COMMISSION OF THE EUROPEAN COMMUNITIES

COM(91) 304 final

Brussels, 30 July 1991

Proposal for a

COUNCIL REGULATION (EEC)

introducing Community measures
for the control of avian influenza

(presented by the Commission)

EXPLANATORY MEMORANDUM

Avian influenza in its highly pathogenic form is a contagious and serious disease of poultry. It is caused by a virus which may show a great variation in pathogenicity and in the clinical signs produced in susceptible birds. The disease has a worldwide distribution and the virus can be harboured by migrating birds, in particular by certain migratory waterfowls.

Article 19 of Council Directive 90/539/EEC of 15 October 1990 on animal health conditions governing intra-Community trade in, and imports from third countries of, poultry and hatching eggs[1], foresee the adoption of control rules for combating avian influenza before 1 July 1991.

The proposed measures have the aim of eradicating and preventing the spread of avian influenza in the event outbreaks should occur. This will be done by "stamping-out" with or without the use of vaccine and carefully controlling the movement of poultry, poultry products, vehicles and any other substance liable to transmit avian influenza virus. Measures must be introduced as soon as the presence of avian influenza virus is suspected so that immediate and effective action can be taken.

To ensure such actions the present proposal consists of obligations to Member States, which include

- to arrange for an investigation to confirm or rule out the presence of avian influenza when poultry is suspected of being infected;

- to place holdings under surveillance and prohibit movements to and from holdings during the surveillance period when avian influenza is suspected;

- to kill and destroy infected birds when avian influenza has been confirmed;

(1) OJ No L 303, 31.10.1990, p. 6

- to perform a thorough epizootiological inquiry when avian influenza is suspected and confirmed;

- to establish protection zones (3 km) and surveillance zones (10 km) around infected holdings;

- the establishment of laboratories providing the technical assistance necessary for correct implementation of disease control measures;

- to inform the Commission about vaccination programmes;

- to prepare a contingency plan.

PROPOSAL

for a

COUNCIL REGULATION (EEC)

introducing Community measures

for the control of avian influenza

THE COUNCIL OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Economic Community, and in particular Article 43 thereof,

Having regard to the proposal from the Commission,

Having regard to the opinion of the European Parliament,

Having regard to the opinion of the Economic and Social Committee,

Whereas poultry is listed in Annex II of the Treaty; whereas the marketing of poultry constitutes an important source of revenue for the agricultural population;

Whereas, it is necessary to establish at Community level the control measures to be taken in the event of outbreak of the highly pathogenic form of avian influenza, caused by an influenza virus with specific characteristics, and hereafter termed by avian influenza in order to ensure national development of the poultry sector and contribute to the protection of animal health in the Community;

Whereas an outbreak of avian influenza can quickly take on epizootic proportions, causing mortality and disturbances on a scale liable to reduce sharply the profitability of farming or poultry as a whole;

Whereas action must be taken as soon as the presence of the disease is suspected so that immediate and effective control measures can be implemented when its presence is confirmed;

Whereas it is necessary to prevent any spread of the disease as soon as an outbreak occurs, by carefully monitoring movements of animals and the use of products liable to be contaminated, and where appropriate, by vaccination;

Whereas diagnosis of the disease must be carried out under the auspices of a responsible laboratory, the coordination of which must be ensured by a Community reference laboratory;

Whereas common measures for the control of avian influenza form a basis for maintaining a uniform standard of animal health;

Whereas it is appropriate to confer upon the Commission the task of taking the necessary applicatory measures;

HAS ADOPTED THIS REGULATION:

Article 1

1. This Regulation defines the Community control measures to be applied in the event of an outbreak of avian influenza in poultry without prejudice to the Community provisions governing intra-Community trade.

2. This regulation shall not apply, in the event of detection of avian influenza in other birds, however, in this case, Member States shall inform the Commission of any measure they take.

Article 2

1. For the purpose of this Regulation, the definitions given in Article 2 of Council Directive 90/539/EEC on animal health conditions governing intra-Community trade in, and imports from third countries of poultry and hatching eggs[1] shall apply as appropriate.

2. The following definitions shall also apply:

(a) "infected poultry" means any poultry:

- in which the presence of avian influenza, in the meaning of Annex I, has been officially confirmed following an examination by an approved laboratory, or
- in the case of second and subsequent outbreaks in which clinical signs or post-mortem lesions consistent with avian influenza are present;

(b) "poultry suspected of being infected" means any poultry showing clinical signs or post-mortem lesions which are such that the presence of avian influenza may reasonably be suspected; or the presence of influenza A virus of subtype H5 or H7 has been demonstrated.

(c) "poultry suspected of being contaminated" means any poultry which may have been directly or indirectly exposed to the avian influenza virus, or influenza A virus of H5 subtype or H7 subtype.

(d) "competent authority": veterinary authority appointed for the purpose by the national administration of the country concerned, being directly responsible to the national administration within the scope of this Regulation, and reporting through the national administration.

(e) "official veterinarian": the veterinarian designated by the competent authority of the Member State.

(1) OJ No L 303, 31.10.1990, p. 6

Article 3

Any suspected case of avian influenza shall be immediately notified to the competent authority.

Article 4

1. When poultry are suspected of being infected or contaminated with avian influenza, the official veterinarian shall immediately arrange an investigation to confirm or rule out the presence of the disease and, in particular, must take or have taken the samples necessary for laboratory examination.

2. As soon as the suspected infection is notified, the competent authority shall have the holding placed under official surveillance and shall in particular require that:

   (a) a record be made of all categories of poultry on the holding showing in respect of each of the categories the numbers of poultry which have died, which show clinical signs, and which show no signs. The record shall be kept up-to-date and be produced on request and may be checked at each visit;

   (b) all poultry on the holding must be kept in their living quarters or some other place where they can be isolated and without contact with other birds;

   (c) no poultry enter or leave the holding;

   (d) all movement

   - of persons, other animals and vehicles to or from the holding,

   - of poultry meat or carcasses, or of animal feed, implements, waste and litter or any other thing liable to transmit avian influenza virus from the holding

   be prohibited unless authorized by the official veterinarian;

(e) no eggs shall leave the holding with the exception of table eggs which have been disinfected to the satisfaction of the official veterinarian;

(f) appropriate means of disinfection be used at the entrances and exits of buildings housing poultry and of the holding itself;

(g) an epizootiological inquiry be carried out in accordance with Article 7.

3. Until such time as the official measures laid down in paragraph 2 are enforced, the owner or keeper of any poultry in which disease is suspected shall take all reasonable action to ensure compliance with paragraph 2, particularly indents (b), (c), (d) and (e).

4. The competent authority may apply any of the measures provided for in paragraph 2 to other holdings should their location, their configuration, or contacts with the holding where the disease is suspected give reason to suspect possible contamination.

5. The measures referred to in paragraphs 2, 3 and 4 shall not be withdrawn until the suspicion of avian influenza has been ruled out by the official veterinarian.

Article 5

1. As soon as the presence of avian influenza has been officially confirmed on a holding, the competent authority, in addition to the measures listed in Article 4(2) shall require the following measures to be undertaken:

(a) all poultry on the holding shall without delay be killed on the spot. The poultry which have died or have been killed and all eggs shall be destroyed. These operations shall be carried out in a way which minimizes the risk of spreading disease;

(b) any substance or waste, such as animal feed, litter or manures liable to be contaminated, shall be destroyed or treated; this treatment, carried out in accordance with the instructions of the official veterinarian, shall ensure the destruction, of any avian influenza virus present;

(c) where poultry have been slaughtered during the presumed incubation period of disease the meat from those poultry shall wherever possible be traced and destroyed;

(d) hatching eggs laid during the presumed incubation period which have been moved from the holding shall be traced and destroyed; but poultry which have already hatched from the eggs shall be placed under official surveillance; table eggs laid during the presumed incubation period which have been moved from the holding shall wherever possible be traced and destroyed;

(e) after carrying out operations listed in sub-paragraph (a) the buildings used for housing poultry, their surroundings, the vehicles used for transport and all equipment likely to be contaminated shall be cleaned and disinfected in accordance with the provisions of Article 11;

(f) no poultry shall be reintroduced to the holding until at least 21 days after completion of operations provided for in sub-paragraph (e);

(g) an epizootiological inquiry shall be carried out in accordance with Article 7.

2. The competent authority may extend the measures provided for in paragraph 1 to other holdings should their location, their configuration, or contact with the holding where the disease has been confirmed given reason to suspect possible contamination.

Article 6

1. In the case of holdings which consist of two or more separate flocks the competent authority may grant a derogation from the requirements of Article 5(1)(a), for healthy flocks of a holding which is infected, provided that the official veterinarian has confirmed that the operations carried out there are such that the flocks are completely separate as regards housing, keeping and feeding, so that the virus cannot spread from one flock to another.

2. The Commission shall in accordance with the procedure in Article 21 lay down the criteria to be applied for granting a derogation as referred to in paragraph 1.

Article 7

1. The epizootiological inquiry shall deal with:

   - the length of time during which avian influenza may have existed on the holding;

   - the possible origin of the avian influenza on the holding and the identification of other holdings on which there are poultry which may have become infected or contaminated from the same source;

   - the movement of persons, poultry or other animals, vehicles, eggs, meat and carcasses and any implement or substance likely to have carried avian influenza virus to or from the holding in question.

2. In order to provide full coordination of all measures necessary to ensure eradication of avian influenza as quickly as possible and for the purpose of carrying out the epidemiological inquiry, a crisis unit shall be be established.

The general rules concerning National Crisis units and Community crissis units are as laid down in Council Regulation ../.../EEC.

Article 8

1. Where the official veterinarian has reason to suspect that poultry on any holding may have been contaminated as a result of the movement of persons, animals or vehicles or in any other way, that holding shall be placed under official control in accordance with paragraph 2.

2. The purpose of the official control shall be to detect immediately any suspicion of avian influenza, count the poultry and monitor their movements and, where appropriate, to take the action provided for in paragraph 3.

3. When a holding is subject to the official control under the provisions of paragraph 2, the competent authority shall prohibit removal of poultry from the holding other than for transport directly to a slaughterhouse under official supervision for the purpose of immediate slaughter. Prior to granting such authorization, the official veterinarian must have carried out a clinical examination of all the poultry to exclude presence of avian influenza on the holding. The above movement restrictions shall be imposed for a period of 21 days from the latest date of potential contamination. However, such restrictions must apply for a period of at least 7 days.

4. Where it considers that conditions permit, the competent authority may limit the measures provided for in this article to a part of the holding and to the poultry contained therein, provided that the poultry there have been housed, kept and fed completely separately by separate staff.

Article 9

1. Once the diagnosis of avian influenza has been officially confirmed, the competent authority shall establish around the infected holding an infected area involving a protection zone based on a minimum radius of 3 km and a surveillance zone based on a minimum radius of 10 km. The establishment of these zones must take account of natural boundaries and the epidemiology of the outbreak.

2. The measures applied in the protection zone shall include:

   (a) the identification of all holdings having poultry within the zone;

   (b) periodic visits to all the holdings having poultry, a clinical examination of those poultry including, if necessary, the collection of samples for laboratory examination; a record of visits and findings must be kept;

   (c) the keeping of all poultry in their living quarters or some other place where they can be isolated;

   (d) the use of appropriate means of disinfection at the entrances and exits of the holding;

   (e) the control of movements of persons handling poultry, poultry carcasses and eggs and vehicles carrying poultry, carcasses and eggs within the zone; in general transport of poultry is prohibited except for transit by major highways or railways;

   (f) a prohibition on removing poultry and hatching eggs from the holding on which they are kept unless the competent authority has authorized the transport:

(i) of poultry for immediate slaughter to a slaughterhouse preferably located in the infected area or, if that is not possible, to a slaughterhouse designated by the competent authority outside the infected area. The special health mark provided for in Article 6(1) of Regulation ../..../EEC on animal health conditions governing intra-Community trade in and imports from third country of fresh poultry meat and fresh meat of reared game birds[1] must be applied to this poultry meat;

(ii) of day-old chicks or ready to lay pullets to a holding within the infected area at which there are no other poultry. This holding must be placed under the official control provided for in Article 8, paragraph (2);

(iii) of hatching eggs to a hatchery situated inside the infected area or one outside the area designated by the competent authority; before dispatch eggs and their packing must be disinfected;

Movements allowed in (i), (ii) and (iii) shall be directly executed, under official control. They shall be authorized only after the official veterinarian has carried out a health inspection of the holding. The means of transport used must be cleaned and disinfected before and after use.

(g) a prohibition on removing or spreading poultry manure or litter without authorization;

(h) the prohibition of fairs, markets, shows or other gatherings of poultry or other birds.

3. The measures applied in the protection zone shall be maintained for at least 21 days after the carrying out, of preliminary cleaning and disinfection operations on the infected holding in accordance with Article 11. The protection zone shall thereafter be part of the surveillance zone.

---

(1) COM (89) 507 final

4. The measures applied in the surveillance zone shall include:

(a) the identification of all holdings having poultry within the zone;

(b) the control of poultry and hatching egg movement within the zone;

(c) a prohibition on the movement of poultry out of the zone during the first 15 days, except for movement directly to a slaughterhouse outside the surveillance zone designated by the competent authority. The special health mark provided for in Article 6 of Regulation ../..../EEC on animal health conditions governing intra-Community trade and imports from third countries of fresh poultry meat and fresh meat of reared game birds must be applied to this poultry meat;

(d) a prohibition on the movement of hatching eggs out of the surveillance zone unless to a premises designated by the competent authority. Before dispatch the eggs and their packing must be disinfected;

(e) a prohibition on the movement of poultry manure and litter out of the zone;

(f) a prohibition of fairs, markets, shows or other gatherings of poultry and other birds;

(g) without prejudice to provisions of (b) and (c) the prohibition of transport of poultry, except for transit by major highways or railways.

5. The measures applied in the surveillance zone shall be maintained for at least 30 days after the carrying out of preliminary cleaning and disinfection operations on the infected holding in accordance with Article 11.

Article 10

1. The competent authority shall determine the arrangements allowing them to trace the movement of eggs and poultry.

2. The owner or keeper of poultry shall be required to supply the competent authority, on request by that authority, with information concerning poultry and eggs entering or leaving his holding.

3. All persons engaged in the transport or marketing of poultry and eggs shall be able to supply the competent authority with information concerning the movements of poultry and eggs which they have transported or marketed and to furnish all the details concerning such information.

Article 11

1. The disinfectants to be used and their concentrations shall be approved by the competent authority.

2. The cleaning and disinfection operations shall be carried out under official supervision, in accordance with instructions given by the official veterinarian.

Article 12

Collection of samples and laboratory testing to detect the presence of avian influenza virus shall be carried out in accordance with Annex I.

Article 13

1. Each Member State shall designate:

   (a) one or more national laboratories at which facilities and expert personnel shall be maintained to permit assessment of the pathogenicity of influenza virus isolates (Annex I Chapter 7) and identification of influenza A viruses of H5 or H7 subtypes;

(b) one or more national laboratories at which reagents for use in regional laboratories are tested;

(c) one or more national institutes or laboratories at which authorized vaccines may be tested in order to verify their conformity with the specifications laid down in the marketing authorization.

2. The national laboratories listed in Annex II are responsible for coordinating standards and methods of diagnosis, use of reagents and testing of vaccines.

3. The national avian influenza laboratories referred to in paragraph 2 shall be responsible for coordinating the standards and diagnostic methods laid down in each avian influenza diagnostic laboratory within the Member State. To this end:

(a) they may provide diagnostic reagents to regional laboratories;
(b) they shall control the quality of all diagnostic reagents used in that Member State;
(c) they shall arrange comparative tests periodically;
(d) they shall hold isolates of avian influenza virus from cases confirmed in that Member State;
(e) they shall ensure the confirmation of positive results obtained in regional diagnostic laboratories.

4. The national laboratories listed in Annex II shall liaise with the Community reference laboratory referred to in Article 14.

Article 14

The Community reference laboratory of avian influenza is mentioned in Annex III. The powers and duties of the laboratory shall be laid down, in so far they are not already covered by Article 28 of Council Decision 90/424/EEC(1) on expenditure in the veterinary field, in accordance with the procedure laid down in Article 21.

(1) OJ No L 224, 18.08.1990, p. 19

Article 15

1. Vaccination against avian influenza with vaccines authorized by the competent authority may be used only to supplement the control measures carried out when the disease appears.

2. The decision to introduce vaccination to supplement control measures shall be taken by the Commission in collaboration with the Member State concerned, acting in accordance with the procedure laid down in Article 21. This decision shall have particular regard to:

   - the concentration of poultry in the affected area;
   - the characteristics and composition of the vaccine to be used:
   - the procedures for supervision of the distribution, storage and use of vaccines;
   - the species and categories of poultry which shall be subject to vaccination;
   - the areas in which vaccination shall be carried out.

3. Where a Member State is authorized, in accordance with the provisions of paragraph 2, to have recourse to emergency vaccination on a limited part of its territory the status of the remainder of the territory shall not be affected, provided that the immobilization measures for the vaccinated animals are effective during a period of 3 months following the end of the vaccination operations.

Article 16

1. When, if a given region, an epizootic of avian influenza is exceptionally serious and is tending to spread, the Member States concerned:

   - shall declare a demarcated territorial area including at least all the protection and surveillance zones in that area to be a "high health risk area";

- shall apply the measures provided for in Article 9(3) in the "high health risk area";

- shall prohibit all live poultry and hatching eggs from leaving the "high health risk area";

- shall inform the Commission and other Member States within the framework of the Standing Veterinary Committee about disease situation and applied control measures.

2. The boundaries of the "high health risk area" may be revised with a progressive elimination of surveillance zones. The measures laid down in the preceding paragraph shall be discontinued after the elimination of the last surveillance zone.

3. If an exceptionally serious situation continues, the measures to be taken by the Member States concerned, in particular determination of the "high health risk area" and recourse to the provisions of Article 15, must be decided in accordance with the procedure laid down in Article 21.

Article 17

1. Each Member State shall draw up a contingency plan, specifying the national measures to be implemented in the event of an outbreak of avian influenza.

   This plan should allow access to facilities, equipment, personnel and all other appropriate materials necessary for the rapid and efficient eradication of the outbreak. It must give a precise indication of the vaccine requirements which each Member State concerned considers it needs in the event of emergency vaccination.

2. The criteria to be applied for drawing up the contingency plan shall be those laid down in Commission Decision 91/42/EEC[1] of 8 January 1991 laying down the criteria to be applied when drawing up contingency plans for the control of foot and mouth disease, in application of Article 5 of Council Directive 90/423/EEC, which shall apply mutatis mutandis.

   The Commission may, in accordance with Article 21, amend or supplement those criteria taking into account the specific nature of avian influenza.

3. Plans drawn up in accordance with the criteria provided for in paragraph 2 shall be submitted to the Commission not later than 12 months after this regulation enters into force.

4. The Commission shall examine the plans in order to determine whether they permit the desired objective to be attained and shall suggest to the Member State concerned any amendments required in particular to ensure that they are compatible with those of the other Member States.

   The Commission shall approve the plans, if necessary amended, in accordance with the procedure laid down in Article 21.

   The plans may subsequently be amended or supplemented, in accordance with the same procedure, to take into account developments in the situation.

Article 18

Veterinary experts from the Commission may, in collaboration with the authorities of the concerned Member State, insofar as is necessary to ensure uniform application of this Regulation, make on-the-spot checks; the Commission shall inform the Member States of the results of the investigation.

(1) OJ No L 23, 29.01.1991, p. 29

A Member State in whose territory a check is being carried out shall give all the necessary assistance to the experts in carrying out their duties.

The general provisions for implementing this Article shall be determined in accordance with the procedure laid down in Article 21.

Article 19

Modalities for financial participation of the Community to the actions arising from this Regulation are fixed in Council Decision 90/424/EEC on expenditure in the veterinary field.

Article 20

The Annexes to this Regulation may be amended by the Commission, in accordance with the procedure laid down in Article 21, in particular in order to take into account the development in diagnostic procedures.

Article 21

1. The Commission shall be assisted by the Standing Veterinary Committee, hereinafter referred to as "the Committee"; set up by Decision 68/361/EEC(1).

2. Where the procedure laid down in this Article is to be followed, the following provisions shall apply.

   The representative of the Commission shall submit to the Committee a draft of the measures to be taken. The Committee shall deliver its opinion on the draft within a time limit which the Chairman may lay down according to the urgency of the matter, if necessary by taking a vote.

   The opinion shall be recorded in the minutes; in addition, each Member State shall have the right to ask to have its position recorded in the minutes.

(1) OJ No L 255, 18.10.1968, p. 23

The Commission shall take the utmost account of the opinion delivered by the Committee. It shall inform the Committee of the manner in which its opinion has been taken into account.

Article 22

This Regulation shall enter into force on 1 July 1991.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels

For the Council

ANNEX I

DIAGNOSTIC PROCEDURES FOR THE CONFIRMATION AND DIFFERENTIAL DIAGNOSIS OF AVIAN INFLUENZA (AI)

The following procedures for the isolation and characterization of avian influenza viruses should be regarded as guidelines and the minima to be applied in the diagnosis of the disease.

For the purpose of the diagnostic procedures for the confirmation and differential diagnosis of avian influenza the following definition shall apply.

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

CHAPTER 1: Sampling and treatment of samples

1. Samples

Cloacal swabs (or faeces) and tracheal swabs from sick birds; faeces or intestinal contents, brain tissue, trachea, lungs, liver, spleen and other obviously affected organs from recently dead birds.

2. Treatment of samples

The organs and tissues listed above may be pooled, but separate treatment of faecal material is essential. Swabs should be placed in sufficient antibiotic medium to ensure full immersion. Faeces samples and organs should be homogenised (in an enclosed blender or using a pestle and mortar and sterile sand) in antibiotic medium and made to 10-20% w/v suspensions in the medium. The suspensions should be left for about two hours at ambient temperature (or longer periods at 4°C) and then clarified by centrifugation (eg. 800 to 1000 x g for 10 minutes).

3. Antibiotic medium

Different laboratories have used various formulations of antibiotic medium with success and National Laboratories will be able to offer advice for a particular country. High concentrations of antibiotics are required for faeces amples and a typical mixture is:

10,000 units/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml gentamycin and 5,000 units/ml mycostatin in phosphate buffered saline. These levels can be reduced up to five-fold for tissues and tracheal swabs. For control of Chlamydia organisms 50 mg/ml oxytetracycline may be added. It is imperative when making the medium that the pH is checked after the addition of the antibiotics and readjusted to pH 7.0-7.4.

CHAPTER 2: Virus Isolation

Virus isolation in embryonated fowls' eggs

The clarified supernatant fluid should be inoculated in 0.1-0.2 ml amounts into the allantoic cavity of each of a minimum of four embryonated, fowls' eggs which have been incubated for 8-10 days. Ideally, these eggs should be obtained from a specific pathogen free flock, but when this is impracticable it is acceptable to use eggs obtained from a flock shown to be free of antibodies to Avian Influenza. The inoculated eggs are held at 37°C and candled daily. Eggs with dead or dying embryos as they arise, and all remaining eggs six days after inoculation should be chilled to 4°C and the allantoic-amniotic fluids tested for haemagglunination activity. If no haemagglutination is detected the above procedure is repeated using undiluted allantoic/amniotic fluid as inoculum.

When haemagglutination is detected the presence of bacteria should be excluded by culture. If bacteria are present the fluids may be passed through a 450nm membrane filter, further antibiotics added and inoculated into embryonated eggs as above.

CHAPTER 3: Differential diagnosis

1. Preliminary differentiation

Because it is important that control measures aimed at limiting the spread of virus should be implemented as soon as possible, each regional laboratory should be in a position to identify any isolated haemagglutinating virus as influenza viruses of H5 or H7 subtype in addition to Newcastle disease virus. The haemagglutinating fluids should be used in a haemagglutination inhibition test as described in Chapters 5 and 6. Positive inhibition ie. 2^4, or more, with polyclonal antisera specific for H5 or H7 subtypes of influenza A and of a titre of at least 2^9 would serve as preliminary identification enabling the imposition of interim control measures.

2. Confirmatory identification

Since there are 13 haemagglutinin subtypes and 9 neuraminidase subtypes of influenza viruses and variations occur within each of these it is not practicable nor cost effective for each national laboratory to hold antisera which will allow full antigenic characterization of influenza isolates. However, each national laboratory should:

(i) confirm that the isolate is an influenza A virus using an immunodoublediffusion test to detect group antigen as described in Chapter 9 of this Annex (immunofluorescence or ELISA techniques to detect group antigens may be used if preferred by the national laboratory);

(ii) determine whether or not the isolate is of H5 or H7 subtype;

(iii) carry out an intravenous pathogenicity index test in six-week-old chickens as described in Chapter 7 of this Annex. Intravenous pathogenicity indices of greater than 1.2 indicate the presence of virus requiring the full implementation of control measures (It would be a useful exercise if national laboratories also carried out tests to determine the capacity of an isolate to produce plaques in cell cultures as specified in Chapter 8).

National laboratories should immediately submit all avian influenza and all H5 and H7 isolates to the Community Reference Laboratory for full characterization.

3. Further typing and characterization of isolates

The Community Reference Laboratory should receive all haemagglutinating viruses from the national laboratories for further antigenic and genetic studies to enable a greater understanding of the epizootiology of the disease(s) within the European Community in keeping with the functions and duties of the reference laboratory.

In addition to these duties the Community Reference Laboratory shall carry out full antigenic typing for all influenza viruses received. For H5 and H7 viruses which do not have intravenous pathogenicity indices greater than 1.2, nucleotide sequencing of the haemagglutinin gene to determine whether or not there are multiple basic animo acids at the cleavage site of the haemagglutinin protein should also be carried out.

CHAPTER 4: Serological tests for avian influenza virus antibodies

1. During eradication programmes where the H subtype of the virus responsible is already known, or by using the homologous virus as antigen, serological monitoring for evidence of infection may be done using haemagglutination inhibition tests as described in Chapters 5 and 6.

   If the haemagglutinin subtype is not know, evidence for infection with influenza A viruses may be obtained by detecting antibodies directed to the group specific antigens. For this purpose either an immunodoublediffusion test (as described in Chapter 9 of this Annex) or an ELISA test may be used (a problem with ELISA is the host specificity of the test since it is dependent on the detection of host immunoglobulins). Waterfowl rarely give positive results in immunodoublediffusion tests and, unless the subtype is known, it is probably only practicable to examine such birds for the presence of antibodies to H5 and H7 subtypes.

2. (a) Samples

   Blood samples should be taken from all birds if the flock size is less than 20 and from 20 birds from larger flocks (This will give a >99% probability of detecting at least one positive serum if 25% or more of the flock is positive, regardless of flock size). The blood should be allowed to clot and serum removed for testing.

   (b) Examination for antibodies

   Individual serum samples should be tested for their ability to inhibit influenza virus haemagglutinating antigen in standard haemagglutination inhibition tests as defined in Chapter 6.

There is some debate as to whether 4 or 8 haemagglutinin units should be used for the HI tests. It would appear that either is valid and the choice should be left to the discretion of the national laboratories. However, the antigen used will affect the level at which a serum is considered positive: - for 4 HAU a positive serum is any showing a titre of 2^4 or greater, for 8 HAU a positive serum is any showing a titre of 2^3 or greater.

CHAPTER 5: Haemagglutination (HA) test

Reagents

1. Isotonic saline buffered with phosphate (0.05M) to pH 7.0-7.4.

2. Red blood cells (RBC) taken and pooled from a minimum of three specific pathogen free chickens (if not available blood may be taken from birds regularly monitored and shown to be free of Avian Influenza antibodies) into an equal volume of Alsever's solution. Cells should be washed three times in PBS before use. For the test a 1% suspension (packed cell v/v) is PBS is recommended.

3. The Community Reference Laboratory will supply or recommend H5 and H7 viruses of low virulence for use as standard antigens.

Procedure

1. Dispensed 0.025ml PBS into each well of a plastic microtitre plate (V-bottomed wells should be used).

2. Place 0.025ml of virus suspension (ie. allantoic fluid) in the first well.

3. Use a microtitration diluter to make two-fold dilutions (1:2 to 1:4096) of virus across the plate.

4. Dispense a further 0.025ml of PBS to each well.

5. Add 0.025ml of 1% red blood cells to each well.

6. Mix by tapping gently and place at 4°C.

7. Plates are read 30-40 minutes later when Red Blood Cells control are settled. Reading is done by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. Wells with no HA should flow at the same rate as the control cells with no virus.

8. The HA titre is the highest dilution that causes agglutination of the RBCs. That dilution may be regarded as containing one HA unit (HAU). A more accurate method for determining the HA titre is to do HA tests on virus from a close range of initial dilutions ie. 1:3, 1:4, 1:5, 1:6, etc. This is recommended for the accurate preparation of antigen for haemagglutination inhibition tests (Chapter 6).

CHAPTER 6: Haemagglutination Inhibition (HI) Test

Reagents

1. Phosphate Buffer Solution (PBS).

2. Virus containing allantoic fluid diluted with PBS to contain 4 or 8 HAU per 0.025ml.

3. 1% chicken RBCs.

4. Negative control chicken serum.

5. Positive control serum.

Procedure

1. Dispense 0.025ml PBS into all wells of a plastic microtitre plate (with V-bottomed wells).

2. Place 0.025ml of serum into first well of plate.

3. Use microtitration diluter to make two-fold dilutions of serum across plate.

4. Add 0.025ml of diluted allantoic fluid containing 4 or 8 HAU.

5. Mix by tapping and place plate at 4°C for a minimum of 60 minutes or room temperature for a minimum of 30 minutes.

6. Add 0.025ml 1% RBCs to all wells.

7. Mix by gentle tapping and place at 4°C.

8. Plates are read after 30-40 minutes when control RBCs are settled. This is done by tilting and observing the presence or absence of tear-shaped streaming at the same rate as control wells containing RBCs (0.025ml) and PBS (0.05ml) only.

9. The HI titre is the highest dilution of antiserum causing complete inhibition of 4 or 8 units of virus (an HA titration to confirm the presence of the required HAU should be included in each test).

10. The validity of the results is dependent on obtaining a titre of less than 2^3 for 4 HAU or 2^2 for 8 HAU with the negative control serum and a titre of within one dilution of the known titre of the positive control serum.

CHAPTER 7: Intravenous Pathogenicity Index (IVPI)

1. Infective allantoic fluid from the lowest passage level available, preferably from the initial isolation without any selection, is diluted 10^{-1} in sterile isotonic saline.

2. 0.1ml diluted virus is injected intravenously into each of 10 six-week-old chickens (specific pathogen free birds should be used).

3. Birds are examined at 24 hour intervals for 10 days.

4. At each observation each bird is recorded normal (0), sick (1), severely sick (2) or dead (3).

5. Record results and calculate index as shown in this example:

Clinical Signs	Day after inoculation										Total Score
	1	2	3	4	5	6	7	8	9	10	
Normal	10	2	0	0	0	0	0	0	0	0	12 x 0 = 0
Sick	0	4	2	0	0	0	0	0	0	0	6 x 1 = 6
Severely sick*	0	2	2	2	0	0	0	0	0	0	6 x 2 = 12
Dead	0	2	6	8	10	10	10	10	10	10	76 x 3 = 228
											TOTAL = 246

Index = mean score per bird per observation = $\frac{246}{100}$ = 2.46

* This has to be a subjective clinical judgement but normally this would involve birds showing more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of exposed skin or wattles, oedema of face and/or head, nervous signs.

CHAPTER 8: Assessment of Plaque-forming Ability

1. It is usually best to use a dilution range of virus to ensure that an optimum number of plaques are present on the plate. Ten-fold dilutions up to 10^{-7} in PBS should be sufficient.

2. Confluent monolayers of chick embryo cells or a suitable cell line (Madin-Darby bovine kidney for example) are prepared in 5cm diameter Petri dishes.

3. 0.2ml of each virus dilution is added to each of two Petri dishes and the virus allowed to absorb for 30 minutes.

4. After washing three times with PBS the infected cells are overlaid with the relevant medium containing 1% w/v agar and either 0.01mg/ml trypsin or no trypsin. It is important that no serum is added to be overlay medium.

5. After 72 hours incubation at 37°C the plaques should be of sufficient size. They are best seen by removing the agar overlay and staining the cell monolayer with crystal violet (0.5% w/v) in 25% v/v ethanol.

6. All viruses should give clear plaques when incubated in the presence of trypsin in the overlay. When trypsin is absent from the overlay only viruses virulent for chickens will produce plaques.

CHAPTER 9: Immunodoublediffusion

The preferred method to show the presence of influenza A virus is to demonstrate the possession of the nucleocapsid or matrix antigens which are shared by all influenza A viruses. This is generally done in immunodoublediffusion tests involving either concentrated virus preparations or extracts from infected chorioallantoic membranes.

Suitable preparations of concentrated virus may be made by simple high speed centrifugation of infectious allantoic fluid and disruption of virus to release the internal nucleocapsid and matrix antigens by treatment with the detergent sodium lauroyl sarcosinate. Acid precipitation may also be used by adding 1N HCL to infective allantoic fluid to give a final pH of 3.5-4.0, chilling for at least one hour at 0°C and low speed centrifugation at 1000g for 10 minutes. The supernatant may be discarded and the virus-containing precipitate resuspended in a minimum volume of glycine-sarkosyl buffer (1% sodium lauroyl sarcosinate buffered to pH 9.0 with a 0.5M glycine). These preparations possess both nucleocapsid and matrix antigens.

Beard (1970) described the preparation of nucleocapsid-rich antigen from chorioallantoic membranes removed from infected eggs. This method involves: removal of the chorioallantoic membranes from infected haemagglutinin positive eggs, grinding or homogenising the membranes, freezing and thawing three times followed by centrifugation at 1000g for 10 minutes. The pellet is discarded and the supernatant treated with 0.1% formalin for use as antigen.

Either of these two antigens may be used in immunodoublediffusion tests using 1% agarose, or agar, gels containing 8.0% sodium chloride made up to 0.1M phosphate buffer pH 7.2. Influenza A virus is confirmed by precipitin lines formed by test antigen and known positive antigen against a known positive antiserum coalescing to give a line of identity.

ANNEX II

LIST OF NATIONAL AVIAN INFLUENZA LABORATORIES

- Belgium	Institut National de Recherches Vétérinaires, Groeselenberg 99, B 1180 Bruxelles
- Denmark	National Veterinary Laboratory, Poultry Disease Division, Hangøvej 2 DK 8200 Aarhus N.
- Germany	Institut für Kleintierzucht der Bundesforschungsanstalt für Landwirtschaft, Braunschweig-Völkenrode, Postfach 280, D 3100 Celle
- France	Centre National d'Etudes Vétérinaires et Alimentation - Laboratoire Central de Recherches Agricoles et Porcines, B.P. 53, F 22440 Ploufragan
- Greece	
- Ireland	Veterinary Research Laboratory, Abbotstown, Castleknock, Dublin 15
- Italy	Istituto Patolgie Aviaire, Facolta di Medicina Veterinaria, Universita di Napoli, Via Aniezzo Falcone 394, I 80127 Napoli F Delpino 1
- Luxembourg	Institut National de Recherches Vétérinaires, Groeselenberg 99, B 1180 Bruxelles
- Netherlands	Centraal Diergeneeskundig Instituut, Vestiging Virologie, Hourtibweg 39, NL 8221 RA Lelystad
- Portugal	Laboratório Nactional de Investigaçao Veterinaria (LNIV), Estrada de Benfica 701, 1500 Lisboa
- Spain	
- United Kingdom	Central Veterinary Laboratory, New Haw, Weybridge, GB-Surrey KT15 3NB

ANNEX III

NAME OF THE COMMUNITY REFERENCE LABORATORY FOR AVIAN INFLUENZA

Central Veterinary Laboratory
New Haw
Weybridge
Surrey KT15 3NB
UNITED KINGDOM

ISSN 0254-1475

COM(91) 304 final

DOCUMENTS

EN 03

Catalogue number : CB-CO-91-344-EN-C

ISBN 92-77-74817-6

Office for Official Publications of the European Communities
L-2985 Luxembourg