# Commission Decision of 4 August 2006 approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC (notified under document number C(2006) 3477) (Text with EEA relevance) (2006/437/EC)

## COMMISSION DECISION

of 4 August 2006

approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC

(notified under document number C(2006) 3477)

(Text with EEA relevance)

(2006/437/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to Council Directive 2005/94/EC of 20 December 2005 on Community measures for the control of avian influenza and repealing Directive 92/40/EEC[(1)], and in particular the second subparagraph of Article 50(1) thereof,

Whereas:

(1) Directive 2005/94/EC provides for certain preventive measures relating to the surveillance and early detection of avian influenza and also minimum control measures to be applied in the event of an outbreak of that disease in poultry and other captive birds.

(2) It is necessary to lay down at Community level diagnostic procedures, sampling methods and criteria for the evaluation of the results of laboratory tests for the confirmation of an outbreak of avian influenza.

(3) Annex VII to Directive 2005/94/EC lays down the functions and duties of the Community reference laboratory for avian influenza in order to coordinate, in consultation with the Commission, the methods employed in the Member States for diagnosing that disease. Those functions and duties include the organisation of periodic comparative tests and the supplying of standard reagents at Community level.

(4) Laboratory tests have recently been developed to ensure a quick diagnosis of avian influenza.

(5) The experience gained in the control of avian influenza in recent years has resulted in the identification of the most suitable sampling procedures and criteria for evaluation of the results of the laboratory tests for a proper diagnosis of this disease in different situations.

(6) The measures provided for in this Decision are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health,

HAS ADOPTED THIS DECISION:

*Article 1*

The diagnostic manual, as provided for in Directive 2005/94/EC and set out in the Annex to this Decision, is approved.

*Article 2*

Member States shall apply the diagnostic manual from the date they transpose Directive 2005/94/EC or from 1 July 2007, whichever date is the earlier.

*Article 3*

This Decision is addressed to the Member States.

Done at Brussels, 4 August 2006.

*For the Commission*

Markos KYPRIANOU

*Member of the Commission*

ANNEX

DIAGNOSTIC MANUAL FOR AVIAN INFLUENZA

CHAPTER I

**Introduction, objectives and definitions**

1. In order to ensure uniform procedures for the diagnosis of avian influenza (AI) in the Community, this diagnostic manual sets out:

(a) guidelines and minimum requirements for diagnostic procedures, sampling methods and criteria for the evaluation of the results of laboratory tests for a proper diagnosis of AI;

(b) the laboratory tests to be used for the diagnosis of AI and the laboratory techniques to be used for the genetic typing of AI virus isolates;

(c) minimum bio-safety requirements and quality standards to be observed by the diagnostic laboratories and for the transport of samples.

2. This diagnostic manual is addressed to the authorities responsible for the control of AI. Therefore, it mainly concerns principles and applications of laboratory tests and the evaluation of their results, as well as laboratory techniques.

3. For the purpose of this diagnostic manual, in addition to the definitions in Article 2 of Directive 2005/94/EC, the following definition also applies:
‘diagnostic specimen’ means any animal material including a whole carcase being transported for diagnostic or investigational purposes, but excluding live infected animals.

4. The confirmation of AI in poultry and other captive birds must be in accordance with the procedures, sampling methods and criteria for the evaluation of the results of laboratory tests set out in this diagnostic manual, and be based on one or more of the criteria in points (a), (b) and (c):

(a) the detection of infectious virus, antigen or specific genetic material in samples of poultry or other birds' tissues, organs, blood or excreta;

(b) the detection of clinical signs and post-mortem lesions of disease in those birds;

(c) the demonstration of a specific antibody response in blood samples of those birds.

5. The confirmation of infection of mammals with an influenza A virus of avian origin which is either highly pathogenic or if low pathogenic of the H5 or H7 subtypes must be based on one or more of the criteria in points (a) or (b):

(a) the detection of the AI infectious virus, antigen or specific genetic material in samples of tissues, organs, blood or excreta from mammals;

(b) the demonstration of a specific antibody response to AI in blood samples from mammals.

6. The procedures, sampling methods and criteria for the evaluation of the results of laboratory tests must be:

(a) those set out in this diagnostic manual; or

(b) those authorised by the competent authority provided that:

(i) the sensitivity and specificity of the authorised laboratory tests have been demonstrated as being effective following a comparative test organised by the Community reference laboratory for avian influenza (Community reference laboratory); or

(ii) where no such evaluation has been organised by the Community reference laboratory for a specific type of laboratory test, the sensitivity and specificity of the authorised laboratory test have been validated by the national reference laboratory so that the laboratory test is fit for the purpose for which it is used; the results of such validation must be submitted to the Community reference laboratory for review.

## CHAPTER II

### Description of AI with emphasis on differential diagnosis

1. Aetiology and virulence

AI is a highly contagious viral infection caused by viruses of the family *Orthomyxoviridae*, genus *influenzavirus A*. Influenza A viruses are the only orthomyxoviruses known to infect birds. Many species of birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir of such viruses, but the overwhelming majority of isolates have been of low pathogenicity in chickens and turkeys, the main birds of economic importance to be affected by the disease.

Influenza A viruses have antigenically related nucleoproteins and antigenically related matrix proteins, but are classified into subtypes on the basis of the antigenic relatedness of the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). At present, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) are recognised. Each influenza A virus has one HA and one NA antigen, apparently in any combination.

Influenza A viruses are divided into two groups on the bases of their ability to cause disease in susceptible poultry:

(a) **highly pathogenic avian influenza (HPAI)** viruses, which cause an extremely serious disease characterised by generalised infection of the infected poultry, where they may induce very high flock mortality (up to 100 %); and

(b) **low pathogenic avian influenza (LPAI)** viruses, which cause a mild, primarily respiratory, disease in poultry, unless there is exacerbation by other co-infections or factors.

Wild birds, especially migratory waterfowl, play a very important role as the influenza A virus reservoir, as shown by the isolation of nearly all possible combinations of HA and NA subtypes from wild birds. Generally, except when there has been spill-over of HPAI from infected poultry, only LPAI viruses are detected in wild birds.

Primary introductions of AI viruses to poultry farms most likely originate from direct or indirect contact with wild birds.

In domestic poultry, there is a possibility that such LPAI viruses introduced from a wild reservoir may circulate undetected, as clinical signs are often mild or absent.

Once introduced into poultry, LPAI virus strains of H5 and H7 subtypes may then mutate into HPAI strains. To date, only viruses of the H5 and H7 subtypes have been shown to cause HPAI.

Although it appears that several mechanisms may be responsible for mutation from the LPAI to the HPAI virus, the factors that bring about this mutation are not known. In some instances mutation seems to have taken place rapidly at the primary site after introduction from wild birds, in other instances the LPAI virus has circulated in poultry for months before mutating. Therefore, it is impossible to predict if and when such mutation will occur. However, it may reasonably be assumed that the more widespread the circulation of LPAI in poultry, the higher the chances that mutation to HPAI will occur.

The incubation period is difficult to estimate and probably varies with the strain of virus and host, usually five to six days is quoted, but the range for individual birds is probably from a few hours to about seven days.

2. Clinical signs in birds infected with HPAI virus

The clinical signs are very variable and are influenced by factors such as the virulence of the infecting virus, the species affected, age, sex, concurrent diseases and environment.

Early signs can include inappetence, reduction in water intake and relatively low mortality. However, alternatively the disease may appear suddenly in a flock and many birds may die either without premonitory signs or with minimal signs of depression, inappetence, ruffled feathers and fever. Generally the longer birds survive the more marked are the clinical signs. The timeline for the development of signs depends on the virus, host and initial infecting dose together with husbandry system. The virus spreads more slowly in caged layers or outdoor birds compared to in broiler houses.

Hens infected with the HPAI virus may at first lay soft-shelled eggs, but soon stop laying. Sick birds often sit or stand in a semi-comatose state with their heads touching the ground. Combs and wattles are cyanotic and oedematous, and may have petechial or ecchymotic haemorrhages at their tips. Profuse watery diarrhoea is frequently present and birds are excessively thirsty. Respiration may be laboured and excessive lacrimation may be seen. Haemorrhages may be seen on unfeathered areas of skin. The flock mortality rates vary from 50 to 100 %.

In broilers, the signs of HPAI are frequently less obvious than for other poultry and usually include severe depression, inappetence, and a very marked increase in mortality may be the first abnormality observed. Oedema of the face and neck and neurological signs such as torticollis and ataxia may also be seen.

HPAI in turkeys is similar to that seen in domestic fowl, but some HPAI viruses appear more virulent in turkeys while others appear less virulent.

In geese infected with the HPAI virus the signs of depression, inappetence and diarrhoea are similar to those in layers, though frequently with swollen sinuses. Younger birds may exhibit neurological signs.

Ducks may show no clinical signs when infected with HPAI viruses, but some strains have been reported to induce signs similar to those in geese with some mortality.

Clinical signs may be absent in HPAI and LPAI infections of ostriches. In HPAI outbreaks, such as those in Italy in 1999 and 2000, guinea fowl and Japanese quail were reported to be susceptible to infections with signs and mortality resembling the disease in chickens or turkeys. However, in some experimental studies quail have been reported to be resistant to some HPAI strains. For all birds, the presence of antibodies to the same H subtype whether by vaccination or natural infection may mean that infection with HPAI virus does not result in overt clinical signs.

3. Post-mortem lesions in birds infected with HPAI virus

Birds that die peracutely may show minimal gross lesions, consisting of dehydration and congestion of viscera and muscles.

In birds that die after a prolonged clinical course, petechial and ecchymotic haemorrhages occur throughout the body, particularly in the larynx, trachea, proventriculus and epicardial fat, and on serosal surfaces adjacent to the sternum. There is extensive subcutaneous oedema, particularly around the head and hocks. The carcase may be dehydrated. Yellow or grey necrotic foci may be present in the spleen, liver, kidneys and lungs. The air sac may contain an exudate. The spleen may be enlarged and haemorrhagic.

AI is characterised histologically by vascular disturbances leading to oedema, haemorrhages and perivascular cuffing, especially in the myocardium, spleen, lungs, brain, pancreas and wattles. Necrotic foci are present in the lungs, liver and kidneys. Gliosis, vascular proliferation and neuronal degeneration may be present in the brain.

4. Differential diagnosis

In the differential diagnosis of HPAI, the following diseases, in particular, must be considered:

(a) other diseases causing sudden high mortality, such as:

  (i) Newcastle disease;

  (ii) infectious laryngotracheitis;

  (iii) duck plague;

  (iv) acute poisonings;

(b) other diseases causing swelling of the combs and wattles, such as:

  (i) acute fowl cholera and other septicaemic diseases;

  (ii) bacterial cellulitis of the comb and wattles.

5. Clinical signs in birds infected with LPAI viruses

The severity of the disease produced by LPAI viruses is greatly influenced by:

(a) the strain of the virus;

(b) the species and age of the host;

(c) the immune status of the host against the virus and particularly the presence of other infectious agents, such as:

  (i) *Pasteurella* spp.;

  (ii) Newcastle disease viruses (including vaccine strains);

  (iii) avian pneumovirus, infectious bronchitis virus;

  (iv) *E. coli*;

  (v) *Mycoplasma* spp.;

(d) immunodeficiency conditions;

(e) environmental factors (such as excess ammonia, dust, hot or cold temperatures).

At one extreme, the clinical signs of the disease seen may be inapparent or slight, producing only mild respiratory signs or egg production problems in laying birds. At the other extreme, infections with LPAI viruses may be associated with severe clinical signs of the disease, especially in turkeys, usually with rales, coughing, swelling of the infraorbital sinuses and a febrile condition associated with loss of appetite and with high mortality.

LPAI may be confused with, or complicated by, many of the diseases with respiratory or enteric signs. AI must be suspected in any disease outbreak in poultry that persists despite the application of preventive and therapeutic measures for other diseases.

6. Clinical signs in captive birds

The spectrum of clinical signs can be very broad and, as with poultry, can range from inapparent to severe signs of the disease resulting in high mortality.

Generally, infection spreads more slowly in a collection of captive birds due to the variety of different species kept, with different susceptibilities, inconsistent levels of virus shedding and often relatively slow transmission due to a low contact rate and relatively low stocking densities.

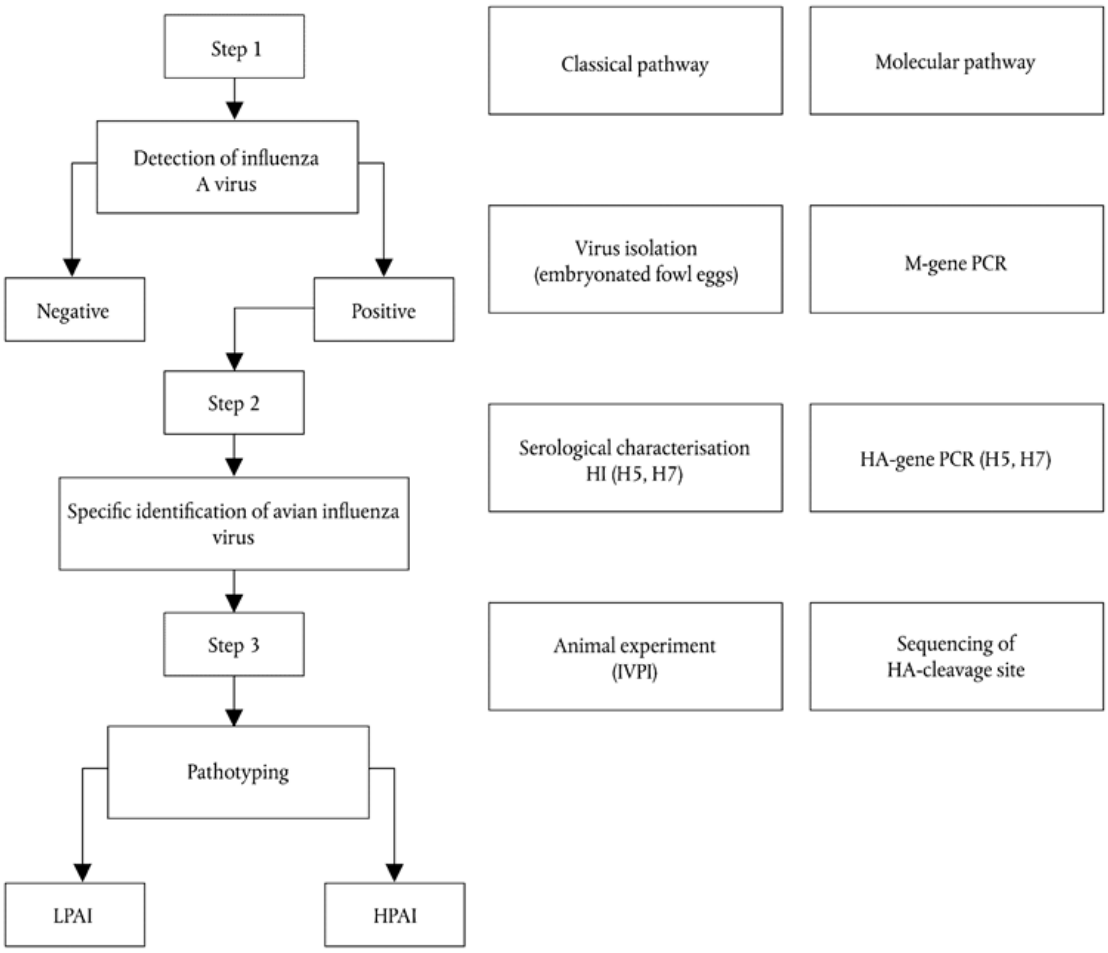
## CHAPTER III

### Guidelines to be considered in the case of suspicion of AI on a holding

The variability of clinical signs for both HPAI and LPAI means that clear-cut guidance for a suspected outbreak is not possible. A sudden, high mortality in poultry with or without any of the associated clinical signs described in Chapter II must be investigated by the submission of samples for laboratory investigation, but in the absence of high mortality, it is more difficult to suspect or exclude the presence of AI.

Since rapid diagnosis of HPAI or LPAI caused by H5 and H7 subtypes is of paramount importance in their early control and eradication, AI must always be considered in the differential diagnosis of respiratory problems, egg production problems and elevated mortality in poultry and the appropriate samples submitted for laboratory investigation.

Figure Schematic overview of diagnostic steps for confirmation of AI




CHAPTER IV

**General procedures for the collection and transport of samples**

1. Directive 2005/94/EC and the diagnostic manual

Where references are made in Directive 2005/94/EC to the diagnostic manual, the investigations, sampling and surveillance procedures set out in this Chapter of the diagnostic manual must be performed.

2. Procedures to be followed where outbreaks of AI are suspected

Where the official veterinarian has a clinical suspicion of an outbreak of AI or where the results of any laboratory test for that disease are not negative, the competent authority must ensure that an investigation, as set out in this Chapter of the diagnostic manual, is performed, in accordance with Article 7 of Directive 2005/94/EC, and satisfactorily completed before the presence of the disease is excluded.

3. Interpretation of virological testing

The competent authority may consider that the presence of the AI virus may be excluded when an appropriate number of the sick or dead birds and tracheal/oro-pharyngeal or cloacal swabs have been submitted, in accordance with this Chapter, for the detection of that virus or its

genome and have given negative results when tested using one of the specified virus detection methods referred to in Chapter V or VI or authorised by the competent authority in accordance with point 6(b) of Chapter I.

4. Standard set of samples for virological or serological laboratory testing

For the investigation of a holding suspected of being infected with the AI virus the standard set of samples for virological or serological testing, as referred to in points (a) and (b) (the standard samples), must be taken and submitted directly for virological and serological laboratory tests.

(a) The standard set of samples for virological testing is:

(i) at least five sick/dead birds, if present; and/or

(ii) at least 20 tracheal/oropharyngeal and 20 cloacal swabs.

Carcases must be taken of birds that have died recently or that are severely sick or moribund and have been killed humanely.

Swabs must be taken from the number of birds referred to in point (a) or from all birds on the suspected holding where a smaller number of birds are present. Birds showing clinical disease signs must be targeted for sampling.

The cloacal swabs must be coated in faeces (optimum 1 g). If for any reason it is impracticable to take cloacal swabs from live birds, carefully collected fresh faeces samples may serve as an alternative.

Frequently, it is most practical to collect tracheal/oropharyngeal swabs from the buccal cavity.

As soon as the growth characteristics of the virus are known, the competent authority may decide to choose either tracheal/oropharyngeal or cloacal swabs rather than to collect both depending on whether the virus replicates better in the respiratory or gastrointestinal tract and also taking into account the species concerned.

(b) The standard set of samples for serological testing is a minimum of 20 blood samples.

Samples must be taken from the number of birds referred to in point (b) or from all birds on the holding where a smaller number of birds are present. Birds appearing sick or that have apparently recovered must be targeted for sampling.

The competent authority may decide that the full range of standard samples need not be taken, but that a subset of the standard samples may be taken instead.

5. Transport of samples

Specific care must be taken for the storage and transport of samples to the laboratory for testing.

The swabs must be chilled immediately on ice or with frozen gel packs and submitted to the laboratory as quickly as possible. The samples must not be frozen unless absolutely necessary. If rapid transport within 24 hours to the laboratory is not guaranteed, the samples must be immediately frozen, stored and then transported on dry ice.

In addition and not as an alternative to chilling, the swabs must be placed in an antibiotic or specific virus transport medium at 4 $^{o}$C so that they are fully immersed. In the absence of such medium, swabs must be returned to their casing and submitted dry to the laboratory for testing.

The storage and transport of samples may be affected by a variety of factors so the method selected for transport must be fit for that purpose.

6. Antibiotic medium

The antibiotic medium referred to in point 5 must be based on phosphate-buffered saline at pH 7,0 to 7,4 (checked after the addition of antibiotics).

Protein-based media, such as brain-heart-infusion or tris-buffered tryptose broth may give added stability to the virus, especially during transport. The antibiotics used and their concentrations may be varied to suit local conditions and availability.

Very high levels of antibiotics may be necessary for faecal samples and appropriate levels are: 10 000 IU/ml penicillin, 10 mg/ml streptomycin, 0,25 mg/ml gentamycin, and 5 000 IU/ml nystatin. Those levels may be reduced by up to five-fold for tissues and tracheal swabs.

If the control of Chlamydophila is desired, 0,05 to 0,1 mg/ml oxytetracycline must be included.

7. Brain-heart-infusion medium

The solution must be prepared in water and contain 15 % w/v brain-heart-infusion broth powder, prior to sterilisation (by autoclaving at 121 $^{o}$C/15 minutes).

Following sterilisation antibiotics must be added as follows: 10 000 IU/ml penicillin G, 20 μg amphotericin B and 1 000 μg/ml gentamycin. Media may be stored at 4 $^{o}$C for a maximum of two months.

8. Procedures to be performed, as regards the relevant provisions of Directive 2005/94/EC

8.A. Suspected outbreaks

8.1. Article 7(1) — Measures to be applied on holdings where outbreaks are suspected

When an official veterinarian inspects a holding where an outbreak is suspected, the following measures must be carried out:

(a) A check of the production and health records of the holding, if such records exist. The daily mortality data and daily data on egg-production, and feed-and/or water intake for the period beginning one week before the date of commencement of clinical signs of AI until the date of the inspection of the holding by the official veterinarian must be documented in the inspection-report by the official veterinarian.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds in particular those that appear sick.

(c) Unless the competent authority is satisfied that a suspected outbreak may be excluded on the basis of the clinical inspection in accordance with points (a) and (b), the standard samples must be taken from each production unit.

(d) Independently of negative results to testing of standard samples and subject to local factors a clinical inspection of the poultry in each production unit must be carried out before the official surveillance may be lifted.

8.2. Article 10(3) — Additional measures based on the epidemiological inquiry

Standard samples must be taken from poultry or other captive birds which are killed, in each production unit.

8.B. Highly pathogenic avian influenza (HPAI)

8.3. Article 11(4) — Measures to be applied in cases of poultry hatched from eggs collected from holdings where outbreaks have been confirmed

When an official veterinarian inspects a holding where poultry are present that have already hatched from eggs collected during the incubation period on a holding where HPAI has been confirmed, the following measures must be carried out:

(a) A check of the production and health records of the holding. The daily mortality data and daily data on feed- and/or water intake, if available, for the period beginning one week before the date of commencement of clinical signs of HPAI until the date of the farm inspectionby the official veterinarian must be documented in the farm inspection-report by the official veterinarian.

(b) A clinical inspection in each production unit and a clinical examination of poultry, in particular those that appear sick or that are not growing as expected.

(c) The standard samples must be taken from the poultry between two and three weeks of age.

(d) The official surveillance of the holding may be lifted following a clinical examination of poultry more than 21 days old and negative results to testing of standard samples.

8.4. Article 13(2)(b) — Derogations concerning certain holdings

When an official veterinarian inspects a holding that has been granted a derogation from the first subparagraph of Article 11(2) of Directive 2005/94/EC, the following measures must be carried out:

(a) A check of the production and health records of the holding, if such records exist.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, in particular those that appear sick.

(c) Instead of the standard samples, the following samples must be taken for laboratory testing, 21 days following the date of the last positive finding of HPAI from each production unit and at 21 days intervals:

  (i) samples of any dead poultry or other captive birds present at the time of sampling;

  (ii) where practical, tracheal/oropharyngeal and cloacal swabs from at least 60 poultry or other captive birds or from all such poultry or other captive birds where less than 60 are present on the holding; or if the birds are small, exotic and not used to being handled or handling them would be dangerous for people, samples of fresh faeces must be collected.

  However, the competent authority may grant derogations from the sample size referred to in (i) and (ii), based on the outcome of a risk assessment.

(d) The sampling referred to in point (c) and laboratory testing of such samples must continue until two consecutive negative laboratory results are obtained which must be at least 21 days apart.

8.5. Article 15(1) and (3) — Measures to be applied in contact holdings

When an official veterinarian inspects a contact holding, the following measures must be carried out:

(a) A check of the production and health records of the holding, if such records exist. The daily mortality data and daily data on feed- and/or water intake, if available, for the period beginning one week before the date of contact with the flock suspected of being infected with AI until the date of the inspection of the holding by the official veterinarian must be documented in the farm inspection-report by the official veterinarian.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, in particular those that appear sick.

(c) If there are clinical signs present in poultry or other captive birds or indications of an increase in daily mortality (> 3 times normal mortality rate of the flock) or a depression in daily egg-production (> 5 %) or a decrease in daily feed and/or water intake (> 5 %), the standard samples must be taken immediately from each production unit.

(d) If there are no signs as referred to in points (b) and (c), the standard samples must be taken 21 days following the date of the last suspected contact with an infected holding or when the poultry or other captive birds are killed.

8.6. Article 18, points (b) and (c) — Census and inspections by the official veterinarian and surveillance in holdings in the protection zone

When an official veterinarian inspects a commercial holding, the following measures must be carried out:

(a) A check of the production and health records of the holding. If there are indications of an increase in daily mortality (> 3 times normal mortality rate of the flock) or a depression in daily egg-production (> 5 %) or a decrease in daily feed and/or water intake (> 5 %), the standard samples must be taken from each production unit.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry and other captive birds, in particular those that appear sick.

(c) Where the species of poultry or other captive birds are not expected to clearly express clinical signs of disease, or in the case of vaccinated birds, the competent authority may decide, based on the outcome of a risk assessment that the standard samples must be taken from each production unit.

(d) Based on the outcome of a risk assessment, the competent authority must decide upon additional official surveillance by clinical inspections and sampling for laboratory tests in targeted holdings, compartments or production types.

8.7. Article 19, point (f) — Measures to be applied on holdings in protection zones

When an official veterinarian inspects a holding where increased morbidity, mortality or change in production data have been reported, the following measures must be carried out:

(a) A check of the production and health records of the holding. If there are indications of an increase in daily mortality (> 3 times normal mortality rate of the flock) or a depression in daily egg-production (> 5 %) or a decrease in daily feed- and/or water intake (> 5 %), the standard samples must be taken from each production unit.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, in particular those that appear sick.

8.8. Article 23, point (b) — Derogations for the direct transport of poultry for immediate slaughter

When an official veterinarian inspects a holding that has been granted a derogation from Article 22 of Directive 2005/94/EC, the following measures must be carried out:

(a) A check of the production and health records of the holding.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of any poultry, in particular those that appear sick less than 24 hours prior to the time of departure of the poultry.

(c) Based on the outcome of a risk assessment by the competent authority and instead of the standard samples, at least 60 tracheal/oropharyngeal and/or 60 cloacal swabs must be taken from poultry from each production unit to be sent to slaughter less than 48 hours prior to the time of departure of the poultry.

8.9. Article 25, point (b) — Derogations for the direct transport of ready-to-lay poultry

When an official veterinarian inspects a holding that has been granted a derogration from Article 22, prior to the direct transport of ready-to-lay poultry, the following measures must be carried out :

(a) A check of the production and health records of the holding.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of the poultry, in particular those that appear sick less than 24 hours prior to the time of departure of the poultry.

(c) Based on the outcome of a risk assessment by the competent authority, and instead of the standard samples, at least 60 tracheal/oropharyngeal and/or cloacal swabs must be taken from the poultry from each production unit to be transported less than 48 hours prior to the time of departure of the poultry.

8.10. Article 26(1)(a) — Derogation for the direct transport of hatching and table eggs

When an official veterinarian inspects a parent flock holding that has been granted a derogation from Article 22, prior to the direct transport of the hatching eggs, the following measures must be carried out:

(a) A check of the production and health records of the holding.

(b) A clinical inspection in each production unit every 15 days.

(c) The standard samples must be taken from each production unit.

8.11. Article 29(1) — Duration of measures

The measures that apply in the protection zone in accordance with Section 3 of Chapter IV of Directive 2005/94/EC may be lifted no earlier than 21 days following the date of preliminary cleaning and disinfection of infected holdings provided that:

(a) All commercial holdings situated in the protection zone have been inspected by an official veterinarian and all checks and clinical inspections and laboratory tests, as set out in point 8.6(a), (b) and (c) and point 8.7 have given negative results.

(b) All identified non-commercial holdings in the protection zone have been inspected by an official veterinarian and neither the clinical examination nor the results of any laboratory tests undertaken have led to a suspicion of infection of AI.

(c) Any additional official surveillance that was carried out, as set out in point 8.6(d) has given negative results.

8.12. Article 30, point (g) — Measures to be applied in the surveillance zones

When an official veterinarian inspects a holding in which increased morbidity, mortality or change in production data have been reported, the following measures must be carried out:

(a) A check of the production and health records of the holding.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, in particular those that appear sick.

(c) The standard samples must be taken from each production unit.

8.13. Article 35 — Investigation of suspected presence of HPAI in slaughterhouses and in means of transport

When an official veterinarian inspects a holding of origin of birds in slaughterhouses or means of transport, the following measures must be carried out:

(a) A check of the production and health records of the holding, if such records exist.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, taking into account the consultation with the official veterinarian at the slaughterhouse who must provide details of any previous inspection data and results of ante- and post-mortem examinations.

(c) Unless the competent authority is satisfied that the suspected presence of HPAI may be excluded on the basis of the veterinary investigation in accordance with points (a) and (b), the standard samples must be taken from each production unit.

(d) In addition to the standard samples, samples of at least five sick, dead or slaughtered birds in the slaughterhouse with pathological findings must be submitted for laboratory tests.

8.14. Article 36(1) — Measures to be applied in slaughterhouses

Following the completion of investigations referred to in point 8.13 and provided that the results of the laboratory tests are negative and that there is no clinical suspicion of the presence of HPAI on the holding of origin and in the slaughterhouse, the official supervision may be lifted.

8.15. Article 37(1) and (2) — Measures to be applied in border inspection posts or means of transport

8.15.1. When an official veterinarian examines poultry or other captive birds kept in isolation, which have been moved from a border inspection post or means of transport, due to suspicion or confirmation of HPAI, the following measures must be carried out:

(a) A check of the relevant documents and records, if such documents or records exist.

(b) A clinical examination of such poultry or other captive birds that are kept in isolation and a clinical inspection of any other poultry or other captive birds, in particular those that appear sick.

(c) The standard samples must be taken from poultry or other captive birds selected from different transport crates or cages.

8.15.2. When an official veterinarian inspects an identified holding of origin in case the poultry or other captive birds are slaughtered, the following measures must be carried out:

(a) A check of the production and health records of the holding, if such records exist.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds taking into account the consultation with the official veterinarian at the slaughterhouse who must provide details of any previous inspection data and results of ante- and post-mortem examinations.

(c) Unless the competent authority is satisfied that the suspected presence of HPAI may be excluded on the basis of the veterinary investigation in accordance with points (a) and (b), the standard samples must be taken from each production unit.

(d) In addition to the standard samples referred to in point (c), samples of at least five sick, dead or slaughtered birds in the slaughterhouse with pathological findings must be submitted for laboratory tests.

(e) Provided that the results of the laboratory tests of the samples referred to in points (c) and (d) are negative and that there is no clinical suspicion of HPAI on the holding of origin and in the slaughterhouse, the official supervision may be lifted.

8.C. Low pathogenic avian influenza (LPAI)

8.16. Article 39(6)(b) and (h) — Measures to be applied on holdings where outbreaks of LPAI are confirmed

When an official veterinarian inspects a holding, prior to the transport of poultry to a slaughterhouse, or a holding where poultry already hatched from eggs collected during the incubation period are present, the following measures must be carried out:

(a) A check of the production and health records of the holding.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of the poultry or other captive birds.

(c) The standard samples must be taken from birds from each production unit to be sent to slaughter less than 48 hours prior the time of departure of the poultry.

(d) The standard samples must be taken from each production unit from poultry already hatched from eggs collected during the incubation period.

8.17. Article 40(2)(b) — Derogation for certain holdings from measures to be applied where outbreaks are confirmed

When an official veterinarian inspects a holding that has been granted a derogation from Article 39(2) and point (b) of Article 39(5) of Directive 2005/94/EC, the following measures must be carried out:

(a) A check of the production and health records of the holding, if such records exist.

(b) A clinical inspection in each production unit at regular intervals, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, in particular those that appear sick.

(c) Instead of the standard samples the following samples must be taken for laboratory testing, 21 days following the date of the last positive findings of LPAI from each production unit and at 21 days intervals:

(i) samples of any dead poultry or other captive birds present at the time of sampling;

(ii) tracheal/oropharyngeal and cloacal swabs from 60 poultry and other captive birds or from all poultry and other captive birds where there are less than 60 present on the holding; or if the poultry or other captive birds are small, exotic and not used to being handled or handling them would be dangerous for people, samples of fresh faeces must be collected.

However, the competent authority may grant derogations from the sample size referred to in (i) and (ii) based on the outcome of a risk assessment.

(d) The sampling referred to in point (c) and laboratory testing of such samples must continue until two consecutive negative laboratory results are obtained which must be at least 21 days apart.

8.18. Article 42(1) and (3) — Measures to be applied in contact holdings

When an official veterinarian inspects a contact holding, the following measures must be carried out:

(a) A check of the production and health records of the contact holding, if such records exist.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, in particular those that appear sick.

(c) The standard samples must be taken from each production unit or when poultry or other captive birds are killed.

8.19. Article 44(1)(b) — Measures to be applied in the restricted zones

When an official veterinarian inspects a commercial holding in a restricted zone, the following measures must be carried out:

(a) A check of the production and health records of the holding.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of poultry or other captive birds, in particular those that appear sick.

(c) The standard samples must be taken from each production unit.

(d) Based on the outcome of a risk assessment, the competent authority, must decide upon additional official surveillance by clinical inspections and sampling for laboratory tests in targeted holdings, compartments or production types.

8.20. Article 45, points (a) and (b) — Duration of measures

The measures that apply in the restricted zone, in accordance with Section 3 of Chapter V of Directive 2005/94/EC, may be lifted no earlier than 21 days following the date of preliminary cleaning and disinfection of infected holdings following depopulation of the holding or no earlier than 42 days following the date of confirmation of LPAI provided that:

(a) all commercial holdings in the restricted zone have been inspected by an official veterinarian and all laboratory tests for the samples referred to in points (c) and (d) of point 8.13 have been carried out and are available;

(b) the results of any additional clinical inspections and laboratory tests, which may include non-commercial holdings to determine the risk of LPAI spread, are available;

(c) the competent authority is satisfied, based on the outcome of a risk assessment taking into account the epidemiological situation and the results of the laboratory tests referred to in points (a) and (b), that the risk of the spread of LPAI is negligible; such assessment may conclude that in the case of positive serological findings and negative virological findings, the restrictions may be lifted.

8.D. Measures aimed at avoiding the spread of influenza viruses of avian origin to other species

8.21. Article 47(1) and (6) — Laboratory tests and other measures concerning pigs and other species

When an official veterinarian inspects a holding where pigs are kept, following confirmation of AI, the following measures must be carried out:

(a) A check of the production and health records of the holding, if such records exist.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of the pigs, in particular those that appear sick.

(c) Nasal/oropharyngeal swabs from at least 60 pigs from each production unit or from all pigs where less than 60 pigs are present in the production unit, must be taken before or on the day the infected poultry or other captive birds are culled. At least 60 blood samples must be collected from the pigs, two to four weeks from the date of the cull. Samples must be collected in such a way that at least one sample is obtained from groups of pigs that are in direct contact with each other.

(d) the movement of pigs to other holdings may be authorised if at least 60 nasal/oropharyngeal swabs and 60 blood samples from pigs, from each production unit, 14 days following the date of the positive findings of the presence of AI have given negative results.

The movement of pigs to a slaughterhouse may be authorised if at least 60 nasal/oropharyngeal swabs, from each production unit, 14 days following the date of the positive findings of the presence of AI have given negative results.

In the case of inconclusive or positive laboratory results, any further investigations required to exclude the infection or transmission of AI amongst pigs.

(e) Where the official veterinarian has a suspicion that other domestic mammals on the holdings, in particular those with identified susceptibility to infection with AI viruses of H5 and H7 subtypes, may have been in contact with the infected poultry or other captive birds, samples for laboratory tests must be taken.

8.E. Re-population

8.22. Article 49(3)(b) and (c) — Re-population of holdings

When an official veterinarian inspects a commercial holding, which has been re-populated, the following measures must be carried out:

(a) A check of the production and health records of the holding.

(b) A clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of the poultry or other captive birds, in particular those that appear sick.

(c) Instead of the standard samples, the following samples must be taken from each production unit:

  (i) at least 20 blood samples as soon as the poultry have been placed in the holding except in the case of day-old chicks; if appropriate such sampling may be performed on the holding of origin of the poultry before movement to the holding for re-population;

  (ii) samples of dead poultry or swabs taken from their carcasses from a maximum of 10 dead birds per week during the 21 day period from the date of the re-population.

(d) Where the holding has previously been infected with HPAI 20 tracheal/oropharyngeal and 20 cloacal swabs must also be taken from waterfowl (ducks/geese) from each production unit, if appropriate, within the last week of the 21 day period from the date of re-population.

(e) Where the holding has previously been infected with LPAI, 20 tracheal/oropharyngeal and 20 cloacal swabs and 20 blood samples from each production unit must be taken.

8.F. Vaccination

8.23. Article 56(2)(i) — Preventive vaccination in poultry or other captive birds

Laboratory tests, as provided for in Chapter IX of Directive 2005/94/EC, must be performed on the vaccinated poultry or other captive birds using approved DIVA assays, where the field virus is known.

When sentinel birds are used they must be present in each vaccinated flock, inspected clinically and be tested using the haemagglutination inhibition test (HI). For that purpose, 20 blood samples from the unvaccinated sentinels in each vaccinated holding must be taken at least every 60 days.

8.24. Annex IX — Requirements for movements of poultry or other captive birds and poultry products applicable in relation to emergency vaccination

Strict monitoring measures must be applied to the movement of live poultry and other captive birds and their eggs in order to minimise the risk of a further spread of infection of AI.

For that purpose, at the beginning of an emergency vaccination campaign the same monitoring measures must be applied in relation to the movement of live poultry and other captive birds and their eggs in order to minimise the risk of a further spread of AI infection within and out of the vaccination area.

(a) Before the first movement within and out of the vaccination area of hatching eggs and table eggs and thereafter at least every 30 days, the official veterinarian must carry out the following measures:

(i) a clinical inspection of unvaccinated parent or layer poultry in each production unit, including an evaluation of its clinical history and clinical examinations of the poultry, in particular those that appear sick; the standard samples must be taken from poultry from each production unit; or

(ii) a clinical inspection of vaccinated parent or layer poultry in each production unit, including an evaluation of its clinical history and clinical examinations of the sentinel birds present in those flocks; the standard samples must be taken from those sentinel birds.

(b) For the movement of live vaccinated poultry or other captive birds to other holdings or for the movement of live vaccinated poultry within and out of the vaccination area, the official veterinarian must carry out the following measures:

(i) a check of the production and health records of the holding.

(ii) a clinical inspection in each production unit, including an evaluation of its clinical history and clinical examinations of the poultry or other captive birds within 72 hours prior to the time of departure with particular attention to the sentinel birds;

(iii) where the results of the checks and clinical inspection and examinations in (i) and (ii) are not satisfactory, the standard samples must be taken from the sentinels; however, where those results are satisfactory, the following samples must be taken from:

— the vaccinated poultry or other captive birds: at least 20 tracheal/ oropharyngeal and 20 cloacal swabs and 20 blood samples for using an appropriate DIVA assay within 72 hours prior to the time of departure, and

— sentinel birds: 20 tracheal/oropharyngeal and 20 cloacal swabs and 20 blood samples for serology using the HI test prior to the time of departure.

## CHAPTER V

### Diagnostic virological tests and evaluation of results

1. Until the advent and development of molecular-based tests, virus isolation by the inoculation of embryonated fowls' eggs was considered the most sensitive diagnostic test for AI by far and essential for subsequent identification and characterisation of the infecting virus. The essential steps are set out in this Chapter.

2. Sample processing

Swabs if submitted ‘dry’ must be placed in sufficient antibiotic medium to ensure full immersion. Samples may be pooled in batches in batches of five provided they are derived from the same species, time and epidemiological unit.

Carcases submitted to the laboratory must be subjected to post mortem examination and samples of the following organs must be taken: faeces or intestinal contents, brain tissue, trachea, lungs,

liver, spleen and other obviously affected organs. Those organs and tissues may be pooled, but separate treatment of faecal material is essential.

Faeces samples and organs must be homogenised (in an enclosed blender or using a pestle and mortar and sterile sand) in antibiotic medium and made to 10 to 20 % w/v suspensions in the medium.

The immersed swabs and suspensions must be left for approximately two hours at ambient temperature (or longer periods at 4 $^{o}$C) and then clarified by centrifugation (for example, 800 to 1 000 x g for 10 minutes).

3. Virus isolation in embryonated fowls' eggs

The clarified supernatant fluid must be inoculated in 0,1 to 0,2 ml amounts into the allantoic cavity of each of a minimum of four embryonated fowls' eggs that have been incubated for nine to 11 days. Ideally, these eggs must be obtained from a specific pathogen free (SPF) flock, but when this is impracticable eggs obtained from a flock shown to be free of antibodies to AI (serum antibody negative — SAN), may be used.

The inoculated eggs must be held at 37 $^{o}$C and candled daily. Eggs with dead or dying embryos as they arise, and all remaining eggs six days after inoculation must be chilled to 4 $^{o}$C and the allantoic- amniotic fluids tested for haemagglutination activity. If no haemagglutination is detected, this procedure must be repeated using undiluted allantoic/amniotic fluid as inoculum. When haemagglutination is detected the presence of bacteria must be excluded by culture. If bacteria are present, the fluids may be passed through a 450 nm membrane filter, further antibiotics added and inoculated into embryonated eggs as above.

To expedite diagnosis some laboratories have used two 3-day passages or 2-day and 4-day passages and reported comparable results to two 6-day passages, but this has not yet been fully evaluated.

Positive fluids need to be tested for freedom from bacteria. If bacteria are present the fluids may be passed through a 450-nm membrane filter or centrifuged to remove bacteria and re-passaged in eggs after the addition of more antibiotics.

4. Differential diagnosis

(a) Preliminary differentiation

As it is important that control measures aimed at limiting the spread of the AI virus must be implemented as soon as possible, each national reference laboratory that has isolated a haemagglutinating virus must be in a position to identify it if it is an influenza A virus of H5 or H7 subtype or Newcastle disease virus. The haemagglutinating fluids must be used in haemagglutination inhibition tests as described in Chapter IX. Positive inhibition, such as a titre within 2 to 3 $\log_2$ of a positive control, with polyclonal antisera specific for H5 or H7 subtypes of influenza A may serve as a preliminary identification enabling the imposition of interim control measures.

(b) Confirmatory identification

Since there are 16 haemagglutinin subtypes and 9 neuraminidase subtypes of influenza viruses and variations occur within each of these it is neither practicable nor cost effective for each national reference laboratory to hold antisera that may allow a full subtype identification of influenza isolates. However, each national reference laboratory must as a minimum:

(i) confirm that the isolate is an influenza A virus using an immunodoublediffusion test to detect group antigens;

(ii) determine whether or not the isolate is of H5 or H7 subtype, positive identification requires implementation of the control measures for LPAI of H5 and H7 subtypes;

(iii) immediately submit all HPAI and all H5 and H7 isolates to the Community reference laboratory for confirmation and full characterisation, unless a derogation is granted in accordance with point (d).

In addition it is desirable in laboratories with appropriate facilities to:

(iv) carry out an intravenous pathogenicity index test in six-week-old chickens as set out in Chapter VII. Intravenous pathogenicity indices of greater than 1,2 indicate the presence of virus requiring a full implementation of control measures for HPAI.

National reference laboratories must also consider putting in place expertise and equipment that allow nucleotide sequencing of the haemagglutinin gene to determine whether or not there are multiple basic amino acids at the cleavage site of the haemagglutinin precursor protein for any LPAI H5 or H7 virus. Although the Community reference laboratory will conduct pathogenicity determination as a high priority, as part of the duties referred to in Annex VII, point 2(b) to Directive 2005/94/EC, such virus characterisation at the national level will greatly decrease the time taken for diagnosis and, when positive, for the full implementation of control measures for HPAI.

(c) Further typing and characterisation of isolates

The Community reference laboratory must receive all haemagglutinating viruses from the National reference laboratories for further antigenic and genetic studies to enable a greater understanding of the epizootiology of the disease(s) within the Community in accordance with the functions and duties of the Community reference laboratory as set out in Annex VII to Directive 2005/94/EC.

In addition to those functions and duties, the Community reference laboratory must carry out full antigenic typing for all influenza viruses received. For H5 and H7 viruses that 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 amino acids at the cleavage site of the haemagglutinin precursor protein must also be carried out immediately and the national reference laboratory and the competent authority in the country of origin must be informed as soon as the results are available so that control measures for HPAI can be fully implemented.

(d) Given the changing epidemiological situation in relation to HPAI/LPAI, it may be possible by agreement with both the Commission and the Community reference laboratory for a derogation to be given to laboratories with full capabilities for rapid virus characterisation, to submit a subset of these viruses following inspection of data and for the Community reference laboratory to make a relevant selection. This derogation must only be permitted where data can be rapidly generated by the national reference laboratory and shared with the Community reference laboratory.

## CHAPTER VI

### Molecular tests and evaluation of results

The current definition of HPAI, allows the molecular identification of virulence factors and confirms the use of molecular techniques in the diagnosis of AI. Recently there have been developments in their application for detection and characterisation of AI virus directly from clinical specimens from infected birds. Conventional RT-PCR techniques on clinical specimens could, with the correctly defined primers, result in rapid detection and subtype (at least of H5 and H7) identification, plus a PCR amplicon product that could be used for nucleotide sequencing and have been demonstrated to have an important application by rapidly identifying subsequent outbreaks once the primary infected premises has been detected and the virus characterised. 'Real time' single-step RT-PCR using primer/fluorogenic probe systems (rRT-PCR) allow even more rapid and sensitive diagnosis with detection of AI viruses and determination of subtype H5 or H7 in clinical samples.

An important problem with RT-PCR and rRT-PCR systems is that, to date, different laboratories have developed different systems, which, although perfectly legitimate, have not been validated or subjected to tests with large numbers of samples in different laboratories. The Community reference laboratory and specified national reference laboratories have been addressing this problem as part of an Community-funded project (EU AVIFLU) to produce ratified protocols for conventional RT-PCR and rRT-PCR that could be adopted by other national reference laboratories. If test parameters, such as cycling and ramp times, are varied from those recommended in the specified protocols they must be demonstrated as fit for purpose prior to use in accordance with point 6 of Chapter I of this diagnostic manual.

The standard protocols for those molecular tests and their evaluation as applied by the Community reference laboratory can be found on the following website:

http://www.defra.gov.uk/corporate/vla/science/science-viral-ai-reflab.htm

## CHAPTER VII

### In vivo pathogenicity test and the evaluation of results

The virulence for chickens of influenza A viruses isolated from birds must be estimated using the intravenous pathogenicity index (IVPI) test which must be carried out as follows:

(a) Fresh infective allantoic fluid with a HA titre >1/16 ($>2^4$ or $>\log_2 4$ when expressed as the reciprocal) from the lowest passage level available, preferably from the initial isolation without any selection is diluted 1/10 in sterile isotonic saline.

(b) 0,1 ml of the diluted virus is injected intravenously into each of ten six-week-old SPF or SAN chickens.

(c) Birds are examined at 24-hour intervals for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. The judgement of sick and severely sick birds is a subjective clinical assessment.

Normally, 'sick' birds show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead birds must be scored as 3 at each of the remaining daily observations after death.

On welfare grounds when birds are too sick to eat or drink, they must be killed humanely and scored as dead at the next observation since they will die within 24 hours without intervention. This approach is acceptable to accreditation authorities.

(d) The IVPI is the mean score per bird per observation over the 10-day period. An index of 3,0 means that all birds died within 24 hours, and an index of 0,0 means that no bird showed any clinical sign during the 10-day observation period.

A simple method for recording results and calculating indices is shown in the following 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 |

Notes:

10 birds observed for 10 days = 100 observations

Index = mean score per bird per observation = 246/100 = 2,46

Any influenza A virus, regardless of subtype, giving a value of greater than 1,2 in an IVPI test is considered to be a HPAI virus.

## CHAPTER VIII

### Serological tests and evaluation of results

The preferred method used to show the presence of influenza A virus is to demonstrate the possession of the nucleoprotein or matrix antigens which are shared by all influenza A viruses.

It may be done in immunodoublediffusion tests involving either concentrated virus preparations or extracts from infected chorioallantoic membranes.

The preferred methods used for serological tests for AI virus antibodies are haemagglutination (HA) and haemagglutination inhibition (HI) tests.

Chapter 2.7.12 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health (OIE) contains detailed information on the laboratory techniques and the evaluation of the results.

The standard protocols for serological tests and the evaluation of their results as applied by the Community reference laboratory can be found on the following website:

http://www.defra.gov.uk/corporate/vla/science/science-viral-ai-reflab.htm

## CHAPTER IX

### Monitoring systems associated with vaccination

1. Directive 2005/94/EC and the diagnostic manual

Sections 2 and 3 of Chapter IX, of Directive 2005/94/EC allow the use of emergency and preventive vaccination under certain conditions. One of those conditions is that a 'DIVA' (Differentiating Infected from Vaccinated Animals) strategy is employed.

Vaccination must be aimed at preventing the infection and subsequent spreading of virus between flocks. There is indisputable evidence to show vaccination increases the amount of virus needed to infect birds and decreases the amount of virus excreted. However, although vaccinated birds do no longer develop clinical signs they can still spread virus when challenged. Thus HPAI viruses of H5 and H7 subtypes could circulate unnoticed for some time in a flock with suboptimal levels of immunity in the same way that LPAI viruses could do in an unvaccinated flock. There is therefore a need to be able to identify virus positive vaccinated flocks that have become infected with field virus so that other control measures, such as stamping out, can be implemented.

2. Use of sentinels for monitoring infection

At the flock level, a simple method is to regularly monitor sentinel birds left unvaccinated in each vaccinated flock, but this approach does have some management problems, particularly in identifying the sentinels especially in large flocks. The contact between the sentinels and the vaccinated birds must be ensured.

3. DIVA laboratory test for monitoring infection

As an alternative or in addition, testing for field exposure may be performed on the vaccinated birds itself by using DIVA laboratory tests. Several test systems have been developed in recent years that also allow the detection of field challenge of vaccinated birds. One method that has proved workable is the use of a vaccine containing a virus of the same haemagglutinin (H) subtype but a different neuraminidase (N) from the prevailing field virus. Antibodies to the N of the field virus act as natural markers of infection.

That system was used in Italy following the re-emergence of a LPAI H7N1 virus in 2000. In order to supplement direct control measures, a DIVA strategy was implemented using a vaccine containing H7N3 to combat an H7N1 field infection. Vaccinated and field exposed birds were differentiated using a serological test to detect specific anti-N1 antibodies. The same strategy was used to control LPAI caused by H7N3 in Italy in 2002 to 2003, in this case with an H7N1 vaccine and a serological test detecting antibodies against N3 specifically. In both cases vaccination with stamping out using this DIVA strategy resulted in eradication of the field virus.

Problems with that system arise if a field virus emerges that has the same N antigen as the existing field virus but are of another H subtype than H5 or H7 or if subtypes with the same N antigens are already circulating in the field. Particularly ducks are known to be carriers of more than one subtype. There was also a need to develop a suitable test that would allow the routine monitoring of flocks for anti-neuraminidase antibodies. In Italy an 'ad hoc' serological test based on an indirect fluorescent antibody assay, using as an antigen N proteins expressed by baculovirus recombinants was developed and used. That may have wider and easier application when an ELISA test is developed.

The use of vaccines containing only HA, such as recombinant vector vaccines, allows classical AGID tests or ELISA tests based on nucleoprotein, non-structural protein or matrix proteins to be used to detect infection in vaccinated birds.

For inactivated vaccines, a test that detects antibodies to the non-structural virus protein that are only produced during natural infection has been described. Such a system is yet to be validated in the field, but has the limitation that natural infection of a flock with any influenza virus, irrespective of subtype results in the production of antibodies directed against the non-structural protein.

The development of rapid and sensitive virus-detection methods, especially those that can be automated, such as real time RT-PCR, means that these could be used for simple widespread and regular testing of vaccinated birds for the presence of field virus. Agent detection, however, will be limited to a short window in the acute phase of the infection and cannot be used to conclude that a flock was not exposed to the virus in the past. This approach is most appropriate to the testing of vaccinated birds prior to movement to demonstrate freedom from active infection.

The number of samples to be tested by the systems of choice must enable the exclusion of a prevalence of AI virus infection in a flock of more than 15 % with confidence level of 95 %.

## CHAPTER X

### Strategies in the diagnosis of AI

As set out in Annex IV to Directive 2005/94/EC, decisions to apply measures in specific areas or contact holdings and the severity of those measures may vary greatly with the magnitude of the risk. Similarly, the required diagnostic confirmation of disease is likely to be balanced against the prevailing situation, the magnitude of the hazard and the degree of risk. Veterinary authorities need to make decisions on diagnostic evidence that balance the rapid control and eradication of disease against the potential impact of misdiagnosis. Such judgements have to be made against the background of many factors at the time, but certain situations can be predicted.

| Disease situation | Potential problem | Diagnostic criteria |
|---|---|---|
| Non specific signs, no official suspicion | Isolated holding | Carry out rapid detection based on M gene RT-PCR. Differential diagnosis as required |
| Primary suspected outbreak | Isolated holding | Carry out full diagnostic testing, virus isolation and characterisation |
| Primary suspected outbreak | Holding in densely populated poultry area | Carry out full diagnostic testing, virus isolation and characterisation, but concentrate on rapid detection and characterisation methods especially those based on RT-PCR and sequencing[a]. |

**a** For these full sampling should be carried out and samples stored for later evaluation.

| | | |
|---|---|---|
| Second and subsequent suspected outbreaks | Isolated holdings, epidemiologically linked to primary suspected outbreak | Concentrate on rapid detection and characterisation methods especially those based on RT-PCR and sequencing[a]. |
| Second and subsequent suspected outbreaks | Holdings in densely populated poultry area or with many epidemiological links | Rely on rapid detection methods that give the earliest evidence of the presence of any AI virus[a]. |
| Multiple suspected outbreaks or disease spreading rapidly including surveillance | Spread will become uncontrollable without rapid intervention | Rely on rapid detection methods that give the earliest evidence of the presence of any AI virus or rely on clinical signs[a]. |

**a** For these full sampling should be carried out and samples stored for later evaluation.

## CHAPTER XI

### Diagnosis of infection with AI viruses in pigs and other mammals

1. AI in pigs

AI viruses readily infect pigs and although replication is in most cases relatively restricted, there is a potential that infected pigs could transmit the disease to poultry and other susceptible animals. To date, there is no evidence from the field that infected pigs transmit AI viruses of H5 and H7 subtypes.

Experience gained during the outbreak in the Netherlands in 2003 indicated that H7N7 infected pigs did not show clinical signs that could be attributed to the H7N7-infection. Moreover, apparently no diseased pigs have been reported to date during the H5N1 outbreak in Asia and elsewhere.

Therefore, clinical signs may not be relied upon to indicate whether pigs are infected although clinical presentation due to infection of pigs with other influenza viruses of avian origin, can occur once a virus has become adapted to the host. The diagnosis of AI virus infections of pigs is essentially similar to diagnosis for avian species, relying on virus isolation, molecular techniques and detection of specific antibodies using haemagglutination inhibition tests. There are, however, certain differences and none of the tests are fully validated for the use in pigs to confirm infection with AI viruses.

2. Samples for virus isolation

AI virus infections in pigs are usually restricted to the respiratory tract and samples must be respiratory tract tissues and, if appropriate, oropharyngeal or nasal swabs, preferably taken from pigs showing signs of that disease. These samples and swabs may be processed for virus isolation or molecular detection of virus, using the same techniques described above for samples from birds. However, when using PCR techniques, proper controls must be used to ensure that the amplification is not inhibited by substances in the samples from pigs.

3. Inoculation and incubation of eggs

To isolate mammalian influenza viruses in 9- to 11-day-old embryonated fowls' eggs it is usual practice to inoculate each egg via the allantoic cavity and into the amniotic cavity. However, when testing pigs in contact with AI viruses, when virus has had little opportunity to adapt, allantoic cavity inoculation is probably sufficient.

Similarly, 35 $^{o}$C is usually recommended for the incubation temperature for isolation of mammalian influenza A viruses, but again for viruses poorly adapted to pigs, 37 $^{o}$C is not be detrimental to virus isolation.

4. Test for specific antibodies in HI tests

Virus isolation or molecular detection are likely to be the most sensitive for determining AI virus infections of pigs. However, serological responses in pigs have been detected in the absence of virus isolation or detection. HI tests, using pig sera, require some modifications to the test used for avian sera referred to in Chapter VIII.

Pig sera are notorious for their property of non-specific inhibition in HI tests and therefore each serum sample must be treated with a receptor destroying enzyme (RDE) to prevent this from occurring. The following method must be used:

(a) To 100 µl of pig antiserum add 400 µl RDE (predetermined working dilution) and mix thoroughly.

(b) Incubate at 37 $^{o}$C for one hour.

(c) Then incubate for 30 minutes at 56 $^{o}$C.

(d) Cool samples at 4 $^{o}$C for a minimum of 15 minutes.

(e) Add 10 µl of 30 % (v/v packed cells) chick red blood cells and mix vigorously.

(f) Incubate at 4 $^{o}$C overnight. Alternatively, if it is essential to use samples same day, incubate at 37 $^{o}$C for one hour and centrifuge at 300 x g for five minutes.

The treated serum is then used in HI tests as described for avian sera in paragraph [..], the initial dilution is 1:10. A set of sera of pigs with a known sero-negative status regarding AI must be used to assess the specificity of the HI test for the virus strain to be used (see use of virus strain for serology derived from the outbreak; Chapter VIII). During the outbreak in the Netherlands in 2003 up to 2,6 % non-specific reactors were detected in the HI test using pig sera that were collected independently of the outbreak

5. Sampling of pigs

Particularly on farms that keep both pigs and poultry, either mixed or in separate houses, pigs are at risk of becoming infected with AI directly or indirectly via contact with poultry or poultry products. To exclude such infection, oropharyngeal or nasal swabs and blood samples must be collected according to the procedures described in point 8.21 of Chapter IV. Samples must be obtained from pigs that show clinical signs of the disease. However, when they do not show any clinical signs, samples may be collected at random over all sections of the house. If available in the laboratory, swabs must be tested in rapid molecular tests and/or virus isolation. The RT-PCR must have been appropriately validated and have a sensitivity at least equivalent to virus isolation in eggs for influenza A viruses.

Two to four weeks after culling of the AI infected poultry, at least 60 blood samples must be collected from pigs in such a way that at least some samples are obtained from groups of pigs that are in direct contact with each other. Samples must be tested in the HI test using virus

derived from the poultry outbreak. Samples from both the acute and convalescent phases must be tested in the same test. Positive samples can be confirmed using virus neutralisation and/or Western blot analyses.

When any of those samples test positive, an epidemiological investigation on all pig farms located within the protection zone must be carried out, irrespective whether they are of mixed type or not.

6. **AI viruses in other mammals other than pigs**

Investigations in other mammals other than pigs which are susceptible to AI including cats must be undertaken. With specific reference to HPAI H5N1, the following must be carried out for testing cats:

Gross pathological lesions, associated with viral replication, concentrate on the lungs and liver, therefore samples for virological investigations must preferably be taken from these organs of dead animals. In living animals, preferably tracheal/oropharyngeal swabs must be taken for virus detection. In addition, faecal swabs can be taken separately.

Blood samples to be examined in HI assays require heat treatment for 56 $^{o}$C for 30 minutes and RDE treatment can be omitted.

## CHAPTER XII

### Minimum safety requirements for the transport of samples

1. The transport of samples in which pathogens are known to be present or suspected of being present are the subject of strict national and international regulations that must be adhered to at all times. Virus isolates are not classified as diagnostic samples but must be packaged in accordance with international standards.

The instructions set out in this chapter are for air transport, but similar packaging must be used for land or sea transport of samples.

2. Packing diagnostic specimens for transport

Diagnostic specimens transported under the IATA Regulations are assigned to UN identification number 2814, 2900, or 3373, as relevant.

The shipper and not the transport company is responsible for the shipment until the package reaches the consignee.

3. Primary packaging

(a) Primary receptacle(s) must be water tight, for example, screw caps must be sealed with parafilm or adhesive tape or similar protections taken.

(b) Multiple primary receptacles must be wrapped individually to prevent breakage.

(c) When determining the volume of diagnostic specimens being shipped, the viral transport media must be taken into account.

(d) Primary receptacle(s) must not contain more than 500 ml or 500 g.

The entire contents of the primary receptacle is the diagnostic specimen.

4. Secondary packaging

(a) Enough absorbent material in the secondary container to absorb the entire contents of all primary receptacles in case of leakage or damage must be used.

(b) Secondary packaging must meet the IATA packaging requirements for diagnostic specimens including 1,2 metres (3,9 feet) drop test procedure. Since infectious substance packaging surpasses the requirements for diagnostic specimen packaging, in the IATA Packing Instruction 602, it may be used.

(c) Infectious substance packaging must have the required specification markings on packaging (UN will be in a circle), for example:

**UN 4G/CLASS 6.2/99/GB/2450**

(d) Secondary packaging must be watertight. The packaging manufacturer or other authorised party's packing instructions, included with the secondary packaging, must be followed.

(e) Secondary packaging must be at least 100 mm (four inches) in the smallest overall external dimension.

(f) Secondary packaging must be large enough for shipping documents, such as an air waybill.

5. Outer packaging

(a) The outer packaging must not contain more than 4 l or 4 kg.

(b) If required, either dry ice or wet ice must be placed outside the secondary packaging. If using dry ice the packaging must permit the release of carbon dioxide gas and not allow a build-up of pressure that could rupture the packaging. If using wet ice, the packaging must be leak-proof.

Each package and the air waybill must be marked with the following exact wording:

**UN 3373 DIAGNOSTIC SPECIMEN**

**PACKED IN COMPLIANCE WITH**

**IATA PACKING INSTRUCTION 650**

(c) An itemised list of contents must be enclosed between the secondary packaging and the outer packaging.

(d) The outer packaging must be placed in a sealed plastic bag to protect from moisture.

(e) A Shipper's Declaration for Dangerous Goods is not required.

## CHAPTER XIII

### Dispatch of viruses and samples to the Community reference laboratory

1. The samples to be sent to the Community reference laboratory need to comply with the recommendations for the transport of dangerous pathogens within the Community and also regulations and legislation in force in the United Kingdom.

The instructions set out in this chapter must be followed.

2. Dispatch of viruses or other materials to the Community reference laboratory

(a) All materials must be packed in accordance with the instructions set out in this Chapter.

(b) The outer packaging must be marked as follows:

ANIMAL PATHOGEN — PACKAGE ONLY TO BE OPENED AT THE AVIAN VIROLOGY SECTION, VLA, WEYBRIDGE. IMPORTATION AUTHORISED BY LICENCE NUMBER ....*..... ISSUED UNDER THE IMPORTATION OF ANIMAL PATHOGENS ORDER.

(c) One of the following licence numbers must be inserted:

| | | |
|---|---|---|
| (i) | for AI viruses: | '**AHZ/2232/2002/5***' |
| (ii) | for tissues and other materials: | '**AHZ/2074C/2004/3***' |

As those licence numbers are changed from time to time laboratories sending samples must ensure that they use the current licence numbers before sending packages.

(d) The package must be addressed to:

Avian Virology

VLA Weybridge,

New Haw, Addlestone,

Surrey KT15 3NB

United Kingdom

(e) A letter must accompany the parcel with as much history about the isolates as possible, such as the species and age, area/country of isolation, any clinical history.

(f) Packages must be sent by air mail or air freight.

If packages are sent by air freight, the airway bill number must be given to the Community reference laboratory by fax, telephone, or e-mail before arrival of the materials.

Packages sent by air freight, must be clearly marked as follows:

'**CARE OF TRANSGLOBAL**' to ensure rapid processing at the airport.

*Community reference laboratory contacts*

Ian H. Brown, Director of the Reference Laboratory

Direct tel. (44-1932) 35 73 39;

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## CHAPTER XIV

### Minimum safety requirements for an AI diagnostic laboratory

1. The safety requirements in diagnostic laboratories working with AI viruses must cover both the containment of the viruses as a threat to animal health and the protection of those working in the laboratory (and those outside) from any zoonotic risk.

In the Community, the minimal safety requirements for laboratories are set out in the several Directives. In addition, operational aspects are described and set out in underlying European Norms (EN). For the operation of laboratories for diagnostic purposes there are additional regulations (EN), such as good laboratory practice.

2. Community directives on laboratories

**Council Directive 89/391/EEC** of 12 June 1989 on the introduction of measures to encourage improvements in the safety and health of workers at work (OJ L 183, 29.6.1989, p. 1).

**Council Directive 90/679/EEC** of 26 November 1990 on the protection of workers from risks related to exposure to biological agents at work (seventh individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC) (OJ L 374, 31.12.1990, p. 1).

If there is diagnostic done by Polymerase Chain Reaction (PCR) and cloning of PCR-products into a bacterial plasmid for propagation, for example, for the purpose of DNA-sequencing, the following Directive and European Norms (EN) apply in addition to those two Directives:

**Council Directive 90/219/EEC** of 23 April 1990 on the contained use of genetically modified micro-organisms (OJ L 117, 8.5.1990, p. 1).

3. In addition to the Community Directives, European Norms (EN) must be recognised:

**EN 12128** Biotechnology. Laboratories for research, development and analysis. Containment levels of microbiology laboratories, areas of risk, localities and physical safety requirements

**EN 12738** Biotechnology. Laboratories for research, development and analysis. Guidance for containment of animals inoculated with micro-organisms in experiments

**EN 12740** Biotechnology. Laboratories for research, development and analysis. Guidance for handling, inactivating and testing of waste

**EN 12741** Biotechnology. Laboratories for research, development and analysis. Guidance for biotechnology laboratory operations

For operating/managing a laboratory, the following conditions apply:

4. Requirements for Laboratories (containment levels 1 to 4)

In accordance with **Directive 2000/54/EC** of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual Directive within the meaning of Article 16(1) of Directive 89/391/EEC) (OJ L 262, 17.10.2000, p. 21), Directive 90/219/EEC and European Norms: EN 12128; EN 12740; EN 12741.

| **Containment measures** | **Containment level** | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |

| | | | | |
|---|---|---|---|---|
| Laboratory suite: isolation | no | yes | yes | yes |
| Laboratories separated by doors | no | yes | yes | yes |
| An observation window or alternative must be present so that occupants can be seen | optional | optional | optional | yes |
| Hand washing facilities must be provided for personnel | yes | yes | yes | yes |
| Disinfecting facilities (hands) must be provided | optional | yes | yes | yes |
| Restricted access | no | yes | yes | yes |
| Specific measures to control aerosol | no | Yes<br>minimise | Yes<br>prevent | Yes<br>prevent |
| Biohazard sign on the door | no | yes | yes | yes |
| Shower | no | no | optional | yes |
| Eye irrigation | yes | yes | yes | yes |
| Laboratory: sealable for fumigation | no | no | yes | yes |
| Surfaces resistant to water, acids, alkalis, solvents, disinfectants, decontamination agents and easy to clean | Yes (bench) | Yes (bench) | Yes (bench, floor) | Yes (bench, floor) |
| Entry to lab via airlock | no | no | optional | yes |
| Negative pressure relative to the pressure of the immediate environment | no | no | optional | yes |

| | | | | |
|---|---|---|---|---|
| Extract and input air from laboratory must be HEPA-filtered | no | no | yes (extract air) | yes |
| Autoclave | on site | in the building | en suite | in lab, double ended |
| Protective clothing | Suitable protective clothing | Suitable protective clothing | Suitable protective clothing (optional footwear) | Complete change of clothing |
| Gloves | no | optional | yes | yes |
| Efficient vector control (e.g. for rodents and insects) | optional | yes | yes | yes |
| Safe Storage of a biological agent | yes | yes | yes | yes |
| Laboratory to contain its own equipment | no | no | Recom-mended | yes |

There are additional European Norms which deal with the management and organisation of laboratories.

There are other national and international regulations and recommendations which must be followed. The WHO has published its Laboratory Biosafety Manual 3rd Edition on its website:

http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/

5. Containment relating to animal health

Regulations concerning the containment of AI viruses especially HPAI, but also including all AI viruses of H5 and H7 subtypes must be put in place by the veterinary authorities in Member States. Some guidance is provided by the World Organisation for Animal Health (OIE) in Chapter 1.4.5 of the Terrestrial Animal Health Code 2005 and HPNAI is regarded as an OIE Containment Group 4 pathogen.

Although the regulations governing the handling of AI viruses will be put in place by the veterinary authorities of the Member State.

The Minimum Safety Requirements applied by the Community reference laboratory, which are the national rules of the United Kingdom, can be viewed on the following website:

http://www.defra.gov.uk/corporate/vla/science/science-viral-ai-reflab.htm

6. Containment relating to human health

Laboratories working with AI viruses must be aware at all times that these are at least potential human pathogens and conduct the running of the laboratory to avoid infecting those working in the laboratory and any escape of virus to those outside.

Guidelines for handling specimens suspected of containing AI A virus can be found on the website of the World Health Organization (WHO):

http://www.who.int/csr/disease/avian_influenza/guidelines/handlingspecimens/en/

(**1**) OJ L 10, 14.1.2006, p. 16.

**Changes to legislation:**
There are outstanding changes not yet made to Commission Decision of 4 August 2006 approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC (notified under document number C(2006) 3477) (Text with EEA relevance) (2006/437/EC). Any changes that have already been made to the legislation appear in the content and are referenced with annotations.
View outstanding changes

**Changes and effects yet to be applied to :**

- Annex Ch. 13 substituted by S.I. 2018/1410 reg. 10(11)
- Annex Ch. 6 words substituted by S.I. 2018/1410 reg. 10(8)
- Annex Ch. 8 words substituted by S.I. 2018/1410 reg. 10(9)
- Art. 2 words substituted by S.I. 2018/1410 reg. 10(2)
- Art. 3 omitted by S.I. 2018/1410 reg. 10(4)

**Changes and effects yet to be applied to the whole legislation item and associated provisions**

- Annex Ch. 5 para. 4(c) omitted by S.I. 2018/1410 reg. 10(7)(b)
- Annex Ch. 5 para. 4(d) omitted by S.I. 2018/1410 reg. 10(7)(b)
- Annex Ch. 5 para. 4(b)(iii) substituted by S.I. 2018/1410 reg. 10(7)(a)(i)
- Annex Ch. 14 para. 1 word substituted by S.I. 2018/1410 reg. 10(12)(a)
- Annex Ch. 1 para. 6(b)(i) words omitted by S.I. 2018/1410 reg. 10(6)(b)(i)
- Annex Ch. 1 para. 6(b)(ii) words omitted by S.I. 2018/1410 reg. 10(6)(b)(ii)(aa)
- Annex Ch. 1 para. 6(b)(ii) words omitted by S.I. 2018/1410 reg. 10(6)(b)(ii)(bb)
- Annex Ch. 5 para. 4(b) words omitted by S.I. 2018/1410 reg. 10(7)(a)(ii)
- Annex Ch. 1 para. 1 words omitted by virtue of S.I. 2018/1410, reg. 10(6)(a) (as substituted) by S.I. 2020/1388 reg. 25(7)(b)
- Annex Ch. 1 para. 1 words substituted by S.I. 2018/1410 reg. 10(6)(a) (This amendment not applied to legislation.gov.uk. Reg. 10(6)(a) substituted immediately before IP completion day by virtue of S.I. 2020/1388, regs. 1(2)(a), 25(7)(b))
- Annex Ch. 14 para. 5 words substituted by S.I. 2018/1410 reg. 10(12)(b)(i)
- Annex Ch. 14 para. 5 words substituted by S.I. 2018/1410 reg. 10(12)(b)(ii)
- Annex Ch. 14 para. 5 words substituted by S.I. 2018/1410 reg. 10(12)(b)(iii)(iv) (This amendment not applied to legislation.gov.uk. Reg. 10(12)(b)(iii) substituted and (iv) omitted immediately before IP completion day by virtue of S.I. 2020/1388, regs. 1(2)(a), 25(7)(c)(i)(ii))
- Annex Ch. 14 para. 5 words substituted by S.I. 2018/1410, reg. 12(b)(iii) (as substituted) by S.I. 2020/1388 reg. 25(7)(c)(ii)
- Art. 2a inserted by S.I. 2018/1410 reg. 10(3)
- Art. 2a(a)(ii)(iii) Art. 2a(a)(iii)(iv) renumbered as Art. 2a(a)(ii)(iii) in earlier amending provision S.I. 2018/1410, reg. 10(3) by S.I. 2020/1388 reg. 25(7)(a)(i)
- Art. 2a(a)(ii) omitted in earlier amending provision S.I. 2018/1410, reg. 10(3) by S.I. 2020/1388 reg. 25(7)(a)(i)
- Art. 2a(b) words substituted in earlier amending provision S.I. 2018/1410, reg. 10(3) by S.I. 2020/1388 reg. 25(7)(a)(ii)