COMMISSION DECISION

of 26 May 2003

approving an African swine fever diagnostic manual

(notified under document number C(2003) 1696)

(Text with EEA relevance)

(2003/422/EC)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Council Directive 2002/60/EC of 27 June 2002 laying down specific provisions for the control of African swine fever and amending Directive 92/119/EEC as regards Teschen disease and African swine fever [1], and in particular Article 18(3) thereof,

Whereas:

(1) It is necessary pursuant to Directive 2002/60/EC to lay down uniform diagnostic procedures, sampling methods and criteria for the evaluation of the results of laboratory tests for the confirmation of African swine fever.

(2) In accordance with that Directive, the Community Reference Laboratory for African swine fever is to coordinate, in consultation with the Commission, the methods employed in the Member States for diagnosing the disease, by organising, *inter alia*, periodic comparative tests and the supplying of standard reagents at Community level.

(3) African swine fever virus is not considered to be a hazard for human health.

(4) Laboratory tests have been developed to ensure rapid confirmation of African swine fever.

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

(6) It is therefore appropriate to approve the manual laying down those procedures and criteria.

(7) The national diagnostic laboratories should be authorised to modify the approved laboratory tests or use different tests, provided that equal sensitivity and specificity can be demonstrated.

(8) 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

1. The African swine fever diagnostic manual in the Annex is approved.

2. Member States shall ensure that the confirmation of African swine fever is carried out in accordance with the procedures, sampling methods and criteria for evaluation of laboratory test results laid down in the manual and based on:

(a) the detection of clinical signs and post-mortem lesions of disease;

(b) the detection of the virus, antigen or genome in samples of pig tissues, organs, blood or excreta;

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

3. By way of derogation from paragraph 2, the national diagnostic laboratories referred to in Annex IV to Directive 2002/60/EC may apply modifications to the laboratory tests referred to in the manual, or use different tests, provided that an equal sensitivity and specificity can be demonstrated.

If modified or different tests are applied, their sensitivity and specificity must be evaluated in the framework of the periodic comparative tests organised by the Community Reference Laboratory for African swine fever.

Article 2

This Decision shall apply from 1 July 2003.

Article 3

This Decision is addressed to the Member States.

Done at Brussels, 26 May 2003.

For the Commission

David BYRNE

Member of the Commission

[1] OJ L 192, 20.7.2002, p. 27.

ANNEX

AFRICAN SWINE FEVER DIAGNOSTIC MANUAL

Chapter I

Introduction, objectives and definitions

1. In order to ensure uniform procedures to diagnose African swine fever (hereinafter 'ASF'), this manual:

   (a) provides guidelines and minimum requirements on diagnostic procedures, sampling methods and criteria for the evaluation of the results of clinical and post-mortem examinations and laboratory tests for a correct diagnosis of ASF ([1]);

   (b) establishes minimum bio-safety requirements and quality standards to be observed by the ASF diagnostic laboratories and for transport of samples;

   (c) establishes the laboratory tests to be used for the diagnosis of ASF and the laboratory techniques to be used for the genetic typing of ASF virus isolates.

2. This manual is principally directed towards the authorities responsible for the control of ASF. Therefore, emphasis is on the principles and applications of laboratory tests and evaluation of their results and not on detailed laboratory techniques.

3. For the purpose of this manual, in addition to the definitions referred to in Article 2 of Directive 2002/60/EC, the following definitions shall apply:

   (a) 'suspected holding' means any pig holding which contains one or more pigs suspected of being infected with the ASF virus or a contact holding as defined in Article 2(k) of Directive 2002/60/EC;

   (b) 'epidemiological subunit' or 'subunit' means the building, place or land nearby where groups of pigs within a holding are kept in such a way that they have frequent direct or indirect contact with one another but, at the same time, they are kept separate from other pigs in the same holding;

   (c) 'in-contact pigs' means the pigs which lived in a holding in direct contact with one or more pigs suspected to be infected with the ASF virus within the previous 21 days.

Chapter II

Description of ASF with emphasis on differential diagnosis

A. INTRODUCTION

1. ASF is caused by an enveloped DNA virus which belongs to the genus *Asfivirus* of the family *Asfarviridae*. ASF virus strains differ in virulence, although different serotypes cannot be identified.

2. The ASF virus is very stable in excretions of infected pigs, pig carcases and fresh pig meat and some pig meat products. Appropriate disinfectants must be used to ensure its inactivation in the environment.

3. The main natural route of infection of pigs in Europe is oro-nasal by direct or indirect contact with infected pigs or by feeding of virus-contaminated feed. However, in those areas where vectors ([2]) exist, transmission via these vectors plays a very important role in virus persistence and spread. ASF may also spread via indirect contact with contaminated materials and via biting insects which mechanically transport the ASF virus. Disease transmission may also occur via semen of infected boars.

4. The incubation period in individual animals is about five to 15 days, but under field conditions clinical symptoms may only become evident in a holding several weeks after virus introduction or even more if mild strains of the virus are concerned.

([1]) When deciding the number of samples to be taken for laboratory testing, the sensitivity of the tests that will be used shall also be considered. The number of animals to be sampled shall be higher than the one indicated in this manual, if the sensitivity of the test to be used is not very high.

([2]) As defined in Article 2(r) of Directive 2002/60/EC.

5. Acute, subacute and chronic forms of ASF occur, the difference depending mainly on virus virulence.

6. In pigs which clinically recover after infection, viraemia persists for 40 to 60 days and these pigs become virus carriers. The ASF virus has been isolated from carrier pigs up to six months after infection.

B. ACUTE FORM

1. The development of high fever (more than 40 °C) is usually the first clinical sign of the disease, which is accompanied by depression, loss of appetite, rapid and difficult breathing, and discharges from the nose and eyes. Pigs show uncoordinated movements and huddle together. Sows may abort at all stages of pregnancy. Some pigs may show vomiting and constipation, while others may develop bloody diarrhoea. Congested or haemorrhagic subcutaneous areas become evident, in particular at the extremities and ears. A coma may develop before death, which occurs one to seven days after development of clinical signs. The morbidity and mortality rate within a holding may be 100 %.

   Post-mortem findings indicate a typical haemorrhagic syndrome, with generalised congestion of the carcase, bloody fluid in the chest and abdominal cavities, enlarged dark spleen, haemorrhagic lymph nodes which resemble blood clots, especially renal and gastrohepatic lymph nodes, petechial haemorrhages in the kidneys (cortical and medullary pyramids and renal pelvis), abdominal serosae, gastric and intestinal mucosa and heart (epicardium and endocardium), hydrothorax and petechial haemorrhages of the pleura.

2. In general the acute form of classical swine fever leads to a clinical and pathological picture very similar to that of African swine fever. When present, haemorrhages on the skin and ears are quite easy to detect and lead to suspicion of acute classical or African swine fever. Few other diseases cause similar lesions.

   Acute African swine fever must also be considered in cases of suspected erysipelas, porcine reproductive and respiratory syndrome, coumarin poisoning, purpura haemorragica, post-weaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome, salmonella or *Pasteurella* infections or any enteric or respiratory syndromes with fever which do not respond to antibiotic treatment.

C. SUBACUTE FORMS

Subacute forms of the disease are more common in endemic areas. Subacute infection is characterised by fluctuating fever, depression and pneumonia. Death may occur due to heart failure. Lesions in subacute forms are similar to those in the acute form but milder. Characteristic lesions are large haemorrhages in the lymph nodes, kidneys and spleen, pulmonary congestion and oedema and in some cases interstitial pneumonia.

D. CHRONIC FORMS

Chronic forms of the disease are rare. In chronic forms, secondary bacterial infections can be observed. As clinical signs of chronic ASF are rather non-specific, many other diseases must be considered for differential diagnosis. Increased body temperature is not necessarily present in every animal, but in an infected holding fever can be detected at least in some pigs.

Clinical symptoms of chronic ASF may include respiratory problems, abortions, arthritis, chronic skin ulcers or necrosis, not resembling the typical clinical picture of ASF virus infections. The lesions may be minimal or absent. Histopathological findings are characterised by enlarged lymph nodes and spleen, pleuritis and fibrinous pericarditis and infiltrated pneumonitis. Focal caseous necrosis and mineralisation of the lung have also been described.

Chapter III

Guidelines on main criteria to be considered for the recognition of a holding as an ASF suspected holding

1. The decision to recognise a holding as a suspected holding will be taken on the basis of the following findings, criteria and grounds:

   (a) clinical and pathological findings in pigs. The main clinical and pathological findings to be considered are:

   — fever with morbidity and mortality in pigs of all ages,

   — fever with haemorrhagic syndrome; petechial and ecchymotic haemorrhages, especially in the lymph nodes, kidneys, spleen (which is enlarged and dark, particularly in the acute forms) and urinary bladder and ulcerations on the gall bladder;

(b) epidemiological findings. The main epidemiological findings to be considered are:

— where pigs had direct or indirect contact with a pig holding proven to have been infected with the ASF virus,

— where a holding has supplied pigs that were subsequently shown to be infected with the ASF virus,

— where sows have been artificially inseminated with semen originating from a suspect source,

— where there has been indirect or direct contact with feral pigs in a population where ASF occurs,

— where pigs are kept outdoors in a region where feral pigs are infected with the ASF virus,

— where pigs have been fed with swill and there is the suspicion that this swill has not been treated in such a way as to inactivate the ASF virus,

— where possible exposure might have occurred, for example due to persons entering the holding, transports, etc. coming from holdings suspected to be infected or infected with the ASF virus,

— where vectors occur in the area of the holding.

2. In any case, a holding must be considered as a suspected holding if a suspicion of classical swine fever has been raised in the holding due to clinical or pathological findings but clinical, epidemiological and laboratory investigations have not led to the confirmation of this disease or to the identification of other disease sources or agents in the holding in question.

Chapter IV

Checking and sampling procedures

A. GUIDELINES AND PROCEDURES FOR CLINICAL EXAMINATION OF AND SAMPLING ON PIGS IN SUSPECTED HOLDINGS

1. Member States shall ensure that appropriate clinical examinations, sampling and laboratory investigations are carried out in suspected holdings to confirm or exclude ASF, in accordance with the guidelines and procedures laid down in points 2 to 6.

Irrespective of the adoption of the measures referred to in Article 4(2) of Directive 2002/60/EC in the holding in question, these guidelines and procedures shall also apply in cases of disease whenever ASF is considered in the differential diagnosis. This will include occasions when the clinical signs and epidemiological pattern of disease that are observed in pigs suggest a very low probability of occurrence of ASF.

In all other cases where one or more pigs are suspected of being infected with the ASF virus, the measures referred to in Article 4(2) of Directive 2002/60/EC shall be adopted in the suspected holding in question.

In case of suspicion of ASF in pigs in a slaughterhouse or means of transport, the guidelines and procedures laid down in points 2 to 6 shall also apply *mutatis mutandis*.

2. When an official veterinarian visits a suspected holding to confirm or rule out ASF:

— a check of the production and health records of the holding must be carried out, if these records are available; an inspection in each subunit of the holding must be carried out to select the pigs to be clinically examined.

The clinical examination must include taking the body temperature and must primarily concern the following pigs or group of pigs:

— sick or anorexic pigs,

— pigs recently introduced from confirmed outbreaks or from other suspected sources,

— pigs kept in subunits recently visited by external visitors who had recent close contact with ASF-suspected or infected pigs or for whom other particularly risky contacts with a potential source of the ASF virus have been identified,

— pigs already sampled and serologically tested for ASF, in case the results of these tests do not allow ASF to be ruled out, and in-contact pigs,

— pigs recently recovered from the disease.

If the inspection in the suspected holding has not indicated the presence of the pigs or group of pigs referred to in the above subparagraph, the competent authority, without prejudice to other measures that may be applied in the holding in question in accordance with Directive 2002/60/EC and taking into account the epidemiological situation, shall:

— carry out further examinations in the holding in question in accordance with point 3, or

— ensure that blood samples for laboratory tests are taken from the pigs in the holding in question (in this case the sampling procedures laid down in point 5, and in section F(2) shall be used for guidance purposes), or

— adopt or maintain the measures laid down in Article 4(2) of Directive 2002/60/EC, pending further investigations in the holding in question, or

— rule out the suspicion of ASF.

3. When reference is made to this paragraph, the clinical examination in the holding in question must be carried out on pigs selected at random in the subunits for which a risk of introduction of the ASF virus has been identified or is suspected.

The minimum number of pigs to be examined must allow for the detection of fever if it occurs at a prevalence of 10 % with 95 % confidence in these subunits.

4. If dead or moribund pigs are detected in a suspected holding, post-mortem examinations must be carried out, preferably on at least five of these pigs and in particular on pigs that have:

— shown very evident signs of disease before death,

— high fever,

— died recently.

If these examinations have not shown lesions suggesting ASF but, due to the epidemiological situation, further investigations are deemed necessary:

— a clinical examination, as laid down in point 3, and blood sampling, as laid down in point 5 must be carried out in the subunit where the dead or moribund pigs were kept, and

— post-mortem examinations may be carried on three to four in-contact pigs, particularly if these pigs are showing clinical signs.

Irrespective of the presence or absence of lesions suggesting ASF, samples of the organs or tissues from pigs that have been subjected to post-mortem examination must be collected for virological tests in accordance with Chapter V(B)(1). These samples must preferably be collected from recently dead pigs.

When post-mortem examinations are carried out the competent authority must ensure that:

— the necessary precautions and hygiene measures are taken to prevent any disease spread, and,

— in case of moribund pigs, they are killed in a humane way in accordance with Council Directive 93/119/EEC of 22 December 1993 on the protection of animals at the time of slaughter or killing [1], as last amended by Regulation (EC) No 806/2003 [2].

5. If further clinical signs or lesions that may suggest ASF are detected in a suspected holding, but the competent authority deems that these findings are not sufficient to confirm an outbreak of ASF and that laboratory tests are therefore necessary, blood samples for laboratory tests must be taken from the suspected pigs and from other pigs in each subunit in which the suspected pigs are kept, in accordance with the following procedures:

(a) the minimum number of samples to be taken for serological tests must allow for the detection of 10 % seroprevalence with 95 % confidence in the subunit in question;

(b) the number of samples to be taken for virological tests will be in accordance with the instructions of the competent authority, which will take into account the range of tests that can be performed, the sensitivity of the laboratory tests that will be used and the epidemiological situation.

[1] OJ L 340, 31.12.1993, p. 21.
[2] OJ L 122, 16.5.2003, p. 1.

6. If, after the examination carried out in a suspected holding, clinical signs or lesions suggestive of ASF are not detected, but further laboratory tests are deemed necessary by the competent authority to rule out ASF, the sampling procedures laid down in point 5 shall be used for guidance purposes.

B. SAMPLING PROCEDURES IN A HOLDING WHEN PIGS ARE KILLED FOLLOWING CONFIRMATION OF DISEASE

1. In order that the manner of introduction of the ASF virus into an infected holding and the length of time elapsed since its introduction may be established, when pigs are killed on a holding following confirmation of an outbreak in accordance with Article 5(1)(a) of Directive 2002/60/EC, blood samples for serological tests must be taken at random from the pigs when they are killed.

2. The minimum number of pigs to be sampled must allow for the detection of 10 % seroprevalence with 95 % confidence in pigs in each subunit of the holding ([1]).

   Samples for virological tests must also be taken in accordance with the instructions of the competent authority, which will take into account the range of tests that can be performed, the sensitivity of the laboratory tests that will be used and the epidemiological situation.

   In those areas where the presence of vectors infected with the ASF virus has been previously demonstrated, appropriate collections of soft ticks for virological tests must also be taken in accordance with the instructions of the competent authority and Annex III to Directive 2002/60/EC.

3. However, in case of secondary outbreaks, the competent authority may decide to derogate from points 1 and 2 and establish other sampling procedures, taking into account the epidemiological information already available on the source and means of virus introduction into the holding and the potential spread of disease from the holding.

C. SAMPLING PROCEDURES WHEN PIGS ARE KILLED AS A PREVENTIVE MEASURE ON A SUSPECTED HOLDING

1. In order that ASF may be confirmed or ruled out and additional epidemiological information is gained, when pigs are killed as a preventive measure on a suspected holding in accordance with the provisions of Article 4(3)(a) or 7 (2) of Directive 2002/60/EC, blood samples for serological tests as well as blood samples for virological tests must be taken in accordance with the procedure laid down in point 2.

2. Sampling must primarily concern:

   — pigs showing signs or post-mortem lesions suggesting ASF and their in-contact pigs,

   — other pigs which might have had risky contacts with infected or suspected pigs or which are suspected to have been contaminated with the ASF virus. These pigs must be sampled in accordance with the instructions of the competent authority, which will take into account the epidemiological situation.

   Furthermore, pigs proceeding from each of the subunits of the holding must be sampled at random ([2]). In this case, the minimum number of samples to be taken for serological tests must allow for the detection of 10 % seroprevalence with 95 % confidence in the subunit in question.

   The type of samples to be taken for virological tests and the test to be used will be in accordance with the instructions of the competent authority, which will take into account the range of tests that can be performed, the sensitivity of these tests and the epidemiological situation.

([1]) However, if the derogation provided in Article 6(1) of Directive 2002/60/EC has been applied, sampling must concern the subunits of the holding where pigs have been killed, without prejudice to the further examinations and sampling to be carried out on the remaining pigs in the holding, which shall be carried out in accordance with the instructions of the competent authority.

([2]) However, if the competent authority has limited the application of preventive killing only to the part of the holding where the pigs suspected of being infected or contaminated with ASF virus were kept, in accordance with Article 4(3)(a) of Directive 2002/60/EC, sampling must concern the subunits of the holding where this measure has been applied, without prejudice to the further examinations and sampling to be carried out on the remaining pigs in the holding, which will be carried out in accordance with the instructions of the competent authority.

D. CHECKING AND SAMPLING PROCEDURES BEFORE AUTHORISATION IS GIVEN TO MOVE PIGS FROM HOLDINGS LOCATED IN PROTECTION OR SURVEILLANCE ZONES AND IN CASE THESE PIGS ARE SLAUGHTERED OR KILLED (ARTICLES 10 AND 11 OF DIRECTIVE 2002/60/EC)

1. Without prejudice to the provisions of Article 11(1)(f), second subparagraph, of Directive 2002/60/EC, in order that authorisation may be given to move pigs from holdings located in protection or surveillance zones in accordance with Article 10(3) of the said Directive, the clinical examination to be carried by an official veterinarian must:

   — be carried out within the 24-hour period before moving the pigs,

   — be in accordance with the provisions laid down in A(2).

2. In the case of pigs to be moved to another holding, in addition to the investigations to be carried out in accordance with point 1, a clinical examination of pigs, including taking the temperature of a proportion of pigs, must be carried out in each subunit of the holding in which the pigs to be moved are kept.

   The minimum number of pigs to be checked must allow for the detection of fever if it occurs at a prevalence of 10 % with 95 % confidence in these subunits.

3. In case of pigs to be moved to a slaughterhouse, to a processing plant or to other places to be then killed or slaughtered, in addition to the investigations to be carried out in accordance with point 1, a clinical examination of pigs must be carried out in each subunit in which the pigs to be moved are kept. In case of pigs older than three to four months, this examination must include taking the temperature of a proportion of pigs.

   The minimum number of the pigs to be checked must allow for the detection of fever if it occurs at a prevalence of 20 % with 95 % confidence in the subunits in question.

4. When the pigs referred to in point 3 are slaughtered or killed, blood samples for serological tests or blood or organ samples such as tonsil, spleen or lymph nodes for virological tests must be taken from pigs proceeding from each of the subunits from which pigs have been moved.

   The minimum number of samples to be taken must allow for the detection of 10 % seroprevalence or virus prevalence with 95 % confidence in each subunit.

   The type of samples to be taken and the test to be used will be in accordance with the instructions of the competent authority, which will take into account the range of tests that can be performed, the sensitivity of these tests and the epidemiological situation.

5. However, if clinical signs or post-mortem lesions suggesting ASF are detected when the pigs are slaughtered or killed, by way of derogation from point 4, the provisions for sampling laid down in section C shall apply.

6. The derogation provided for in Article 10(5) and Article 11(4) of Directive 2002/60/EC may be granted if the competent authorities ensure that an intensive sampling and testing scheme is also applied on the groups of pigs to be checked or sampled referred to in points 2, 3 and 4. In the context of this scheme, the minimum number of blood samples to be taken must allow for the detection of 5 % seroprevalence with 95 % confidence in the group of pigs in question.

E. CHECKING AND SAMPLING PROCEDURES IN A HOLDING IN RELATION TO REPOPULATION

1. When pigs are reintroduced into a holding in accordance with Article 13(3) of Directive 2002/60/EC, the following sampling procedures must be applied:

   — blood samples must be collected at the earliest 45 days after the reintroduction of the pigs,

   — in case sentinel pigs are reintroduced, blood samples for serological tests must be taken at random from a number of pigs that allow for the detection of 10 % seroprevalence with 95 % confidence in each subunit of the holding,

   — in case of total repopulation, blood samples for serological tests must be taken at random from a number of pigs that allow for the detection of 20 % seroprevalence with 95 % confidence in each subunit of the holding.

2. When pigs are reintroduced into a holding in accordance with Article 13(4) of Directive 2002/60/EC, the following sampling procedures must be applied:

   — blood samples must be collected at the earliest 45 days after the reintroduction of the pigs,

   — in case sentinel pigs are reintroduced, blood samples for serological tests must be taken at random from a number of pigs that allow for the detection of 5 % seroprevalence with 95 % confidence in each subunit of the holding,

   — in case of total repopulation, blood samples for serological tests must be taken at random from a number of pigs that allow for the detection of 10 % seroprevalence with 95 % confidence in each subunit of the holding.

   Then, the procedure laid down in the third indent above must be repeated at the earliest 60 days after total repopulation.

3. After any reintroduction of pigs, the competent authority shall ensure that in case of any disease or death of the pigs in the holding due to unknown reasons, the pigs in question are immediately tested for ASF.

   These provisions shall apply until the restrictions to pig movements referred to in Article 13(3)(a), (b) and (4) of Directive 2002/60/EC are lifted in the holding in question.

F. SAMPLING PROCEDURES IN HOLDINGS IN THE PROTECTION ZONE BEFORE LIFTING RESTRICTIONS

1. In order that the measures referred to in Article 10 of Directive 2002/60/EC may be lifted in a protection zone, in all holdings in the zone:

   — a clinical examination must be carried out in accordance with the procedures laid down in section A(2) and (3),

   — blood samples for serological tests must be taken as laid down in point 2.

2. The minimum number of blood samples to be taken must allow for the detection of 10 % seroprevalence with 95 % confidence in pigs in each subunit in the holding.

   However, the derogation provided for in Article 10(5) and Article 11(4) of Directive 2002/60/EC may only be granted if the competent authority ensures that the number of blood samples taken allow for the detection of 5 % seroprevalence with 95 % confidence in each subunit in the holding.

G. SAMPLING PROCEDURES IN HOLDINGS IN THE SURVEILLANCE ZONE BEFORE LIFTING RESTRICTIONS

1. In order that the measures referred to in Article 11 of Directive 2002/60/EC may be lifted in a surveillance zone, a clinical examination must be carried out in all holdings in the zone in accordance with the procedures laid down in section A(2).

   In addition, blood samples for serological tests must be taken from pigs:

   — in any other holding where sampling is deemed necessary by the competent authority,

   — in all semen collection centres.

2. Whenever blood sampling for serological tests is carried out in holdings located in the surveillance zone, the number of blood samples to be taken in these holdings must be in accordance with section F(2), first sentence.

   However, the derogation provided for in Article 10(5) and Article 11(4) of Directive 2002/60/EC may only be granted if the competent authority ensures that in each holding in the zone blood samples for serological tests are taken. The minimum number of blood samples to be taken must allow for the detection of 5 % seroprevalence with 95 % confidence in each subunit in the holding.

H. SEROLOGICAL MONITORING AND SAMPLING PROCEDURES IN AREAS WHERE ASF IS SUSPECTED TO OCCUR OR HAS BEEN CONFIRMED IN FERAL PIGS

1. In case of serological monitoring in feral pigs in areas where ASF has been confirmed or is suspected to occur, the size and the geographical area of the target population to be sampled should be previously defined in order to establish the number of samples to be taken. Sample size must be established as a function of the estimated number of living animals and not as a function of the number of animals shot.

2. If data on population density and size are not available, the geographic area within which to sample must be identified taking into account the continuous presence of feral pigs and the presence of natural or artificial barriers that will efficiently prevent large and continuous movement of the animals. When such circumstances do not occur, or in case of large areas, it is recommended to identify sampling areas of about 200 km^2, where a population of about 400 to 1 000 feral pigs may usually live.

3. Without prejudice to the provisions of Article 15(2)(c) of Directive 2002/60/EC, the minimum number of pigs to be sampled within the defined sampling area must allow for the detection of 5 % seroprevalence with 95 % confidence. For this purpose at least 56 animals must be sampled in each area which has been identified.

4. Collection of samples for virological tests from feral pigs shot or found dead must be carried out as laid down in Chapter V(B)(1).

   When virological monitoring on shot feral pigs is deemed necessary, it must be primarily carried out on animals less than one year old.

5. All samples to be sent to the laboratory must be accompanied by the questionnaire referred to in Article 16(3)(h) of Directive 2002/60/EC.

Chapter V

General procedures and criteria for collection and transport of samples

A. GENERAL PROCEDURES AND CRITERIA

1. Before sampling is carried out in a suspected holding, a map of the holding must be prepared and the epidemiological subunits of the holding must be identified.

2. Each time that it is deemed that re-sampling of pigs might be necessary, all pigs which are sampled must be uniquely marked in such a way that they can be easily re-sampled.

3. All samples must be sent to the laboratory accompanied by the appropriate forms, in accordance with the requirements established by the competent authority. These forms will include details of the history of the pigs sampled and the clinical signs or post-mortem lesions observed.

   In case of pigs kept in holdings, clear information on age, category and holding of origin of the pigs sampled must be provided. It is recommended that the location of each pig sampled in the holding be recorded together with its unique identification mark.

B. COLLECTION OF SAMPLES FOR VIROLOGICAL TESTS

1. For detection of the ASF virus, antigen or genome from dead or humanely destroyed pigs, tonsils, lymph nodes (gastrohepatic, renal, submandibular and retropharyngeal), spleen, kidney and lung tissues are the most suitable samples ([1]). In case of autolysed carcases, an entire long bone or the sternum is the specimen of choice.

2. Anticoagulated blood and/or clotted blood samples must be collected from pigs showing signs of fever or other signs of disease, in accordance with the instructions of the competent authority.

([1]) It is recommended to collect also samples of ileum, as they may be useful for the diagnosis of classical swine fever.

C. TRANSPORT OF SAMPLES

1. It is recommended that all samples:

   — are properly identified,

   — are transported and stored in leak-proof containers,

   — are kept cool at refrigerator temperature; however, if it is expected that the samples arrive at the laboratory in more than 48 hours, the laboratory should be contacted to obtain instructions regarding the most appropriate temperature conditions during transport,

   — are delivered to the laboratory as quickly as possible,

   — are kept in a package containing ice packs or dry ice to keep them cool,

   — of tissues or organs are placed in separate sealed plastic containers and properly labelled. They must be then placed in larger containers and packed with sufficient absorbent material to protect them from damage and absorb any leakage,

   — whenever possible, are directly transported to the laboratory by a competent person in order that rapid and reliable transport is ensured.

2. The outside of the package must be labelled with the address of the recipient laboratory and the following message should be prominently displayed:

   'Animal pathological material; perishable; fragile; do not open outside an ASF laboratory.'

3. The responsible person in the laboratory receiving the samples must be informed in due time of the arrival of the samples.

4. For air transport of samples to the Community Reference Laboratory for ASF [1] the package must be labelled according to IATA regulations.

Chapter VI

Principles and use of virological tests and evaluation of their results

A. DETECTION OF VIRUS ANTIGENS

1. Direct immuno fluorescent test (DIFT)

   The principle of the test is the microscopic detection of viral antigens on impression smears or thin cryosections of organ material from pigs suspected of being infected with the ASF virus. Intracellular antigens are detected using FIT-conjugated [2] specific antibodies. Fluorescent inclusion bodies or granules appear in the cytoplasm of infected cells.

   Suitable organs are kidney, spleen, and various lymph nodes. A smear of bone marrow cells might also be used for feral pigs, if their organs are not available or are autolysed.

   The test can be performed within two hours. As organ samples can only be obtained from dead animals its use for screening purposes is limited.

   This is a highly sensitive test for cases of acute ASF. For subacute or chronic forms, the DIFT presents only a sensitivity of approximately 40 %, probably due to the presence of antigen-antibody complexes, which block the reaction with the ASF-conjugated antibody. Confidence in the test result may be limited by doubtful staining, particularly where considerable experience in performing the test has not been acquired or if the organs tested are autolysed.

2. ELISA for antigen detection

   Viral antigens can be also detected using ELISA techniques, but it is only recommended for acute forms of the disease because of its low sensitivity when antigen-antibody complexes are present. The sensitivity of the antigen ELISA should be high enough to score a positive result from animals showing clinical signs of acute ASF. In any case it is recommended to use this test only as a 'herd' test and in conjunction with other virological tests.

[1] The Community Reference Laboratory has an open licence to receive diagnostic samples and ASF virus isolates from any other Member State. If the sample proceeds from outside the EU, a copy of the import permit may be requested from this laboratory before transport and attached in an envelope to the outside of the package.

[2] Fluorescein isothiocyanate.

B. VIRUS ISOLATION AND IDENTIFICATION BY THE HAEMADSORPTION TEST (HAD)

1. Virus isolation is based on the inoculation of sample material on susceptible primary cell cultures of porcine origin, monocytes and macrophages cells. The preferred samples for isolation of the ASF virus are whole blood and leucocytes obtained from non-coagulated blood samples or the organs referred to in section A(1). If the ASF virus is present in the sample, it will replicate in the cells and a characteristic cytopathic effect will be produced in the infected cells.

2. The HAD technique is recommended for the identification of ASF virus isolates due to its high sensitivity and specificity. HAD is based on the capability of the ASF virus to replicate in pig macrophages and induce haemadsorption in the presence of pig erythrocytes. A characteristic 'rosette' of erythrocytes develops around the infected macrophages. However, a small number of field ASF virus strains may not induce haemadsorption, but they produce a cytopathic effect. These strains may be specifically identified using the DIF test on the sediments of the cell cultures or by PCR.

3. Virus isolation is best suited for the investigation of samples from small numbers of animals rather than mass surveillance. The virus isolation procedure is labour-intensive and requires one to three days before results are available. Two further cell culture passages may be necessary in order to detect small amounts of the virus in the sample. This may lead to an investigation time of up to 10 days before a final result is obtained. Autolysed samples can be cytotoxic to the cell culture and consequently of limited use.

4. Virus isolation and identification by HAD are recommended as a reference test for the confirmation of positive results of a prior ELISA, PCR or DIFT. They are also recommended when ASF has already been confirmed by other methods, particularly in case of a primary outbreak or case of ASF.

   ASF viruses isolated in pig macrophages can be used for virus characterisation and molecular epidemiology.

5. All ASF virus isolates from all primary outbreaks, primary cases in feral pigs or cases in slaughterhouses or means of transport must be characterised by a National Reference Laboratory in the Member States, or by any other laboratory authorised by the Member State in question or by the Community Reference Laboratory, in accordance with section E.

   In any case, these virus isolates must be sent to the Community Reference Laboratory for virus collection without delay.

C. DETECTION OF THE VIRUS GENOME

1. The polymerase chain reaction (PCR) is applied to detect the virus genome in blood, serum, tissues or organ samples. Small fragments of viral DNA are amplified by PCR to detectable quantities. A wide range of isolates belonging to all the known virus genotypes, including both non-haemadsorbing viruses and isolates of low virulence, can be detected by using primers from a highly conserved region of the genome. Since this test detects only a genome sequence of the virus, the PCR may be positive, even when no infectious virus is detected by virus isolation (e.g. in autolysed tissues or samples from convalescent pigs or from pigs which have recovered and become clinically normal).

2. PCR can be used on a limited number of samples which have been carefully selected from suspected animals. It is the recommended method for organ samples which are cytotoxic, where virus isolation is therefore not possible (for example, samples from feral pigs).

3. Suitable sample material for the PCR are the organs described for virus isolation and serum. Tick homogenates may also be analysed by PCR.

4. The PCR can be performed within a working day. It requires appropriate laboratory equipment, separated facilities and skilled staff. An advantage is that the infectious virus need not be replicated in the laboratory. The PCR is highly sensitive, but contamination may easily occur, which leads to false positive results. Therefore stringent quality control procedures are essential.

D. RECOMMENDED VIROLOGICAL TESTS AND EVALUATION OF THE RESULTS

Virological tests are essential for the confirmation of ASF.

Virus isolation and HAD must be considered as the reference virological tests and must be used as confirmatory tests when necessary. Their use is particularly recommended where positive DIF or PCR results are not associated with the detection of clinical signs or lesions of disease and in any other doubtful cases.

However, a primary outbreak of ASF can be confirmed if clinical signs or lesions of disease have been detected in the pigs in question and at least two distinct antigen, genome or antibody detection tests have given a positive result on samples taken from the same suspected pig.

A secondary outbreak of ASF can be confirmed if, in addition to the epidemiological link to a confirmed outbreak or case, clinical signs or lesions of disease have been detected in the pigs in question and an antigen, genome or antibody detection test has given a positive result.

A primary case of ASF in feral pigs can be confirmed by virus isolation or when at least two antigen, genome or antibody detection tests have given a positive result. Further cases of ASF in feral pigs for which an epidemiological link with previously confirmed cases has been found can be confirmed if an antigen, genome or antibody detection test has given a positive result.

E. GENETIC CHARACTERISATION OF ASF VIRUS ISOLATES

1. Genetic characterisation of ASF virus isolates is achieved by determining restriction enzyme patterns and nucleotide sequences of portions of the virus genome. The similarity of these restriction patterns or sequences with those already obtained from previous virus isolates may indicate whether outbreaks of the disease are caused by viruses that follow a European or an African molecular model.

   Genetic characterisation of ASF virus isolates is of major importance to improve the current knowledge on the molecular epidemiology of ASF and the genetic variation of viruses. The molecular data allow new isolates to be classified and provide information on their possible origin.

2. If virus molecular characterisation cannot be performed in a national laboratory or in any other laboratory authorised to diagnose ASF within a short delay, the original sample or the virus isolate must be sent to the Community Reference Laboratory for molecular characterisation as soon as possible.

   The data from restriction enzyme analysis and sequencing of ASF virus isolates available to the laboratories authorised to diagnose ASF must be forwarded to the Community Reference Laboratory so that this information can be put onto the database kept by this Laboratory.

   The information included in this database must be available to all national reference laboratories in the Member States. However, for the purpose of publication in scientific journals, if requested by the laboratory in question, the Community Reference Laboratory shall guarantee confidentiality of these data until they are published.

Chapter VII

Principles and use of serological tests and evaluation of their results

A. BASIC PRINCIPLES AND DIAGNOSTIC VALUE

1. ASF-specific antibody detection is recommended for subacute and chronic forms as well as for large-scale testing and ASF eradication programmes, for several reasons:

   (i) antibodies are rapidly produced in the infected pig. In these pigs antibodies are usually detectable in serum samples from seven to ten days after infection;

   (ii) no vaccines are available against ASF. This means that ASF-specific antibodies are only induced by ASF virus infection;

   (iii) the long-lasting antibodies response. In pigs that have recovered from the disease, specific antibodies can be detected at high levels for many months or even for the lifetime of some of these pigs.

Specific ASF antibodies of maternal origin can be detected in piglets during the first weeks of life. The half-life of maternal antibodies in piglets is about three weeks. If found in piglets older than three months, ASF antibodies are very unlikely to be of maternal origin.

2. The detection of antibodies against the ASF virus in serum or plasma exudates from organs submitted is carried out to assist the diagnosis of ASF in suspected holdings, to estimate the date of introduction of the infection in case of a confirmed outbreak and for monitoring and surveillance purposes.

   The location of seropositive pigs on the holding can provide valuable information on how and where the ASF virus entered the holding.

   However, an accurate evaluation of the results of the serological tests must be carried out, taking into account all the clinical, virological and epidemiological findings, in the framework of the enquiry to be carried out in case of suspicion or confirmation of ASF, in accordance with Article 8 of Directive 2002/60/EC.

B. RECOMMENDED SEROLOGICAL TESTS

1. The ELISA, indirect immunofluorescence test (IIFT) and immunoblotting (IB) tests are the tests of choice for the serological confirmation of ASF.

   The quality and efficiency of the serological diagnosis performed by the national laboratories must be regularly checked in the framework of the inter-laboratory comparison test periodically organised by the Community Reference Laboratory.

2. The ELISA test is the most reliable and useful test for large-scale serological studies. It is based on the detection of ASF virus antibodies bound to the viral proteins which are attached to a solid phase by addition of protein A conjugated with an enzyme that produces a visible colour reaction when it reacts with the appropriate substrate.

3. Quality control on sensitivity and specificity of each batch of ELISA reagents must be regularly performed by the national laboratories, making use of the panel of reference sera provided by the Community Reference Laboratory. This panel shall include:

   — sera from pigs in the early phase of ASF virus infection (less than 17 days post infection),

   — sera from convalescent pigs (more than 17 days post infection).

   The ELISA to be used for the serological diagnosis of ASF must detect all reference sera from the convalescent pigs. All results obtained with the reference sera must be reproducible. It is recommended all positive sera from the early phase are also detected. The results obtained with the reference sera from pigs in the early phase of infection give an indication of the sensitivity of the ELISA.

4. The IIFT is a rapid technique with high sensitivity and specificity for the detection of ASF antibodies from either sera or tissue exudates. It is based on the detection of ASF antibodies that bind to a monolayer of MS cells infected with an adapted ASF virus. The antibody-antigen reaction is detected by a labelled fluorescein A-protein. Positive samples show specific fluorescence near the nucleus of the infected cells.

   DIFT and IIFT used in combination to test organ, blood and exudates collected from animals showing clinical signs of the ASF may lead to a rapid and reliable confirmation of the disease.

5. The IB test is a highly specific and sensitive technique based on the use of nitrocellulose strips containing viral proteins as antigens. The specific antibody-antigen reaction is detected by addition of a protein A-peroxidase conjugate and an appropriate substrate. It is very useful to test sera that are inconclusive in the ELISA test.

Chapter VIII

Minimum safety requirements for ASF laboratories

1. The requirements laid down in table 1 must be fulfilled in any laboratory where the ASF virus is to be amplified by replication in cell cultures. However, post-mortem examinations, processing of tissues for DIFT or PCR and serology using inactivated antigens, may be carried out at a lower containment level, provided that the minimum requirements of table 1 are fulfilled, basic hygiene is used and post-operational disinfection with safe disposal of carcases, tissues and sera are carried out.

2. The requirements laid down in table 2 must be fulfilled by any laboratory where animals are inoculated with the ASF virus.

3. All stocks of the ASF virus must be kept in secure storage, whether frozen or freeze-dried. All individual ampoules must be clearly labelled, and comprehensive records maintained of virus stocks together with dates and results of quality control checks. Records must also be kept of viruses added to stock, with details of the source, and of viruses issued to other laboratories.

4. It is recommended that the biosecurity unit for ASF virus work should be supported by areas where the ASF virus is not manipulated. These other areas should be available for the preparation of glassware and media, the maintenance and preparation of non-infected cell cultures, the processing of sera and serological testing (other than methods using live ASF virus), and the provision of administrative and clerical support.

Table 1

Principles of biological containment appropriate for diagnostic laboratories

	Minimum requirements	Additional requirements
General environment	Normal atmospheric pressure. Dedicated rooms limited to defined procedures.	Normal atmospheric pressure. One HEPA filtration of exhaust air. Dedicated rooms, used exclusively for classical swine fever or ASF diagnostic procedures. Potentially contaminated effluents treated to inactivate ASF virus (heat or chemical).
Laboratory clothing	Dedicated outer clothing used only in the ASF virus unit. Disposable gloves for all manipulations of infected material. Outer clothing sterilised before removal from unit, or washed at a high temperature within unit.	Complete change of clothes on entry. Laboratory clothing used only in the ASF virus unit. Disposable gloves for all manipulations of infected material. Clothing sterilised before removal from unit, or washed at a high temperature within unit.
Control of personnel	Entry to unit limited to named, trained personnel. Wash and disinfect hands on leaving unit. Personnel not permitted to visit premises with pigs for 48 hours after leaving unit.	Entry to unit limited to named, trained personnel. Wash and disinfect hands on leaving unit. Personnel not permitted to visit premises with pigs for 48 hours after leaving unit.
Equipment	Biological safety cabinet (class I or II) used for all manipulations of live virus. Cabinet should have double HEPA filtration of exhaust air. All equipment needed for laboratory procedures to be available within the dedicated laboratory suite.	

Table 2

Bio-safety requirements for experimental animal rooms

	Requirements
General environment	Negative-pressure-controlled ventilation. One HEPA filtration of exhaust air. Facility for complete decontamination or fumigation at end of experiment. All solid and liquid waste effluents treated to inactivate ASF virus (heat/incineration or chemical).

	Requirements
Laboratory clothing	Complete change of clothes on entry. Clothing sterilised before removal from unit, or washed at a high temperature within unit.
Control of personnel	Entry to unit limited to named, trained personnel. Leave clothes inside before shower. Full shower on exit from unit. Personnel not permitted to visit premises with pigs for 48 hours after leaving unit.
Equipment	All equipment required for animal procedures to be available within the unit. All materials to be sterilised on removal from unit or, in the case of animal samples, to be double-wrapped in leakproof container which is surface disinfected for transport to the ASF laboratory.
Animals	All animals to be slaughtered before leaving the unit, post-mortem examinations to be completed within the bio-safe area, and carcases incinerated on completion of examinations.