicate that the antibodies detected by our blocking ELISA can be correlated with the protection achieved with this type of immunization. Further investigation must be carried out to evaluate this correlation considering, for example, the age of the vaccinated birds, the dose and the route of immunization.

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Figure 1. Correlation between HI and ELISA titres in experimentally infected six-week-old birds

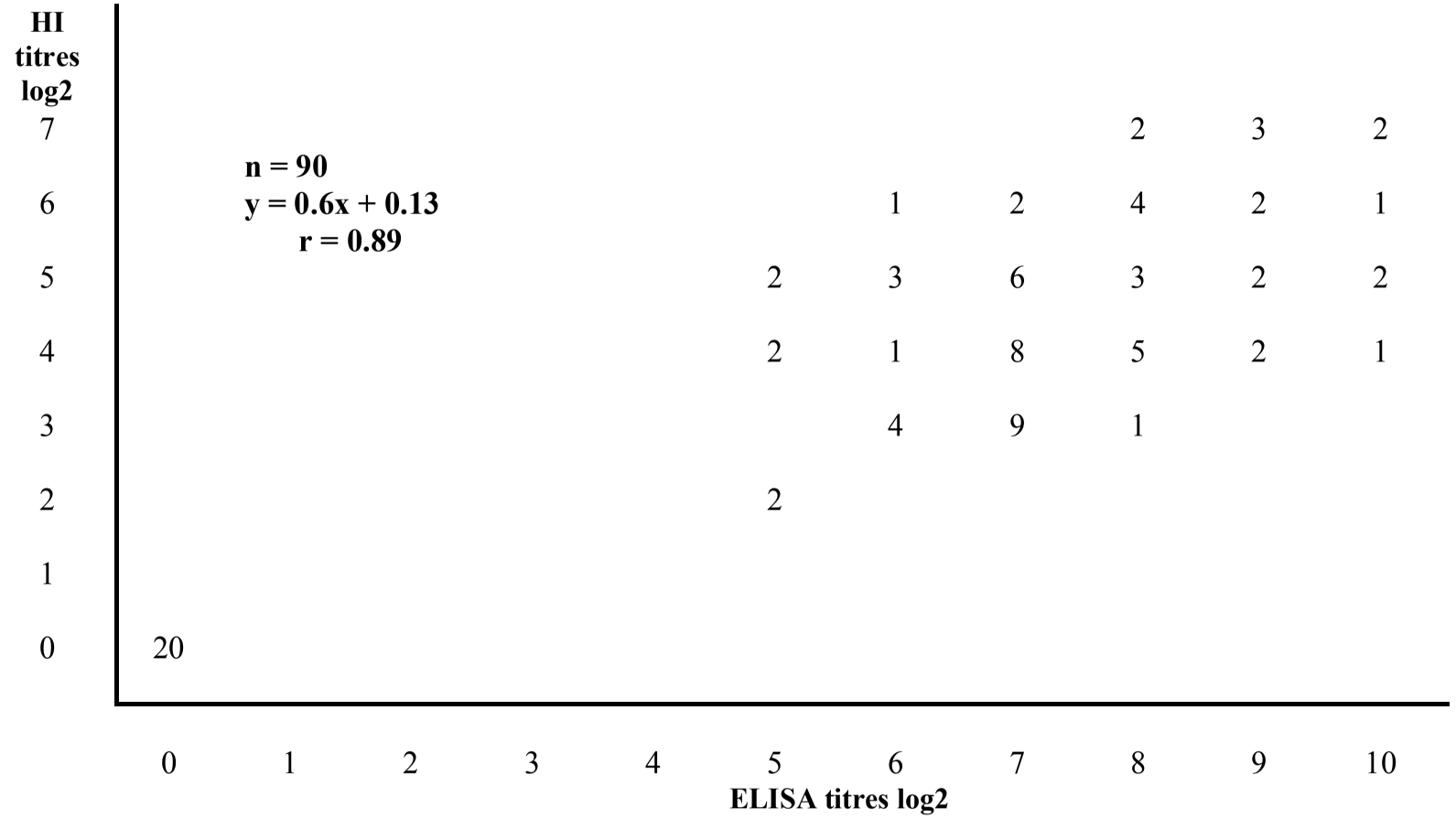

Figure 2. Correlation between HI and ELISA titres in birds vaccinated at 0-3 weeks of age, and sera collected at 6-9 weeks of age.

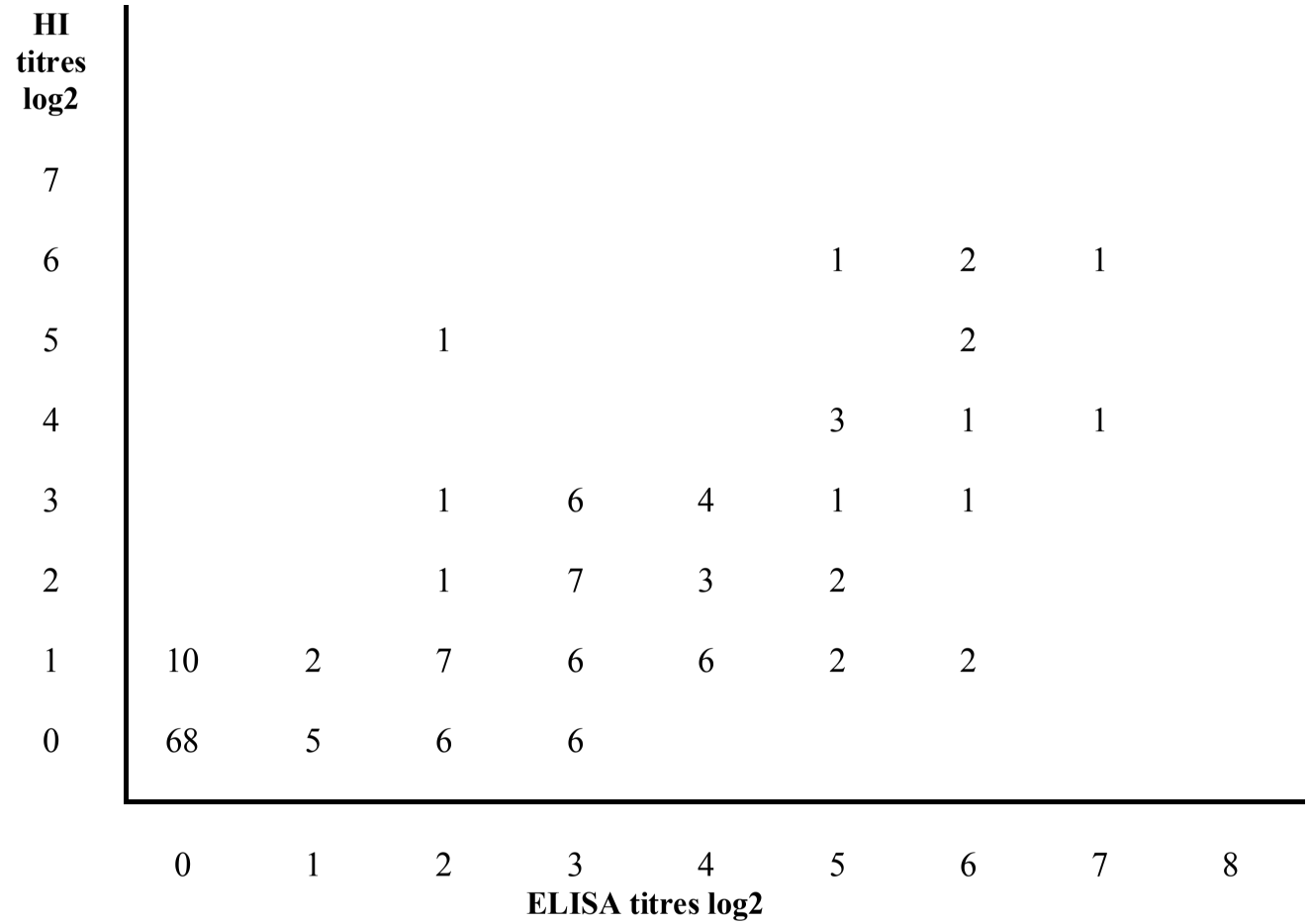

Table 2. Comparison of HI test and ELISA with routine samples from Sweden (S) and Hungary (H)

	ELISA (1:2)	**Positive (PI > 50 %)**				**Negative (PI < 50 %)**			
Serum samples from	**HI titre (log2)**	**> 3**	**= 3**	**< 3**	**0**	**> 3**	**= 3**	**< 3**	**0**
vaccinated large-scale flocks (H)		233	33	40		9		1	
unvaccinated large-scale flocks (S)									276
vaccinated backyard birds (H)		87	41	28					
vaccinated turkeys (H)		16	11	5					
unvaccinated turkeys (H)									45
quarantine case (S)		47	19			1	1		

QUALITY CONTROL OF THE NEWCASTLE DISEASE HI TEST AND THE INFLUENZA TYPE A AGP TEST IN FRENCH LABORATORIES

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1 - Introduction

The technical facilities for the control of Newcastle disease (ND) and avian influenza in France are the responsibility of the National Laboratory (CNEVA, Ploufragan) in association with several local diagnostic laboratories. To assess the performance of the latter the National Laboratory organises inter-laboratory testing biannually which is limited to serological tests, i.e. :

- Newcastle Disease, by haemagglutination inhibition (HI) ;
- Avian Influenza, by agar gel precipitation.

Each inter-laboratory study comprises two trials, the last study having been run as follows :

- First trial in 1993 : for all candidate laboratories (labs) ;
- Second trial in 1994 : only for labs with no satisfactory results in 1993.

We will consider successively the planning, the protocols, and the results of the inter-laboratory tests.

2 - Planning of the Inter-laboratory Tests :

At the beginning of December, invitations to participate in the tests are sent to all the French veterinary diagnostic laboratories (both private and regional laboratories may compete), with an indication of the reference techniques to be used and of the costs.

In January answers are received and the list of candidates is established.

During the year, at a date unknown to the candidates, the inter-laboratory test is started, as follows :

Week 0, Day 0: the samples to be tested are sent by mail. An explanatory note and documents to acknowledge receipt of the parcel and return the results are supplied with the samples.

Week 0, Day 2: this is the maximum delay allowed for the arrival of the parcels within the "colissimo system".

Week 0, Day 4 : this is the maximum delay allowed for returning the acknowledgements of receipt of the samples to the monitoring laboratory.

Week 2, Day 2 : this is the maximum delay for transmitting responses to the monitoring laboratory.

The identification of the sera and the results obtained by each candidate compared with those of the monitoring laboratory are returned to the corresponding participants within one month of their response. The collective results and conclusions are given within three months to all the participants (anonymously) and to the National Veterinary Service.

3 - Protocols :

3.1 - Origin of the sera :

All the sera used in both inter-laboratory tests came from SPF chickens and were prepared by the National Reference Laboratory as follows:

3.1.1 - Negative sera :

These originated from more than 200 birds 6 to 11 weeks old, tested individually for :

- AGP/Influenza :	all were negative	
- HI/Newcastle :	some had titres < 1/4	= Pool 1
	some had titres 1/4	= Pool 2

Each pool was heated at 56°C for 30 minutes, filtered through 0.45µ and 0.02% NaN_3 (sodium azide) added as a conservative.

Pool 1, after dilution 1/2 in PBS and distribution in tubes, constituted the negative serum "E" in HI/Newcastle (see Table 1).

Pool 2, after distribution in tubes, constituted the negative serum "F" in AGP/Influenza (see Table 2).

3.1.2 - HI positive Newcastle disease sera :

Sixty 4-week-old SPF chickens were vaccinated with HB_1 administered ocularly at the rate of $10^{6.5}EID_{50}$ per bird. Three weeks later they were challenged with a velogenic NDV (strain "Ploufragan") inoculated intramuscularly

at the rate of 10^5 EID_{50} per bird. Bleeding of the birds was done two weeks after the boost and sera were tested individually for ND/HI antibodies :

- Sera whose HI titre = 512 were pooled = pool P_1
- All other HI positive sera were pooled = pool P_2.

Each pool was heated at 56°C for 30 minutes, filtered through 0.45 µ and 0.02% N_aN_3 added.

3.1.3 - AGP positive Influenza sera :

Forty 8-week-old SPF chickens were inoculated intramuscularly two or more times, each time with 4 x 0.25 ml of the following formalin-inactivated viruses :

- H7N3 at weeks 0 and 3 (respectively 6400 and 1280 HAU per bird) ;
- H11N9 at week 6 (11400 HAU per bird) ;
- H10N7 at week 9 (1200 HAU per bird).

Birds were tested for influenza AGP antibodies 10 to 11 days after each of the second, third and fourth inoculations, and 2 days after these tests, strongly and moderately positive birds were bled :

- Sera strongly positive were pooled = pool S ;
- Sera moderately positive were pooled = pool M.

Each pool was filtered through 0.45 µ, then 0.02 % N_aN_3 added.

3.2 - Preparation of definitive sera :

Samples from the respective Newcastle and Influenza positive sera were tested by making dilutions in PBS to estimate the dilutions to make later in negative serum (from SPF birds) to obtain the expected titre or reaction for inter-laboratory testing (see figure 1).

According to the results obtained, the dilutions were made and the sera were distributed in tubes as follows (see Tables 1 and 2) :

- pool S = sera A, A/2, A/4, A/8, etc... ;
- pool M = serum B ;
- pool P_1 = sera C, C/2, C/4, C/8, etc... ;
- pool P_2 = sera D, D/2, D/4, D/8, etc...

Tubes were stocked at -20°C for several weeks.

3.3 - Determination of the "reference" and "required" results :

At three different times during their freezing period, samples of each serum type was tested by 3 operators after a period of 3 days at room temperature preceded or not by an additional freezing/thawing cycle (Figure 2).

The titre or reaction most constantly obtained allowed definition of the "reference result" (or reference value) and, consecutively, the "result required" by adding or removing one dilution tolerance to the former (see Tables 1 and 2).

In addition to these criteria (reproducibility and specificity), other parameters were taken into account in column "result required", such as coherence in dilutions (see Figures 3 and 4) and repeatability when several samples of the same sera were present.

4 - Results :

Results obtained in both inter-laboratory tests are summarised in Table 3 (HI/Newcastle) and Table 4 (AGP/Influenza).

4.1 - HI/Newcastle :

Of the 22 laboratories tested in the first trial, 17 gave satisfactory responses to all the parameters defined in Table 1, column "result required". The failures for the 5 remaining labs were identified:

- The use of an inadequate technique and dilution scale for one lab;
- Failure in reproducibility, for two labs ;
- Failure in reproducibility and incoherence in dilutions, for one lab ;
- Failure in reproducibility and specificity, and incoherence in dilutions, for one lab.

Of these 5 labs, only one was still found not satisfactory after the second trial (which was more complicated and difficult for the participants than the first trial): this laboratory continued to fail in reproducibility.

4.2 - AGP/Influenza :

Of the 15 laboratories tested in the first trial, 11 gave satisfactory responses to all the parameters defined in Table 2, column "result required". The failures for the 4 remaining labs were:

- Failure in repeatability for two labs ;
- Failure in repeatability and specificity for two labs.

Of these 4 labs, only one was still found to be unsatisfactory after the second trial, with the same double failing in repeatability and sensitivity.

5 - Conclusion :

Such inter-laboratory studies are difficult to organise, and time consuming for the monitoring laboratory. But results obtained by the laboratories tested have improved continuously since we started these quality control comparisons, several years ago. Thus, the balance-sheet of the last trials can be considered as excellent since 21 laboratories out of 22 for Newcastle HI and 14 out of 15 for Influenza AGP have given satisfactory responses to all the parameters. It could be retorted that it was easy to achieve the result required in Newcastle HI, especially for serum D whose accepted titre ranged from 64 to 512. But besides the fact that an HI titre is always accepted to be plus or minus one $\log_2$ dilution, with serum D titrating exactly between 128 and 256 we had the choice to be either too severe, or too tolerant. In addition to the fact that such a problem concerned only serum D, it must be considered that to pass the trials, one laboratory had to be satisfactory on all parameters among which "coherence in dilutions" was one of the most important. As an example of this, 512 and 64 were accepted values respectively for sera C and C/2 in Newcastle HI (see Table 1), but if obtained by the same laboratory they constitute an anomaly in dilution effect and the corresponding laboratory was failed.

The number of samples of the negative sera was relatively high and was statistically justified to increase the probability of detecting one anomaly in specificity.

The results obtained by the majority of the tested laboratories tend to confirm the "reference results" proposed by the National Laboratory to be reliable. Those relating to Newcastle HI had been defined by using a modified technique, compared with the method proposed for the EU. Some of these modifications were shown in preliminary trials to improve the reproducibility of the test (precise adjustment of the RBC suspension to 80 x 10^6 cells per ml; reading of the HA test horizontally and of the HI by sloping) and others to improve the specificity (dilution of sera directly in the antigen solution, which considerably decreases the effect of non specific inhibitors) or the quality of sedimentation (reaction done at room temperature, ranging from 18 to 24°C ; final volume of 50 µl per well). In addition to the two last parameters, we also showed the quality of reading to depend much more on the origin of the microplates (involvement of the plastic type? or the microplate treatment?) than on the use or V- or U-bottom wells.

Lastly, we have used a formalin inactivated La Sota antigen instead of Ulster 2C, the latter seeming to us to give slightly lower and less reproducible titres. It could be of interest to confirm these observations, or not by, for example, comparison tests between National Laboratories.

Table 1 : Protocol for HI / Newcastle

Type of serum	Property tested (1)	Reference value	Result required
C	RP	256	128 to 512
C2	RP, CD	128	64 to 256 and CD
C4	RP, RE, CD	64	32 to 128, RE and CD
C4	RP, RE, CD	64	32 to 128, RE and CD
C8	RP, CD	32	16 to 64 and CD
C16	RP, CD	16	8 to 32 and CD
C32	RP, CD	8	4 to 16 and CD
C64	RP, CD	4	< 4 to 8 and CD
C128	RP, CD	< 4	< 4 or 4 and CD
C256	RP, CD	< 4	< 4 or 4 and CD
D	RP	128 - 256	64 to 512
D2	RP, CD	64 - 128	32 to 256 and CD
D4	RP, CD	32 - 64	16 to 128 and CD
D8	RP, CD	16 - 32	8 to 64 and CD
D16	RP, CD	8 - 16	4 to 32 and CD
D32	RP, CD	4 - 8	< 4 to 16 and CD
D64	RP, CD	< 4 - 4	< 4 to 8 and CD
D128	RP, CD	< 4	< 4 or 4 and CD
D256	RP, CD	< 4	< 4 or 4 and CD
E (8 repeats)	SP, (RE)	all < 4	all < 4 or 4

(1) RP = reproducibility CD = coherence in dilutions RE = repeatability SP = specificity

TABLE 2 : Protocol for AGP/influenza

Type of serum	Property tested (1)	Reference result (2)	Result required
B	SE, (RE)	P	P
B	SE, (RE)	P	P
B	SE, (RE)	P	P
B	SE, (RE)	P	P
B	SE, (RE)	P	P
A	SE, (RE)	P	P
A	SE, (RE)	P	P
A	SE, (RE)	P	P
A2	SE, CD, RE	P	P or D, RE and CD
A2	SE, CD, RE	P	P or D, RE and CD
A4	CD, RE	P or D	RE, CD
A4	CD, RE	P or D	RE, CD
A8	CD, RE	D or N	RE, CD
A8	CD, RE	D or N	RE, CD
A16	CD, RE	N	RE, CD
A16	CD, RE	N	RE, CD
A32	CD, RE	N	RE, CD
A32	CD, RE	N	RE, CD
F (10 repeats)	SP, (RE)	all N	all N

(1) SE = sensitivity, RE = repeatability, CD = coherence in dilutions, SP = specificity

(2) P = positive, D = doubtful, N = negative

Table 3 : HI/Newcastle results obtained by participating laboratories

Type of serum	Reference value (1)	Results obtained			
		Monitor Laboratory	20 out of the 22 laboratories (2)		
			Geometric mean	Minimum	Maximum
C	256	256	247	128	512
C2	128	128	111	64	256
C4	64	64	62	32	128
C4	64	64	60	32	128
C8	32	32	33	16	64
C16	16	16	14	8	32
C32	8	8	8	4	32
C64	4	4	4	< 4	8
C128	< 4	< 4	< 4	< 4	8
C256	< 4	< 4	< 4	< 4	4
D	128 - 256	256	187	64	512
D2	64 - 128	128	97	32	256
D4	32 - 64	64	54	32	128
D8	16 - 32	32	25	16	64
D16	8 - 16	16	13	8	32
D32	4 - 8	8	7	4	16
D64	< 4 - 4	4	< 4	< 4	8
D128	< 4	< 4	< 4	< 4	4
D256	< 4	< 4	< 4	< 4	4
E (8 repeats)	all < 4	all < 4	all < 4	all < 4	all < 4

(1) predetermined by the National Reference Laboratory

(2) two laboratories were discarded in the geometric mean calculation (one highly incoherent in dilution, the other having used inadequate techniques and dilution scale).

Table 4 : Results obtained for AGP/Influenza by participating laboratories.

		Results obtained			
Type of serum	**Reference results***	**Monitoring laboratory**	**The 15 laboratories**		
			Number of :		
			P	**D**	**N**
B	P	P	14	0	1
B	P	P	15	0	0
B	P	P	14	0	1
B	P	P	14	0	1
B	P	P	15	0	0
A	P	P	15	0	0
A	P	P	15	0	0
A	P	P	15	0	0
A2	P	P	14	0	1
A2	P	P	15	0	0
A4	P or D	P	7	1	7
A4	P or D	D	6	1	8
A8	D or N	D	0	2	13
A8	D or N	N	0	1	14
A16	N	N	0	0	15
A16	N	N	0	0	15
A32	N	N	0	0	15
A32	N	N	0	0	15
F (10 repeats)	all N	all N	all 0	all 0	all 15

* predetermined by the National Reference Laboratory
P = positive, D = doubtful, N = negative

CHARACTERISATION OF THE CLEAVAGE SITE OF NDV-F PROTEIN OF VIRUSES ISOLATED IN 1993 AND 1994 FROM CHICKENS AND PIGEONS - METHOD AND INITIAL RESULTS

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Introduction

With the foundation and reorganisation of the reference laboratory in our institute we were interested in the introduction of modern methods for the diagnosis of Newcastle disease (ND). It was our aim to apply molecular biological methods for detection and characterisation of ND virus (NDV) nucleic acid. The polymerase chain reaction (PCR) is a sensitive method for the detection of nucleic acids. The application of this method makes it possible to detect the nucleic acid of NDV by amplification of small amounts of material. The PCR product is then a source for further characterisation such as hybridisation and sequencing. By amplification of a nucleic acid section with virulence determining properties and sequencing of this DNA fragment we will be able to assess the virulence of the isolate and the relationship to other isolates.

NDV is an enveloped virus with a negatively polarised single stranded RNA of ~15 kb length. The RNA encodes the virus proteins in the order 5' L-HN-F_O-M-P-NP 3' (1). Viral infection is initiated by the interaction of the HN protein with a sialic acid-containing receptor. The subsequent fusion of viral and cellular membranes required for penetration is mediated by the fusion protein. This protein is also responsible for fusion between an infected cell and an adjacent cell. The fusion proteins of paramyxoviruses are synthesised as a precursor, F_O, which is activated by proteolytic cleavage, resulting in F_1 and F_2 polypeptides.

The proteolytic cleavage occurs dependent on cell type and strain of virus. Fusion proteins of lentogenic strains are cleaved only in eggs or by an added protease such as trypsin, but those of velogenic ND strains are cleaved in all cell types including tissue cultures. These readily cleaveable fusion proteins are characterised by existence of two pairs of basic amino acid residues near the cleavage site, which are recognised by ubiquitous host cell enzymes (present in the trans-Golgi membranes or trans Golgi network) (2, 3).

Our aim was to amplify a nucleic acid fragment where the cleavage site of the fusion protein is included, to determine the sequence around the cleavage site and to compare that of various isolates. So we had to find primers for the PCR, that included the cleavage site and hybridised to all NDV strains. We looked for a conserved region in the F-sequence left and right from the cleavage site and

selected the following four primers, two forward and two reverse, considering the general demands of primers for good hybridisation.

Primer 1 (forward) :	315-333	5' CTT TGC TCA CCC CCC TTG G 3'
Primer 2 (reverse) :	589-572	5' CTT CCC AAC TGC CAC TGC 3'
Primer 3(forward) :	141-159	5' TTG ATG GCA GGC CTC TTG C 3'
Primer 4 (reverse) :	503-485	5' GGA GGA TGT TGG CAG CAT T 3'

Initially primers 1 and 2, which were already tried and tested in the PCR by Jestin and Jestin [4], were used. The resulting amplification fragments were products of several sizes (188 bp, 274 bp, 362 bp, 448 bp). With these four primers we also had the possibility for nested PCR.

Since RNA can not serve as a template for the Taq-Polymerase directly, RNA must be reverse transcribed to a cDNA prior to amplification. Therefore, a quick method for isolating RNA, that prevented degradation, was needed. We used the acid guanidiniumthiocyanate-phenol-chloroform extraction, reported by Chomczynski and Sacchi [5].

RNA preparation

The pellet, obtained by ultracentrifugation of 2-4 ml allantoic fluid for 10 min at 40,000 rpm (Kontron Rotor TST 60.4), was mixed with 4 M guanidiniumthiocyanate stock solution, and vortexed vigorously to complete homogenisation of the pellet in the shortest time. Next it was extracted by Phenol/Chloroform, ethanol precipitated, resuspended in guanidiniumthiocyanate stock solution and reprecipitated using a double volume of cold ethanol. An RNA pellet was obtained after centrifugation at 10,000 g for 15 min. at 4°C, rinsed with 75 % cold ethanol to remove salts and centrifuged for 5 min at 10,000g. The pellet was dissolved in 20 µl DEPC-treated water.

RT-PCR

2.5 µl of this solution were used for the RT-PCR. The first step was the reverse transcription with the enzyme reverse transcriptase followed by the PCR in 30 cycles with the enzyme Taq Polymerase. PCR conditions are 3 min 95 °C; 30 x (30 sec 95 °C, 30 sec 55 °C, 30 sec 72 °C); 2 min 72 °C.

With the four possible combinations of primer pairs we obtained four fragments of several lengths.

It was possible to amplify these fragments independently of the NDV strain. The PCR method was practicable and we were able to use the PCR product obtained for sequencing and comparison of different virus strains. We used primers 3 and 4 for the PCR to produce fragments of 362 bp length for sequencing.

Direct nucleotide sequencing was the method used for determination of the sequence near the cleavage site of F_0. The advantage of this technique without

cloning strategies is the exclusion of cloning and PCR artefacts in the sequencing results (6).

Sequencing of the PCR product

We cut out the 362 bp fragment from 1.5 % ethidiumbromide stained Nusieve-Agarose gels after electrophoresis and eluted the nucleic acid with the Spin Bind DNA Recovery System (Biozym). The principle of this elution is as follows: the sample DNA binds to Spin binds microporous silica membrane. Contaminating materials pass through the membrane on centrifugation. Following brief washing, DNA is recovered in 50 µl water. This prepared DNA could be used in cycle sequencing according to the Biozym protocol.

We used primer 3 and 4 for sequencing. Each isolate was sequenced with both the forward and the reverse primer. The comparison of these two sequencing reactions is necessary to remove sequencing errors. We were able to determine the sequence of the amplified DNA fragment in two days, if the radioactive labelling was effective.

Results and discussion

With the prescribed methods we were able to determine the sequences of various isolates. In Table 1 you can see the data of the isolates (bird, place of isolation) and the results concerning ICPI, nucleotide sequences and deduced amino acid sequences. There are similarities and differences in the sequences of the isolates examined. All velogenic field isolates from chicken were closely related. The existence of two pairs of basic amino acid residues near the cleavage site is linked to high virulence as indicated by high ICPI values, except for mesogenic strain 8/94 for which the ICPI was 0.95.

Isolates from pigeons showed more differences in the nucleotide sequence both amongst themselves, for instance 33/93 - 3/94 - 64/93, and to the sequences of virus strains from chicken isolated, compare 33/93 and 6/94. The increased number of basic amino acid residues near the cleavage site is not associated with high virulence as indicated by the ICPI values. Possibly the sequence of five consecutive basic amino acids is responsible for this biological characteristic. It is possible that host cell enzymes do not recognise the cleavage site so readily and therefore the precursor fusion protein is not cleaved so effectively into F_2/F_1. Another explanation of the low ICPI could be related to the effect of the host since one-day-old chickens are the test animals and not pigeons. However, there are also pigeon isolates with two distinct pairs of basic amino acid residues near the cleavage site, but with ICPI values lower than 1.5. It may therefore be necessary to look for other differences in the sequence region.

It is known that lentogenic strains are characterised by a leucine (L) at amino acid position 117 instead of phenylalanine (F). We found the same result with the isolate 63/93. The ICPI was only 0.10, which means it is a lentogenic strain and the deduced amino acid sequence was typical for lentogenic strains (Table 1).

It is possible to discuss influence of other amino acid , such as at position 109. Especially if we look at the determined sequence of chicken isolate 8/94 and pigeon isolates 22/93 and 49/93. Here we find two pairs of basic amino acid residues but only an ICPI of 0.95, 0.6, and 1.4 respectively. The only difference in the amino acid sequence of 8/94 to the other sequences of chicken with a high ICPI is the amino acid at position 109. The amino acid at position 109 of the isolate 8/94 is serine instead of proline. Serine is also the amino acid at position 109 in all sequences of pigeon isolates.
Isolate 8/94 was from a chicken, but its close relationship to isolates of pigeon and the available background information (this isolate came from an ornamental fowl from a hobby flock) makes it possible to speculate that transmission from pigeon to chicken occurred.
Our results have shown that the method of PCR with subsequent direct sequencing is a practicable way of diagnosis. We were able to find similarities and differences between different virus isolates. The sequencing of chicken isolates shows good agreement with the results of the ICPI determination. Sequencing results of pigeon isolates are divergent and we have to look for other characteristics in the sequence that may influence virulence, because two pairs basic amino acid residues are not connected with a high ICPI. It is possible that this difference is related to the hosts of origin. The investigation of Collins et al (7) showed that the virulence of the virus with an unaltered motif for chickens was increased by chicken to chicken passage. It may be that a repeated passage of a pigeon strain in chicken and determination of the sequences and ICPI of re-isolated virus will give more information about this complex.

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Table. 1 Nucleotide and deduced amino acid sequences of various isolates

isolate	bird	place of isolation	nucleotide sequence										amino acid sequence	ICPI
													109 118	
6/94	chicken	Neubrandenburg	CCT	GGG	GGA	AGG	AGA	CAG	AAA	CGC	TTT	ATA	PGG RR Q KR FI	1.89
148/93	"	Aulendorf											PGG RR Q KR FI	1.88
163/93	"	Stuttgart											PGG RR Q KR FI	1.71
1/94	"	Jena											PGG RR Q KR FI	1.86
63/93	"	Cottbus	T		A	G	G			GGG		C		SGG GR Q GR LI	0.10
8/94	"	Halle	T	A	A			G		G				SGG RR Q KR FI	0.95
3/94	pigeon	Stendal	T	A	A			G	A	G				SGG RR K KR FI	1.32
5/94	"	Stendal	T	A	A	T		G	A	G				SGV RR K KR FI	1.05
33/93	"	Karlsruhe	T	A	A	T		G	A	G				SGV RR K KR FI	0.98
22/93	"	Frankfurt/O.	T	A	A			G		G				SGG RR Q KR FI	0.60
49/93	"	Krefeld	T	A	A	T		G		G				SGV RR Q KR FI	1.43
64/93	"	Cottbus	T	A	A		A	G		G				SGG GR Q KR FI	1.45

SAFETY AND EFFICACY OF NEWCASTLE DISEASE VACCINES IN GAME BIRDS

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Introduction

In the context of the French Newcastle disease policy there is a requirement that all long-living birds (all breeds of any species, free range broilers and layers) should be vaccinated and well protected. To this aim, the veterinary services required that an experimental trial should be undertaken in game birds, especially to know their sensitivity to Newcastle disease (ND) virus and the possibility of protection of these birds in view of the few publications on the subject. At present, the game bird breeds are not vaccinated, even when they are reared in the same areas as intensive poultry.

In the present study the safety and the efficacy of live and inactivated vaccines were assessed.

Materials and Methods

Birds

Eighty 5-week-old pheasants, eighty 9-week-old grey partridges, eighty 9-week-old red partridges and forty 9-week-old SPF chickens were housed in cages in filtered rooms.

Experimental design

Animal Room	1	2	3	
Treatment	HB1	La Sota	Inactivated vaccine 1 dose	Control
Pheasant	20	18	18	19
Grey partridge	18	20	19	19
Red partridge	16	18	18	17
SPF chicken	10	10	10	10

Vaccines

Vaccines used were bio-commercial products. Live vaccine strains Hitchner B1 (HB1) and La Sota were administered by eye drop of one dose (0.03 ml) per bird. The inactivated oil adjuvanted vaccine was administered by intramuscular injection of 1 dose (0.3 ml) per bird.

Challenge

The ND challenge virus was the Ploufragan (1972) velogenic strain administered 18 days after vaccination by intramuscular route with 10^5 LD_{50}/0.5 ml per bird.

Serology

The haemagglutination inhibition (HI) test (IS 280 - Programme 109 COFRAC) using the inactivated La Sota strain and 4 HA units was used to check the absence of antibody before vaccination and to assess the level of antibody response on the day of challenge.

Morbidity and mortality

The clinical signs and the mortality were recorded daily after vaccination and during the 24 days observation period after challenge.

RESULTS

Serology

None of the species had ND antibody before vaccination (Table 1).

After vaccination the level of antibodies of game birds seemed to be higher with the HB1 strain than with the other vaccines. Even though no statistical analysis was done, HB1 and La Sota produced very similar immunity in grey and red partridges. The grey partridges presented the lowest level of antibodies after HB1 vaccination among the other species. It has to be underlined that game birds have a completely different reaction with regard to inactivated vaccine.

Safety

No vaccine caused any clinical signs.

Protection

All the unvaccinated birds appeared to be fully susceptible to the ND virus challenge. The red partridges shown only 64 % mortality, but the surviving birds all had nervous signs (Table 2).

The pheasants vaccinated with Hitchner B1 presented very slight signs during one day covering the 3-4 day after challenge.. The La Sota strain conferred complete protection on pheasants.

The grey partridges were not fully protected with any of the vaccine strains.

The red partridges were fully protected with live vaccines.

Under the conditions of this experiment the inactivated vaccine has not afforded good protection to the game birds.

Table 1 : Serology (HI Test) geometric means (Log_2) of the groups of birds

Treatment	Species	Before vaccination	Before challenge
Control	Pheasant	< 2	< 2
	G partridge	< 2	< 2
	R partridge	< 2	< 2
	Chicken	2.3	2.3
Inacti-vated	Pheasant	< 2	3.3
	G partridge	< 2	< 2
	R partridge	2.8	5.3
	Chicken	2.3	8.5
HB1	Pheasant	< 2	6.9
	G partridge	2	4.5
	R partridge	< 2	6.4
	Chicken	2.3	7.4
La Sota	Pheasant	< 2	4.9
	G partridge	2.3	4.4
	R partridge	< 2	6.1
	Chicken	2.3	7.3

Table 2 : Percentage morbidity and mortality in challenged birds.

Treatment	Species	Morbidity	Mortality	Morbidity plus Mortality	Day of start and end of signs
Control	Pheasant	0	100	100	(2 - 5)
	G partridge	0	100	100	(2 - 4)
	R partridge	35.3	64.7	100	(4 - 24)
	Chicken	0	100	100	(2 - 5)
Inactivated	Pheasant	0	22.2	22.2	(5 - 18)
	G partridge	0	89.4	89.4	(2 - 17)
	R partridge	5.5	0	5.5	(13 - 24)
	Chicken	0	0	0	
HB1	Pheasant	10.0	0	10.0	(3 - 4)
	G partridge	11.1	22.2	33.3	(3 - 24)
	R partridge	0	0	0	
	Chicken	0	0	0	
La Sota	Pheasant	0	0	0	
	G partridge	5.0	45.0	50.0	(3 - 18)
	R partridge	0	0	0	
	Chicken	0	0	0	

COMPARISON OF HAEMAGGLUTINATION INHIBITION TITRES AND NEUTRALISATION INDICES OF OSTRICH SERA.

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Summary

The titres of 147 ostrich sera were determined in the haemagglutination inhibition (HAI) test and compared to the neutralisation indices (NI) of the sera determined in a microneutralisation test (SNT) using tissue culture as a substrate to detect virus infectivity. Remarkably, no statistically significant association between the NI and HAI titre was detected. However, the predictive value of the HAI test to detect antibodies against NDV at the flock level is high.

Materials and Methods

The haemagglutination inhibition test was performed as described in EEC directive 92/40. Eight haemagglutination units and chicken red blood cells (RBCs) were used in the test. The ostrich sera were not pre-absorbed with RBCs.

Neutralisation index (NI) was determined by mixing ostrich sera diluted 1:10 with 10^7 EID_{50} of virulent NDV, PMV1/Ch/Nl/152608. The mixture was subsequently diluted on monolayers of chicken embryo related (CER) cell line and these monolayers were checked for CPE at 48 hours after infection. The neutralisation index is the difference between the $\log_{10}$ titre of the virus in the absence and presence of serum.

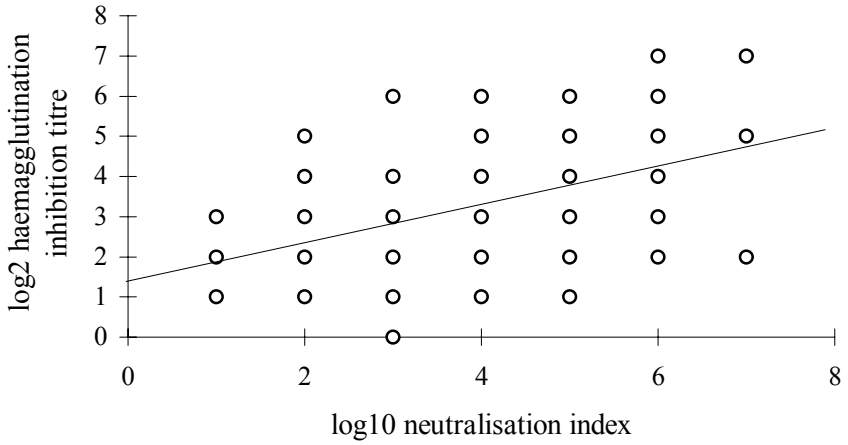
The sensitivity, specificity, predictive value and kappa were calculated using the computer programme EPISCOPE (K. Frankena *et al.* 1990). The sensitivity of a test was defined as the percentage of sera that scored positive in both tests to the total number of sera that scored positive in the reference test. The specificity is defined as the percentage of sera that scored negative in both tests to the total number of sera that scored negative in the reference test.

Results and Discussion

The 147 ostrich sera were tested for freedom of antibodies to NDV, because of intended exportation to the United Kingdom. No signs of disease were observed and according to the owner, animals were never vaccinated. Of the 147 sera, 135 reacted positive in the HAI, i.e. had a titre of ≥2, whereas 146/147 sera had a NI of ≥1. No correlation was found between the NIs and titres obtained in the HAI test, which is

illustrated in Figure 1. Linear regression analysis yielded a calculated correlation of determination (R^2 squared) of only 0.17.

Figure 1: Correlation between neutralisation indices and haemagglutination inhibition titres of ostrich sera



When the association between the NI and HAI titre was tested using the Spearman rank correlation coefficient on an sample of 30 sera, no association between NI and HAI titres was detected at the 1% level ($r_{S,B} = 0.394$).

Arbitrarily, sera with NI >1 were considered to react positively in the SNT; two sera directed against the H5 and H7 subtype of influenza virus that were prepared in SPF chickens and that were included in the test had NIs of 0. Since, obviously, clinical signs can not be used to validate test results, the SNT was used as reference to validate the HAI. The sensitivity of the HAI was 91.8% (Table 1). When the HAI is used as reference test, the sensitivity of the SNT was 99.3% (data not shown). The number of negative sera in the panel was too small to make an estimation of the specificity of both tests and also caused the kappa value (an estimate of the agreement between both tests) to be extremely low. When the SNT was considered positive at a NI values of 2 or larger, the sensitivity of the HAI and SNT was equal, 93.3%, when either the SNT or HAI was used as reference (Table 2). The specificity of both test is 25%. However, the estimation of the specificity is inaccurate because of the low number of negative ostrich sera that are included in the test.

The results show that individual ostriches that scored negative in the HAI test should not be exported when other animals in the flock have positive antibody titres in the HAI test. In this case 12 ostriches would have been exported although all of them had NI of 1 or higher; 4 animals had NI of > 3.

Table 1. Comparison of the haemagglutination with the SNT using the latter as reference test

Haemagglutination inhibition test	Neutralisation test (NI ≥1)	
	+	-
+	134	1
-	12	0
Sensitivity	91.8% (87.3 - 96.2)[a]	
Specificity	0 [b]	
Predictive value	99.3 (97.8 - 100.7)	
Kappa	-0.01	

[a] sensitivity defined as the number of positive sera in the HAI only as percentage of the total number of SNT positive sera. Between brackets 95% confidence.

[b] the number of negative sera is too low to compare the specificity of both tests.

Table 2. Comparison of the haemagglutination with the SNT as reference test

Haemagglutination inhibition test	Neutralisation test (NI ≥ 2)	
	+	-
+	126	9
-	9	3
sensitivity	93.3% (87.4 - 97.5)[a]	
specificity	25 (0.5 - 49.5)	
Predictive value	93.3 (89.1 - 97.8)	
kappa	0.18	

[a] see legend of Table 1

SESSION VI - CONCLUSIONS AND RECOMMENDATIONS

The feeling of the participants was that in terms of conclusions and problems, little had changed since last year and that the recommendations made in the Proceedings of the First Annual Meeting and the extent to which they had been fulfilled should be discussed.

Proposals from Joint First Annual Meetings of EU National Laboratories 1993

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

The representative of the Commission reported that the Commission had made representation to Member States during 1994, but noted the problems in this area discussed at the present meeting.

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

The consensus of the participants was that the role of the National Laboratories was now more fully understood and that this recommendation could be withdrawn.

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

The participants endorsed this recommendation.

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

The representative of the Commission pointed out that this requirement was essentially part of the Directive dealing with trade from third countries.

5. *The Meeting considered that the identified problem of disease in non-commercial poultry [i.e. hobby, show birds etc.] poses an extremely serious*

threat to the poultry industry and current legislation covering trade in and movement of such birds should be reviewed.

The representative of the Commission pointed out that legislation was currently in progress dealing with this problem.

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

The Commission had not done this directly, but this problem had been stressed at meetings of the Standing Veterinary Committee for action by Member States.

7. *The Meeting concluded that it would be extremely helpful if it were possible to distinguish between ND antibody titres produced by vaccination and those produced by field infection. Research leading to this situation, such as vaccines based on the fusion protein alone should be encouraged.*

The participants endorsed this recommendation.

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

The participants endorsed this recommendation.

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

The Meeting noted the advances that had been made, particularly in the demonstration that virulent viruses possessed multiple basic amino acids at the cleavage site of the F0 protein for Newcastle disease virus and the haemagglutinin for influenza viruses. However, it was agreed that there is not yet a practicable alternative to the pathogenicity index tests.

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

The participants discussed this recommendation at length. It was felt that in addition it should be stressed that quarantine should take place at the first country of import and that quarantine regulations should be harmonised throughout the EU.

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

The participants endorsed the first part of this recommendation. The representative of the Commission pointed out that legislation covering disinfectants was currently in progress. Some Member States already published lists of disinfectants approved for NDV and AI.

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

It was agreed that while this was still desirable, current policy in the Commission meant that it was unlikely that meetings outside Brussels would be possible.

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As a result of an editorial error the following report was omitted from the proceedings. The editor apologises to Dr Johann Krogsrud for that omission.

NEWCASTLE DISEASE: COUNTRY REPORT FROM NORWAY

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

During 1993 a total of 121 broiler and layer parent flocks in 113 different farms were tested with the standard HI test in connection with a health certificate programme. Four layer parent flocks in two farms had seropositive animals. Altogether 3,166 blood samples were tested.

In 1993 an avian paramyxovirus-1 (PMV-1) was isolated from seroconverting chicks. The virus was found to have an intracerebral pathogenicity index well below 0.7.

Later the isolate was characterized at the Community Reference Laboratory, Weybridge. The intracerebral pathogenicity index test gave a value of 0.46. HI tests using monoclonal antibodies (Mabs) specific for La Sota and F vaccine strains and for the PMV-1 pigeon variant virus, respectively, were negative. The binding pattern to cells infected with the Norwegian isolate using a panel of 28 mAbs was similar to a group of viruses with low virulence, of which the majority has been isolated from feral ducks and geese.

During 1994 a more comprehensive serological survey has been carried out in the poultry population in addition to the regular health certificate testing. The main purpose of this work has been to contribute to the achievement of ND-free status for Norway within the EU/EFTA community. Every parent flock (epidemiological unit) in the country more than 10 weeks of age at the time of sampling in May/June has been tested (a total of 77 flocks in 59 farms). This investigation programme revealed seven chicken flocks in three farms showing antibody positive birds. In addition seroreactors were found in a goose breeding unit. The duck and turkey breeding flocks were negative for antibodies to PMV-1.

Pullet rearing flocks have also been tested in 1994. Out of 56 flocks in 32 farms, three flocks on one farm were positive for antibodies to PMV-1. This farm

routinely receives day-old chicks from one of the parent farms with seropositive birds.

In this survey 60 blood samples have been taken from each flock. A total of 10,100 sera have been tested so far in 1994.

Antibodies to PMV-1 in chicken have been recorded only in layers. All four farms with seropositive flocks recorded are located within a small area in the south-western part of the country. The virus seems to circulate between flocks in each of these farms due to close contact and suboptimal hygiene regimens. Sanitation programmes individually designed for these farms have now been initiated. All new flocks in these farms will be tested, and restrictions on sale of chicks will be imposed if new seroreactors should be found.

The fact that the pullet rearing flocks, except the three flocks on one farm, have been negative for PMV-1 antibodies indicates that spread of the infection with day-old chicks has been of minor importance.

PMV-1 infection in pigeons has not been recorded since 1985. All racing pigeons are vaccinated with an inactivated vaccine. Vaccination of other poultry is prohibited.