

## Contents

Preamble.....	1
Chapter 1 Basic policies.....	3
I Basic policies .....	3
Chapter 2 Outbreak prevention measures .....	6
Section 1 Outbreak prevention and preparedness for an outbreak.....	6
II-1 Efforts in normal times.....	6
II-2 Establishment and strengthening of systems to prepare for outbreaks .....	9
Section 2 Surveys of viral spreading and wild boar countermeasures.....	12
III-1 Survey of the spread of the virus .....	12
III-2 Preventive vaccination .....	16
Chapter 3 Measures to prevent the spread .....	34
Section 1 Measures for domestic pigs.....	34
IV Detection of abnormal pigs and confirmation by inspection.....	34
V Confirmation of diagnosis .....	46
VI Response to the diagnosis .....	49
VII Control measures on the affected farms .....	54
VIII Restriction or prohibition of traffic (Article 15 of the Act) .....	62
IX Establishment of a movement restriction zone and a shipment restriction zone (Article 32 of the Act) .....	63
X Restriction of events at facilities gathering livestock (Articles 26, 33 and 34 of the Act).....	71
XI Installation of disinfection points (Article 28-2 of the Act) .....	73
XII Confirmation of the status of virus spread.....	75
XIII Emergency Vaccination (Article 31-1 of the Act) .....	85
XIV Reintroduction of domestic animals .....	87
XV Identification of the cause of the outbreak .....	90
Section 2 Control measures for wild boars .....	91
XVI Countermeasures in case infection is suspected .....	91
XVII Confirmation of diagnosis .....	92
XVIII Response to the diagnosis.....	93
XIX Restriction or prohibition of traffic (Article 10 and Article 25-2-3 of the Act) ....	96
XX Establishment of a movement restriction zone (Article 32 of the Act).....	97
XXI Restrictions on events at facilities gathering livestock (Articles 26, 33, and 34 of the Act).....	103
XXII Installation of disinfection points (Article 28-2 of the Act).....	105
XXIII Confirmation of the status of virus spread .....	107
XXVI Spreading oral vaccine .....	109

Chapter 4 Others.....	110
XXV Others.....	110
[Reference] .....	111
Dagnosis Manual of CSF .....	111
Calculation Method of Appraised Value of Pigs .....	123

\*Note

This document includes Notes 1 through 60, which are stipulated in “the Implementation of Spread Control Outbreak and Prevention Measures based on the Guidelines for the Control of Specific Domestic Animal Infectious Disease Caused by Classical Swine Fever (Notice of Food Safety and Consumer Affairs Bureau, 3 Shoan No. 3495, dated October 1, 2021.)

## **Section 2 Control measures for wild boars**

### **XVI Countermeasures in case infection is suspected**

#### **1 Response to case in which infection by CSFV is suspected**

If a CSFV infection is suspected among wild boars during an investigation of wild boars in III-1-4 or infection confirmation tests in XII-6, the prefecture shall immediately report it to the Animal Health Division and thoroughly disinfect the area inhabited by the wild boars (hereinafter referred to as “suspect affected wild boars” in 2) and proceed with the preparation of 2.

Additionally, according to IV-5-(2), based on the consultation with Animal Health Division, they shall simultaneously send the required samples to the NIAH.

#### **2 Preparation for cases in which the diagnosis is positive**

After they send required samples to NIAH as per 1, the prefecture shall take the following measures and report the procedure to the Animal Health Division until the result of the gene analysis in 3 conducted by NIAH is obtained.

- (1) Checking the number of farms and of domestic pigs in an area within a 10 km radius of the point where the suspected affected wild boars were identified.
- (2) Checking personnel and material necessary for control measures such as slaughtering domestic pigs, (including the necessity of staff support from the government or other prefectures) in case CSF occurs in farms in the neighborhood of the area in which suspected affected wild boars were identified.
- (3) Confirming the allocation status of burial sites or incineration facilities (including the use of large-scale quarantine material owned by the MAFF) in farms around the area where suspected affected wild boars were identified.
- (4) Selection of the locations to install disinfection points, as necessary.
- (5) Notification to municipalities where suspected affected wild boars were identified, neighborhood prefectures, and related organizations.
- (6) Necessary instruction such as a voluntary ban on moving live domestic pigs or carcasses within a 10 km radius of the point where the suspected affected wild boars were identified.
- (7) Confirmation of the system to prevent an epidemic from spreading among wild or domestic animals in the area of the point where suspected infected wild boars were identified.

#### **3 Inspection by NIAH**

When the prefecture sends samples to the NIAH through the procedure in 1, the NIAH shall conduct the necessary tests, including genetic analysis, and report the results to the Animal Health Division.

#### **[Note 80] Shipment of samples**

Note 38 shall be applied.

## **XVII Confirmation of diagnosis**

The MAFF shall confirm the diagnosis through the result of gene detection tests by the prefecture and genic analyses by the NIAH, when required samples were sent to NIAH as per XVI-1 (including other cases in which Animal Health Division judges necessary). However, in case where the suspected wild boars were found in the area where other affected wild boars had been confirmed, the MAFF can confirm the diagnosis without waiting for the NIAH's test results. In this case, the results shall be reported to the competent prefectural livestock department via the Animal Health Division.

## **XVIII Response to the diagnosis**

### **1 Notification to the concerned parties**

- (1) When the prefecture is notified that CSF diagnosis is positive in wild boars according to XVII, they shall immediately report the information and location where the wild boars were recognized to the following parties via phone, FAX, or e-mail.
  - (i) Owners and biosecurity managers of domestic pigs in the prefecture
  - (ii) The municipalities in the prefecture
  - (iii) Veterinary medical associations, producer and farmer groups, and other related organizations in the prefecture
  - (iv) Local police, the Self-Defense Forces and other related organizations in the prefecture
  - (v) Adjacent prefectures

#### **[Note 81] Notification to related parties of measures for wild boars**

When CSF diagnosis is positive in wild boars according to XVII of the Guidelines, the Animal Health Division shall notify the Wildlife Division of Nature Conservation Bureau, MOE and the competent Livestock Health Department of prefectures including the area within a 10-km radius of the affected point. The notified competent Livestock Health Department shall notify related sections including those for Wild Animals Damage Prevention Office (MAFF) and Office for Wildlife Management (MOE) in the prefecture, municipalities and related parties such as hunting clubs.

#### **[Note 82] Reporting to related parties and the press in case affected wild boars continue to be confirmed**

When CSFV positive wild boars are continuously found in the prefecture, the prefecture can supply information displaying inspection results on maps instead of those specified in XVIII-1 and 3, upon the confirmed status of affected wild boars.

- (2) When providing information according to (1) above, the prefecture shall explain to the information recipients that information is share only for the purpose to prevent an CSV from spreading, and provide necessary instruction not to use the information for other purposes or not to leak it. In this instruction by prefectures, posting the information to web-site shall be strictly prohibited due to the risk to be widely spread.
- (3) When the prefecture is notified that CSF diagnosis is positive in wild boars according to XVII, they shall notify the parties specified in XVI- 2-(5) and (6).

### **2 Establishment of a Response Headquarters and cooperation among the government and the prefecture**

- (1) After confirming affected wild boars, the MAFF shall immediately organize a

Response Headquarter, and develop the control policy. The Headquarter can be organized before confirmation if it is specifically necessary.

- (2) In collaboration with the NIAH, NLBC or other related organizations, the MAFF shall dispatch the following staff to the prefecture in which an outbreak occurred as necessary.
  - (i) Staff who can appropriately communicate the control policy specified in (1) above to prefectures and make arrangements for the Government and the prefecture to promote close cooperation.
  - (ii) Experts in epidemiology or wild boars, who can accurately grasp the infectious status so that the control policy can be revised (and the Emergency Guidelines can be developed) timely and appropriately.
- (3) Immediately after being notified of a confirmation of affected wild boars, the prefecture shall organize a Prefectural Headquarter in order to smoothly implement the concrete control measures in line with the control policy specified in (1) above. The Prefectural Headquarter can be organized before the confirmation if it is specifically necessary for prompt and appropriate disease controls.
- (4) The prefecture should define each role for disease control, procurement, epidemiological investigation, public relations or cash management in the Prefectural Headquarter so that the Prefectural Headquarter can smoothly and sufficiently fulfill its roles and functions.
- (5) Staff dispatched from the MAFF as specified in (2)(i) shall attend the Prefectural Headquarter, communicate the control policy specified in (1), and make necessary arrangement.
- (6) In order to implement smooth and appropriate control measures, the Prefectural Headquarter shall establish a regime for communication with municipalities, the local police, veterinary medical associations, and producer and farmer groups
- (7) The MAFF shall immediately provide or lend materials/equipment for disease control, which they possess, to the prefecture when receiving request from the prefecture.
- (8) When Response headquarters other than Headquarter or Prefectural Headquarter is organized. In this case, its purpose and scope shall be clarified so that any duplication or confusion of reporting line may be avoided.

**[Note 83] Prefectural Response Headquarters**

Note 44 is applied.

**3 Announcement to the mass media**

- (1) After the MAFF confirms affected wild boars according to XVII, the MAFF and the prefecture shall announce the details and future control measures to the mass media. If deeming it necessary for smooth and appropriate implementation of control

measures, the Animal Health Division may publish it before the confirmation after consulting with the competent prefectural livestock department.

- (2) In principle, the MAFF and the prefecture simultaneously issue the announcement specified in (1) above.
- (3) During issuance of the announcement specified (1) above, accurate information should be provided regarding the risk of virus spread via human or vehicles.
- (4) The mass media shall be asked to take a cooperative approach in the following matters:
  - (i) Give full consideration to privacy protection.
  - (ii) Do not access the area where affected wild boars were confirmed for epidemic prevention and disease control.

**[Note 84] Press release**

Note 45 is applied.

**[Note 85] Seeking the cooperation of mass media**

Note 46 is applied.

### **XIX Restriction or prohibition of traffic (Article 10 and Article 25-2-3 of the Act)**

1 The prefecture or the municipalities shall, immediately after being notified of a confirmation of affected wild boars, in collaboration with the competent police department and other local government(s) concerned, restrict unnecessary and non-urgent entries (including economic activities and tourism activities) to the neighborhood of the area, or restrict or prohibit traffic in the vicinity of the neighboring farms, as necessary in the environment of the surrounding area, for the period of (1) or (2) defined. However, traffic for the purpose of commuting, medical or welfare service may be allowed on condition of sufficient disinfection.

When introducing these measures, prior consultation shall be made with the local police responsible for the area.

- (1) In case where the measure specified in 1 above is taken on farms rearing domestic pigs within a 3 km radius for disease prevention: for the period within 72 hours, based on Article 10-3 of the Act.
- (2) In case where the measure specified in 1 above is taken with no farm rearing domestic pigs in the same area as (1): the period considering the time to identify the status of virus spread in the area, based on Article 25-2-3 of the Act.

2 When deeming it necessary to expand the period for restrictions or prohibitions on traffic due to the infectious status among wild boars, advance consultations with road administrators are needed so that appropriate restrictions from the standpoint of disease prevention may be implemented.

3 When introducing traffic restriction/prohibition in accordance with Articles 3 and 7 of the Order, the prefecture shall endeavor to provide an explanation in advance about the overview and necessity of such restriction/prohibition or marking to the residents of the municipalities concerned, and if this advance explanation is difficult to carry out, an explanation shall be provided promptly after implementation.

## **XX Establishment of a movement restriction zone (Article 32 of the Act)**

### **1 Establishment of a movement restriction zone**

The prefecture shall, in a case of having notified of a confirmation of affected wild boars as per the provision of XVII herein, promptly establish an area within a 10-km radius (as a rule) of the point where the wild boars were identified as the area where movement of livestock (meaning items specified in 7) is prohibited (hereinafter referred to as “movement restriction zone” in the Section 2) in consultation with the Animal Health Division; provided, however, that CSF is considered highly likely, the movement restriction zone is established without waiting for the XVII diagnosis to be determined, in consultation with the Animal Health Division.

### **2 Method to establish the movement restriction zones**

- (1) The outer boundary of the movement restriction zone shall be established based on the administrative units of municipalities, or other landmarks adequate to delineate borders such as roads, rivers and railroads.
- (2) In case where the movement restriction zone is straddle plural prefectures, under the guidance of the Animal Health Division, the prefectures concerned shall carry out sufficient consultation with each other in advance.
- (3) Prior to the establishment of the movement restriction zone, the following measures shall be taken. If taking these measures in advance is difficult, they shall be implemented immediately after establishment.
  - (i) Notifications to the owners of domestic pigs within the movement restriction zone, municipalities, and related organizations.
  - (ii) Publication through press releases.
  - (iii) Posting signs between a major road and the movement restriction zone.

### **3 Contacting pig owners**

When establishing a movement restriction zone, the prefecture shall promptly inform the pig owners within the area of the details and a scheduled on-site inspection specified in XXIII-1(2) via telephone, FAX or e-mail.

### **4 Instructions provided to the pig owners within the movement restriction zone**

When establishing a movement restriction zone, the prefecture shall instruct all pig owners within the zone to closely observe health conditions daily and to thoroughly take biosecurity measures, including prevention the entry of wild animals to the farm. In addition, based on Article 52 of the Act, the prefecture shall ask the owners for daily report about the presence of is any specific symptom and the number of domestic pig deaths every day until the restriction on the area are lifted.

**[Note 86] Instructions that apply in the movement restriction zone**

Prefectural animal health inspectors shall instruct related parties to perform the following activities in the movement restriction zone specified in XX-1 of the Guidelines. Additionally, they shall visit the related facilities as necessary and monitor the implementation status.

1 In requesting reports in accordance with Article 52 of the Act, the minimum necessary items that the prefecture requires from farms are as follows, and if other necessary items are found, additional reports should be requested as appropriate.

- (1) Existence/absence of any specific symptoms
- (2) The number of dead pigs, if any, (i) Location of the dead pigs (name of the premise and the location of the stall), (ii) Animal's age in days (or body weight), (iii) Likely cause of death
- (3) The number of the stillborn piglets
- (4) The number live born piglets
- (5) The number of sows that had abnormal production
- (6) The number of domestic pigs shipped from the farm
- (7) The number of domestic pigs introduced to the farm
- (8) Clinical findings of domestic pigs reared with the dead pigs

2 The farm shall voluntarily ban the entrance and exit of sites where domestic pigs are raised by non-relevant persons and the frequency of entrance and exit by relevant persons shall be minimized.

3 Vehicles and people shall be thoroughly disinfected when coming in and out.

4 In addition to thoroughly implementing control measures, including thorough disinfection of vehicles delivering livestock feed, examination of delivery route, restricted feedstuff delivery areas, the delivery route shall be recorded.

5 When a veterinarian diagnoses domestic animals, he/she shall carry the minimum instruments and drugs, wear and use easily disinfected or disposable medical clothes and medical instruments and thoroughly disinfect the body, instruments, vehicles in coming in or out the farm In addition, he/she shall thoroughly take measures to prevent pathogens spreading such as a voluntary ban on driving medical vehicles into the farm premises, and the route shall be recorded.

6 Vehicle entering and exiting carcass handling plants, rendering plants and slaughterhouses shall be thoroughly disinfected.

7 In areas in which contacts between wild boars and domestic pigs are assumed,

surrounding equipment shall be installed and feedstuff at farms raising domestic pigs shall be separated and stored to prevent contact with wild animals including boars.

- 8 They shall request related agencies such as the Wild Animals Damage Prevention Office (MAFF) and Office for Wildlife Management (MOE) for assistance in asking related parties including municipalities and hunting clubs to process wild boar's carcasses (including those killed by hunting) appropriately by incineration or burial, without leaving them in the field.

## **5 Revisions to the movement restriction zone**

### **(1) Expansion of the movement restriction zone**

If the infectious status among wild boars suggests spread of the epidemic outside the movement restriction zone, the movement restriction zone shall be expanded upon a consultation with the Animal Health Division.

### **(2) Reduction of the movement restriction zone**

If the infectious status among wild boars clearly shows that the spread of the epidemic is focal, the movement restriction can be reduced to within a 3 km radius, upon a consultation with the Animal Health Division.

## **6 Lift of the movement restriction zone**

The movement restriction zone shall be lifted upon a consultation with the Animal Health Divisions, if the infectious status among wild boars suggests that the risk of infection to domestic pigs is limited, the movement restriction zone can be fully or partially lifted based on the opinions of experts, the subcommittee, upon a consultation with the Animal Health Division.

## **7 Items subject to movement restriction**

The following items shall be subject to movement restriction:

- (1) Live domestic pigs;
- (2) Semen and embryos collected in movement restriction zone (excluding those which were collected before 21<sup>st</sup> day prior to the diagnosis confirmed and which were separately managed);
- (3) Carcasses of domestic pigs
- (4) Manure of domestic pigs; and
- (5) Bedding materials, feedstuff and livestock feeding equipment (excluding the movement from non- farms).

## **8 Exclusion from restriction**

The prefecture may, upon a consultation with the Animal Health Division, allow moving of domestic pigs, in which the absence of abnormal symptoms had been

confirmed through the inspection specified in XXIII-1-(2), to specific destination in the following cases. If serum antibody tests are conducted as necessary, the prefecture shall make an arrangement regarding sample shipment with the Animal Health Division and the NIAH.

- (1) Shipping domestic pig directly to slaughterhouses
  - (i) The pig owners shall submit the shipment plan for 1 month (as general rule) to a LHSC in advance. If the plan is changed, he/she shall immediately report to the center.
  - (ii) The administrative veterinarian or the pig owner shall continue clinical check for about one week (in general) before shipment, and he/she shall also measure the body temperatures of all domestic pigs to be shipped and reconfirm clinical signs on the morning of a day preceding shipment. He/she shall report the results with daily report to a LHSC.
  - (iii) The LHSC shall confirm the existence or the absence of fever and clinical signs reported in (ii).
  - (iv) If CSF cannot be denied, such as multiple domestic pigs among the shipped group are recognized to have a fever of 40 degrees or higher in (iii), the center shall enter the farm, collect samples and conduct a further test (blood and gene detection tests).
  - (v) If no abnormality is found in (iii), the center shall notify the owner of a shipment permission.
  - (vi) The LHSC shall confirm in advance that a slaughterhouse properly takes measures to prevent the virus from intruding and spreading.
- (2) Moving live piglets and sows to other farms
  - (i) The owner shall submit the shipment plan for 1 month (as general) to a LHSC in advance.
  - (ii) The movement shall be within a prefecture in principle, but they may be moved outside upon a consultation with the prefecture of acceptance.
  - (iii) As a general rule, all moved domestic pigs shall be confirmed negative via a gene detection test.
  - (iv) A follow-up monitoring of at least 21 days is conducted at a destination farm. During the time, the domestic pigs shall be segregated whenever possible.
- (3) Moving semen or embryo to other farms

Semen and embryos shall be preserved and isolated. Measures shall be taken to prevent pathogens from being brought (i.e., requiring persons entering the isolation area to wear dedicated clothes for that area and to thoroughly disinfect hands)

In addition, tools and equipment to be used for the measures shall be disinfected or sterilized without fail.

  - (i) Semen

In principle, the animals are checked for anomalies after sperm is collected and

the gene detection test is conducted and confirmed negative. Additionally, no semen shall be supplied until the test result is known. Additionally, sperm collected shall be managed separately from sperm already managed in segments until the test result is known.

However, only if blood sampling is problematic, the collected sperm can be used in gene detection tests and shall be confirmed negative.

(ii) Embryo

In principle, the animals are checked for anomalies after embryo is collected and the gene detection test is conducted and confirmed negative. Additionally, embryos shall be managed separately from embryos already managed in segments until the test result is known.

(4) Moving carcasses, manures, bedding materials, feedstuff and livestock feeding equipment

Only when it can be confirmed that the following requirements are met at a farm where the prefectural animal health inspector has confirmed reared domestic pigs have no clinical abnormalities, is it possible to move domestic pig carcasses, manure, bedding materials, feedstuff and livestock feeding equipment to incineration facilities and other necessary facilities for incineration, burial, rendering, composting or disinfection after a consultation with the Animal Health Division.

(i) Measures at the time of movement

- a. The prefectural animal health inspector shall confirm the existence or the absence of abnormalities in the domestic animals in the relevant farm on the day of movement or the previous night, by the daily report.
- b. The prefectural animal health inspector shall instruct the use of enclosed carrier vehicles or sealed containers as a general rule. If neither is available, cover the floor and side surfaces of carrier vehicle with plastic sheets and, after loading the package, cover the upper part of load with a plastic sheet as well, or take other measures necessary to prevent any load spillage.
- c. Disinfect the entire surface of the carrier vehicle before and after loading. In addition, the prefectural animal health inspector shall confirm the status of disinfection whenever possible.
- d. In principle, avoid travelling near other farms, and choose transportation routes that are not used by other livestock-related vehicles.
- e. Avoid delivery to multiple farms in a row.
- f. After transport, immediately disinfect the vehicle and materials used. g. T Record the course of the transportation process and maintain a record of its.

(ii) Measures at the time of incineration, rendering processing or disinfection.

- a. Take measures such as spreading plastic sheets from the carrier vehicle to the location where carcasses are disposed of;
- b. Take measures such as separation of the locations for carcasses and product

storage;

- c. Disinfect the route from the entrance of the disposal facility to the location where carcasses are disposed of, immediately after their introduction into the incineration, rendering or disinfection process; and
- d. A Disinfect livestock feeding equipment by an appropriate disinfection method. In addition, a prefectural animal health inspector should confirm the status of disinfection whenever possible.

## **XXI Restrictions on events at facilities gathering livestock (Articles 26, 33, and 34 of the Act)**

### **1 Restriction within the movement restriction zone**

- (1) The prefecture shall suspend the following businesses or events within the movement restriction zone upon a consultation with the Animal Health Division.
  - (i) Slaughtering domestic pigs in slaughterhouses
  - (ii) Events such as livestock markets, where domestic pigs are gathered
  - (iii) Free range for domestic pigs
- (2) The prefecture shall order the owners of slaughterhouses, rendering facilities in the movement restriction zone to carry out necessary disinfection by setting a time limit, and shall have them install necessary disinfection facilities as necessary.

#### **[Note 87] Period for disinfecting facilities gathering livestock**

In principle, the period should be based on the lifting of the movement restriction zone.

### **2 Resumption of slaughterhouses**

#### **(1) Requirements for resumptions**

As for slaughterhouses in the movement restriction zone, if they satisfy all of the following requirements, the prefecture can allow them to resume operations based on a consultation with the Animal Health Division. In the cases where an outbreak of CSF occurs in the slaughterhouse, disinfection inside the plants shall be completed in addition to these requirements.

- (i) Vehicle disinfection equipment has been installed.
- (ii) The facilities receiving live animals shall be clearly distinguished from other areas in the facilities.
- (iii) Regular cleaning and disinfection are executed.
- (iv) The biosecurity manuals are appropriately prepared and referenced/used by employees.
- (v) A system shall be in place to comply with the provisions described in (2) below.

#### **(2) Matters to be observed after resumption of operations**

After the resumption of operations, the facility shall be thoroughly managed so that following requirements are strictly observed:

- (i) Those entering the facility must wear dedicated outerwear, boots, head cover or gloves.
- (ii) Vehicles are thoroughly disinfected both going into and out of the facility.
- (iii) Animal deliveries are to individual farms only with no stops made at multiple farms.
- (iv) In the case of carrying in domestic pigs from a farm located within the movement restriction zone, adjustment shall be made so that no carrying-in vehicles from other farms are present at the slaughterhouses during the carrying-in operation, and the

facilities receiving live domestic pigs site shall be disinfected before and after carrying in the relevant domestic pigs(v) If domestic pigs are carried in from a farm in movement restriction zones , these pigs shall be carried in at the end of the day and be slaughtered and dressed within the day of being carried in;

(vi) If it is determined that carried-in domestic pigs are unsuitable for slaughter and dressing pursuant to the Slaughterhouse Act (No. 114 of 1953), they shall not be returned to the farm but promptly disposed; (vii) Animals delivered shall be grouped according to farm of origin and managed separately.

(viii) Delivery and shipment of domestic pigs and pork products shall be recorded and the record stored.

**[Note 88] Matters related to events without gathering domestic pigs**

Since it is possible to prevent the spread of CSF by thoroughly disinfecting areas around identified points where wild boars are diagnosed positive, prefectures shall announce that such events will be approved so long as proper disinfection procedures are followed as necessary. In addition, the prefecture will instruct to ensure that those who participate in events, from affected areas of CSF are not subjected to unfair treatment such as restrictions on their participation.

## **XXII Installation of disinfection points (Article 28-2 of the Act)**

- 1 The prefecture shall, in a case of having notified of a confirmation of affected wild boars as per the provision of XVII herein, promptly install disinfection points in collaboration with the municipalities, competent police department, road administrator, with emphasis on the prevention of virus spread.
  
- 2 As for the concrete location of the disinfection points, the entrances of mountain roads where wild boars are diagnosed positive to CSF pursuant to XVII, neighborhoods of the farms around the affected areas, the borders of the movement restriction zone shall be selected in consideration of the following criteria. Additionally, if there are any epidemics of domestic pigs, the location shall be revised accordingly.
  - (1) Conditions of the mountain paths and road system
  - (2) Traffic of personnel or general vehicles
  - (3) Traffic of livestock-related vehicles
  - (4) Topography of the area such as mountains or rivers
  
- 3 In constructing disinfection points, facility structures shall be designed to enable effective disinfection of not only vehicles related to livestock industry or quarantine, but also general vehicles so that the spread of viruses by vehicles can be completely prevented.

Vehicles related to livestock industry or quarantine in particular shall be instructed to drive through to the points for especially thorough treatment including disinfection of the drivers' clothing and the interior of the vehicles.

Additionally, the prefecture shall take measures to prevent cross-contamination between vehicles at the disinfection points, such as installing multiple disinfection points at each location if necessary, paying attention to the location of entrance/exit or traffic flow.

Additionally, if disinfection points are located around mountain paths near locations where wild boars are diagnosed positive to CSF pursuant to XVII, persons passing through shall be thoroughly disinfected to prevent viruses from spreading among wild boars.

### **[Note 89] Items regarding vehicle disinfection**

The Prefecture shall pay attention to the following matters when disinfecting vehicles

#### **1 Disinfection at the disinfection points**

- (1) Location of the disinfection points

In the discussion of where to place disinfection points, in addition to conferring with police superintendents and road administrators, the effect on the residential environment or agriculture shall be sufficiently considered.

- (2) Records of disinfection

If vehicles are processed through disinfection points, a certificate shall be issued as confirmation at destination points. In addition to instructing drivers be in possession of their certificates while on the road, the prefecture shall record and store copies of them so that processed vehicles can be identified.

## **2 Disinfection procedures at the disinfection points**

Disinfection methods at disinfection points, shall be done by wheel dip or vehicle disinfectant mat on the road, or by guiding automobiles into open space such as a parking lot and applying disinfection by power sprayers, and with consideration to the location characteristics. Additionally, personnel for guiding drivers to the disinfection point and those performing disinfection shall be separately arranged.

### **(1) Vehicles related to livestock**

As for the disinfection of vehicles, cationic soap or slaked lime, which are not irritating to the body, shall be used. The entire vehicle shall be disinfected after the mud are removed by power sprayer with focus on wheels and tires. Drivers' seats and cargo beds shall be disinfected by wiping. In these cases, care shall be taken not to leave any point, such as movable parts undisinfected, Hands and shoe soles of drivers shall be also thoroughly disinfected.

### **(2) General vehicles**

At least, drive through wheel dip or disinfectant mats shall be implemented. In these cases, disinfectants shall be regularly replaced so that sufficient effectiveness may be maintained.

## **3 Period for disinfection point operations**

In principle, disinfection points are generally operated until the movement restrictions are lifted from the area. However, depending on the spread of the virus, the operation period shall be reviewed upon a consultation with the Animal Health Division as necessary.

## **4 Providing accurate information and instructions**

Prefectures other than the affected prefecture shall supply precise information and appropriate instructions so that vehicles from the affected prefecture may not be restricted from entering other prefectures after the appropriate disinfection.

## **XXIII Confirmation of the status of virus spread**

### **1 Confirmation of the status of virus spread**

The prefecture shall, in a case of having notified of a confirmation of affected wild boars as per the provision of XVII herein, immediately take the following measures upon a consultation with the Animal Health Division. These measures can be taken before the diagnosis pursuant to XVII as necessary.

#### **(1) Inspections among wild boars**

The prefecture shall conduct antigen tests and antibody tests (as a general rule) on wild boars that died or were captured within a 10-km radius of the point where the said wild boars were identified, to confirm the status of virus spread. Additionally, they shall endeavor to prevent the disease from spreading to other wild boars or domestic pigs.

#### **[Note 90] Inspections on wild boars**

As for the inspection specified in XXIII-1-(1) of the Guidelines, the prefecture shall continue gene detection tests for at least 28 days in principle. Sampling shall be actively conducted within 3-km radius of the point. Serum antibody tests will be conducted as necessary.

The prefecture shall request that parties, including hunting clubs, to notify the department in charge and to cooperate by taking samples from the animals in case they encounter dead wild boars or capture live ones. Depending on the status of virus spread, the subject area shall be expanded and the implementation period of at least 28 days shall be continued based on comments from experts, including commissioners of the subcommittee.

#### **[Note 91] Preventing the spread among wild boars and from wild boars to domestic pigs**

The prefecture shall examine and implement effective measures, including fences, voluntary ban of hunting, coordinating research captures, clearance food materials such as harvest residue around farms as well as reducing wild boar habitat density through capture as necessary, based on the opinions of the government and experts, virus spread status among wild boars in the area, environmental factors (i.e., inhabiting situation of wild boars, number of surrounding farms, rearing density of domestic pigs, and geographical characteristics such as mountains or rivers)

#### **(2) Inspections among domestic pigs**

The prefecture shall conduct on-site inspections on farms (exclusive to farms rearing at least six animals) within the movement restriction zone to confirm the existence or the absence of specific symptoms. In this case, samples for diagnosis shall be taken

and gene detection tests and serum antibody tests conducted as necessary

## **2 Measures to prevent spread of virus among wild boars in the neighborhood**

The prefecture shall thoroughly disinfect the points where wild boars inspected as described in 1-(1) were identified, and require related parties, including hunting clubs to handle carcasses appropriately with immediate incineration or burial.

### **[Note 92] Measures to prevent spread of virus among wild boars**

For information on the appropriate treatment of dead or captured wild boars to prevent the spread of viruses, refer to the Guide.

## **3 Confirmation of the compliance to Biosecurity Standards (Article 34-2 of the Act)**

- (1) In a case of having notified of a confirmation of affected wild boars as per the provision of XVII herein, the prefecture shall confirm the status of compliance with Biosecurity Standards on farm in and around restriction zone, through the results of on-site inspections, the most recent investigation on the status of compliance, and the instruction record on biosecurity management in accordance with I-3-(2).
- (2) In the case when, as a result of (1) above, recognizing that the following items in Biosecurity Standards are not being complied with by the owners of domestic pigs, and that CSF is highly likely to spread unless the situation is not improved immediately, the prefecture shall recommend the pig owners in problem to improve by setting time limits and issuing documents specifying the matters to improve, in accordance with the Biosecurity Instruction Plan:
  - (i) Items regarding methods to prevent the spread of contamination with infectious disease pathogens in domestic animals within the biosecurity area
  - (ii) Items regarding methods of preventing infectious disease pathogens of domestic animals from spreading outside the biosecurity area
- (3) In the event that the owner who has received the recommendation set forth in (2) above fails to comply with the said recommendation, the prefecture shall order the owner to take measures pertaining to the Biosecurity Instruction Plan by setting a time limit and issuing a document specifying the matters to improve

## **XXVI Spreading oral vaccine**

In cases in which CSFV is likely to be spreading among wild boars per results of the investigation in III-1-4, XII-6 or XXIII-1-(1), the government and the prefecture shall, in cooperation with municipalities and related organization such as hunting clubs, take the following measures in principle to prevent further spread of CSF among wild boars and the entry of CSFV into farms.

- 1 The MAFF shall decide whether to use oral vaccines based on the expert opinions including wild boar experts, in consideration to the status of the spread of CSFV among wild boars.
  
- 2 If the use of oral vaccines is decided as per 1, the MAFF shall formulate and publish “Guidelines regarding the implementation of spreading oral vaccine (hereinafter referred to as “Implementation Guidelines” in 3)” which describes oral vaccine usage or methods to analyze and evaluate its effects/ effectiveness of oral vaccine sprays
  
- 3 The prefecture shall formulate the prefectural plan about spreading oral vaccine based on the Implementation Guidelines with the assistance of the government, municipalities, and related organization such as hunting clubs, and conduct it effectively and efficiently.

### **[Note 93] Oral vaccine spreading**

The prefecture shall implement oral vaccination and reduce habitat density by capturing wild boars, and shall, as necessary, review other effective measures based on the opinions of the government and experts.

## **Chapter 4 Others**

### **XXV Others**

- 1 Domestic pigs possessed by related parties in the livestock industry, including genetically important pigs such as sire pigs, are not individually and specially treated at all. On this assumption, related parties in the livestock industry shall distribute risks regularly by keeping the genetic resource with frozen semen and frozen embryos and dispersing the holdings of sire pigs.
  
- 2 The Director-General of the Food Safety and Consumer Affairs Bureau, MAFF, shall, as needed, separately lay down notes when implementing control measures on the basis of this Guidelines.
  
- 3 The MAFF shall promote research and development that will contribute to the improvement of control measures, and when these efforts have produced results, it shall promptly review this Guidelines.
  
- 4 In consideration of the fact pig owners and personnel in charge of control measures may continue to suffer from psychological stress even after completing all control measures, the prefecture shall endeavor to provide support, by visiting farms and maintaining consultation services even after the completion of control measures on the infected farm. Additionally, they shall provide the pig owners, municipalities, and related organizations with the results of epidemiological investigation and information regarding re-introduction of domestic pigs.

## [Reference]

(Attachment 1)

### **Diagnosis Manual of CSF**

CSF is a pestivirus of the family Flaviviridae and antigenically and structurally very similar to the viruses that cause Bovine viral diarrhoea virus (BVDV) and Border disease virus (BDV). Clinical signs of pigs affected with CSF (hereinafter referred to as “this disease”) and autopsy findings vary considerably depending on virus strains and the host pigs. If a pig fetus is infected with a ruminant pestivirus such as BVDV and BDV, the symptoms may resemble those of CSF so closely that it would be impossible to distinguish the two.

Regardless of their stages of development, pigs infected with this disease show the main clinical signs of fever, huddling, decrease or loss of appetite, torpor, weakness, conjunctivitis, constipation, followed by diarrhoea and an unsteady gait. A few days after the onset, purpura may occur in the auricle, abdominal or inner thigh regions. In acute cases, pigs die within one or two weeks, without any showing clinical signs of this disease.

As in cases of different virus strains, either subacute or chronic forms apply depending on the pig’s age in months and condition. Infected animals subsequently die within a period spanning two to four weeks to a few months. In its chronic form, symptoms such as developmental delay, decrease or loss of appetite or intermittent fever or diarrhoea appear. Immunogen and leukopenia are often observed before fever is evident and the immunosuppression effects such as these can cause concurrent infections.

In its acute form, visible pathological changes tend not to emerge. Typical visible symptoms are reddened and enlarged lymph nodes, epicardial bleeding as well as bleeding in the kidneys, bladder, skin, or subcutaneous tissue. In its subacute or chronic form, necrotizing or “button-shaped” ulcers on gastrointestinal, epiglottis, or laryngeal mucosa in addition to the above findings are observed.

In terms of histopathological findings, lesions such as parenchymatous degeneration of lymphoid tissue, cell growth in vascular fibroblasts and nonsuppurative meningoencephalitis with perivascular cell infiltration emerge, none of which are specific to CSF.

Accordingly, although this disease presents multiple clinical signs and lesions, they are not specific to this disease. Making a diagnosis using clinical signs and finding differences from viral diseases such as ASF, postweaning multisystemic wasting Syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS) and salmonellosis, pasteurellosis, actinobacillosis and *Hemophilus parasuis*, which cause sepsis is difficult.

Therefore, laboratory virologic diagnoses are most important. Laboratories employ direct methods to detect antigen factors such as CSFV, its nucleic acid, or viral antigen, as well as indirect methods to detect virus-specific antibodies. Although the latter antibody detection method is subject to problematic cross-reaction with ruminant pestivirus such as BVDV. As in its acute form, pigs show clinical signs and die before detection of the specific antibodies that are used mainly to monitor the cleanliness.

## **A Domestic pigs**

### **I Antigen test**

#### **1 Test policy**

When diagnosing cases of which this disease is suspected, considering the rapidity and the number of processable samples, the CSFV antigen detection method by fluorescent antibody staining of frozen sections is the best. Accordingly, rather than multiple organs collected from a single pig suspected of carrying this disease, it is preferable to test tonsils from many pigs suspected of carrying this disease to prove the viral antigen of this disease.

In addition, a PCR test shall be conducted using blood as a material where there are viral isolations through cell culture and living organisms in tandem with viral antigen detection using the fluorescent antibody method. Although detection is possible by viral isolation method within approximately 24 to 48 hours, provided the virus exists in high concentrations, it is preferable to continue observation for least one week, since the amount of the inoculated virus might have been small. It is important to conduct RT-PCR parallel to viral isolation to confirm the existence of a virus at an early stage. However, since it is necessary to confirm that the amplification product was not derived from another pestivirus (described below) or from PCR products derived from other positive samples or positive control (cross-contamination), a comprehensive judgment, taking also viral isolation results into account, should be implemented.

Additionally, consideration of how cells used in viral tests are maintained and managed in advance, procurement of dry ice to be used to produce frozen sections and precooling of cryostat sections for smooth diagnosis is preferable. In the event that dry ice is not readily available, n-hexane preserved at -80°C can also be substituted.

#### **2 Collection**

- (1) After arriving at a farm, conduct a clinical test and if the signs in IV-2 of the Guideline are confirmed and CSV is suspected, prioritize pigs with the signs and conduct a diagnosis.
- (2) It is desirable to swiftly collect samples from pigs, which are disposed of for diagnosis, or those immediately after death. In addition, when obtaining necropsy materials, live tissue materials should be prioritized, while those remaining for tissue fixing should be kept in formalin. Live tissue materials include the tonsil (all one sides), kidney (including cortex) and spleen (partial) and are used not only to produce emulsion for viral isolation but also frozen sections, striving not to destroy the organizational structures when collecting them. Individually, the collected materials should be placed into a sterilized 6-hole plate, which should then be fixed and have the cover sealed with vinyl tape. The next step would be to place it into a plastic bag, refrigerate (ice) it and bring it back to the examination room. If a pig is infected, its live tissue materials and blood include a high quantity of virus. As used sampling and

dissecting instruments are polluted with high viral loads, they should be handled with due care.

In addition, if a live pig shows signs arousing suspicions of this disease, its blood (blood serum or blood with anticoagulant added) should be collected to use materials for not only antibody and leukocyte counting tests but also viral isolation and PCR tests.

### **3 Producing frozen sections and emulsions**

Materials for producing frozen sections shall not be thawed and fresh materials are used. During each manipulation, measures for litter pathogen control should be taken, such as laying out a cotton cloth impregnated with disinfection liquid on a table.

#### **(1) Processing of live tissue materials**

- a. Cut three portions of tissues respectively around 1 cm x 5 mm in size (tonsil), or 1 cm x 1 cm (kidney and spleen) to create frozen sections.
- b. Place about 1 g of the remaining tissue on a petri dish to produce an emulsion and weigh it. Store it in ice until an emulsion is produced.
- c. Enter the pig number and specimen name on filter paper.
- d. Place a tissue for producing a frozen section with the cutting surface face up on the filter paper. On this occasion, be careful to make a vertical section of the crypt for a tonsil and renal tubules epithelium for a kidney.
- e. Pick up the filter paper with the tissue fragment thereon with tweezers and soak it in n-hexane chilled by dry ice or acetone (around -80°C) for rapid freezing. Note that if it is excessively soaked, the tissue fragment will be broken.
- f. When it is frozen, move it quickly to cryostat storage, place it into a cold-resistant tube and store it in a -80°C deep freezer.

#### **(2) Producing a frozen section specimen**

- a. When frozen tissue is in the cold-resistant tube according to (1)f., take out the tissue fragment from the cold-resistant tube in cryostat storage.
- b. Place the tissue fragment on a sample table with the compound.
- c. Facing.
- d. Produce a 6 µm section.
- e. Place sections on a silicon coat-processed slide glass.
- f. Dry them immediately with a dryer.
- g. Fix it with cold acetone for ten minutes.
- h. Dry it with air to create a slide glass specimen.

#### **(3) Produce emulsion for viral isolation and PCR test (using a homogenizer and cell-crushing apparatus is acceptable)**

- a. Place a tissue fragment in (1)b. in a mortar.
- b. Shred the tissue fragment with scissors in the mortar.
- c. Add silica sand appropriately and lightly grind down the tissue fragment with a pestle.

- d. Place the cultures there so that the weighed tissue fragment can be 10%w/v and emulsify it effectively (for example, when the tissue fragment is 1 g, 9 ml cultures should be added.)
- e. Move the emulsified tissue fragment into a centrifuge tube.
- f. Cool and centrifuge at 3,000 rpm for 15 minutes.
- g. Move the supernatant to a small test tube and create a 10% emulsion.

#### **4 Isolation of the virus (using chamber slides as opposed to coverslips)**

To prepare a cover slip specimen, emulsion is inoculated after producing a cell sheet on a cover slip. The Fetal Bovine Serum (FBS) used in the cell culture shall be negative to BVDV antibody. Additionally, if the individual has both the virus and neutralizing antibody, sometimes virus isolation from the emulsion becomes negative. Therefore, a weak emulsion (as described below) shall be also inoculated. After inoculating the emulsion, cells on the cover slip are sampled every day, immobilized with cold acetone, and the CSFV antibody in the cytoplasm is detected with fluorescent antibody method. Given that the observation period is at least one week, if the viral load of the emulsion is low and consequently the specific fluorescence is not observed in the cell sheet on the cover slip until the day three, the cover slip should be inserted into another six-hole plate to prepare a cell culture. If the specific fluorescence is not observed on the day four, inoculation of the supernatant of the cover slip on the cell culture prepared on the previous day should be done and culturing continued. From days five to seven, the above observation should be performed on the cell culture's cover slip.

Additionally, measures such as laying cotton cloth containing disinfectant solution shall be taken in each operation to prevent pathogen from scattering.

##### **(1) Preparation of cultured cells**

- a. CPK cells are used for viral isolation (note that this differs from the CPK- NS cell in II-4) and subculture cells with three times the amount.
- b. Enter three to four coverslip sheets (6 x 18 mm) into each hole of a six-hole plate to avoid overlapping other sheets.
- c. Place 3 ml of cell-suspended liquid into each hole. On this occasion, note that the coverslip may suspend and be overlapped.
- d. Culture it overnight at 37°C.
- e. Next day, confirm the cell sheets are formed and use them.

##### **(2) Produce an emulsion vaccination and coverslip specimen**

- a. The amygdalae emulsion should be filtrated with a 0.45 µm filter and clogging can be prevented if filtered with a glass filter in advance.
- b. Produce a dilution sequence of emulsion and blood (use a stock solution, 10x or 100x diluted) and vaccinate 0.2 - 0.3 ml of the volume to cell sheets in (1)e. (the stock solution

- should be stored at least until the test is completed.).
- c. Stand them still for viral absorption for 1 hour, during which tilting should be conducted for 15-20 minutes.
  - d. Wash the cell surfaces in PBS or a medium.
  - e. Add culture solution containing 5 % blood serum and incubate at 37°C. Although the serum shall be FBS negative to BVDV antibody, it can be substituted with equine serum. In this case, check in advance whether the CPK cell can be cultured with the equine serum.
  - f. Take out a coverslip chronologically and after washing with PBS, fix it for ten minutes with cold acetone.
  - g. Air dry it to be a coverslip specimen.

## **5 Fluorescent antibody method**

Use a commercially available fluorescent antibody to diagnose CSF for a slide glass specimen in 3-(2) h. and fluorescent staining of a coverslip specimen in 4-(2) g. If a viral antigen is positive in a frozen tonsil section, specific fluorescence, which can be observed only in the cytoplasmic section (the nucleus looks black), is observed in the crypt epithelial cell and fluorescence. On the other hand, if the virus is isolated in the coverslip specimen, specific fluorescence, which is observed within cytoplasm as in the slide glass specimen, is observed either in an entire specimen or part of the cells. It depends on the virus content whether the fluorescence is observed on the entire specimen or a part of it. When the virus amount is small, the infected cells proliferate in focal manner to formulate focuses as the culture time elapses. Since the determination of the test result is done most easily by the focus forming period, it is necessary to observe it for several days. In dyeing either specimen, if GPE-vaccine strain infection coverslip specimen prepared in advance as a positive control of antigen is dyed simultaneously, it will be checked whether there was any problem in the inspection procedure and the determination will be easy. For details of the fluorescent antibody dying method, refer to the manual attached to the fluorescent antibody for Diagnosis Manual of CSF.

## **6 Conventional RT-PCR**

The blood material in 2-(2) and 10% emulsion in 3-(3) g., or culturing supernatant in viral isolation shall be used as specimens. In addition, to accurately determine when cross-contamination has occurred, the test is always conducted via a method using two types of positive control samples. However, if it is not possible to obtain the positive control specimen in (1)(ii), the test is conducted with the CSFV vaccine strain (GPE-strain) as the positive control specimen according to 7.

Although it is possible to test by the same method when testing semen, when the material is undiluted, it shall be diluted to the same extent (50 folds) as a commercially available semen using a diluted solution for semen, PBS or physiological saline.

### (1) Positive control sample

#### (i) Positive control sample 1: BVDV culture supernatant

Culture supernatant of BVDV type 1 or 2 is used. RNA is extracted from the sample in the same way as from the test material and used as a positive control sample to determine the success or failure of the test up to the PCR reaction.

#### (ii) Positive control sample 2: CSFV (GPE-strain) altered DNA

The DNA distributed by the NIAH is used. The sample is a positive control sample used to determine the success or failure of the test from the PCR reaction to the restriction enzyme treatment.

### (2) Extraction of RNA

Commercially available RT-PCR kits or nucleotide (RNA/DNA) extraction kits are easy to use and operate. While refinement with automatic extraction machines is also possible, it should be confirmed whether the inspection in the following items. The materials to extract are blood, emulsion, culturing supernatant and kits suitable for materials shall be selected. Degradation of infectivity titer because of freeze-thaw would not be worried about if appropriate quantity (within the range 50 - 400 µl depending on the kit) of material is dispensed into each of other microtubes than used for virus isolation in preparing specimen for isolating virus. Materials are handled as infectious until denaturant is added and admixed.

In addition, the extraction of RNA must also be carried out for the positive control sample 1. Preferably, the appropriate amount of sample should be dispensed into tubes and stored frozen.

### (3) RT-PCR reaction

Commercially available RT-PCR kits are convenient. Especially, those of one-tube method that can continuously conduct RT and PCR reactions are particularly convenient and capable of mitigating manipulation and cross-contamination problems. However, although some commercially available kits contain UNG enzyme (Uracil-N-Glycosylase) to prevent cross-contamination due to carry-over of products after the PCR reaction, it should be noted that, although this enzyme can be expected to reduce the risk of cross-contamination, it is not suitable for gene analysis after PCR reaction (restriction enzyme treatment, sequence analysis). The target of the test is the 5'-nontranslated region (5'-NTR). However, while the 5'-NTR region is highly preserved and can be detected with high sensitivity, it also detects various types of other pestiviruses other than CSF, such as BVDV, the detected PCR products also need to be analyzed in detail with supplementary tests such as RFLP analysis or gene analysis.

In addition, while positive control sample 2 is placed as a positive control and PBS as a negative control, since there is a risk of cross-contamination, the positive control must be carefully handled with facilities and biosafety in mind.

#### a. Primer and annealing temperature

Upstream primer “324” and downstream primer “326” by Š.Vilček (Arch.Virol,136:309-323,1994) shall be suitable to detect CSFV. As both  $T_m$  values are 56.5°C, PCR annealing (pairing) should be conducted at 55-57°C. The denature (denaturation) and extension (expanding) temperatures and their time and number of cycles are set according to the kit to be used.

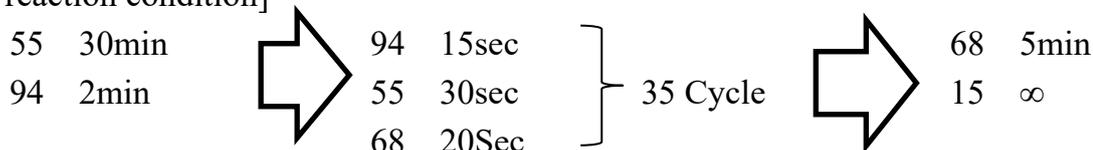
[Primer Sequence]

Upstream primer [324] 5'-ATG CCC (T/A)TA GTA GGA CTA GCA-3'  
 Downstream primer [326] 5'-TCA ACT CCA TGT GCC ATG TAC-3'

[Composition of the reaction liquid] Example for SuperScriptIII One-step RT-PCR kit, Invitrogen

2×Reaction Mix	12.5µl
324 Primer (10pmol/µl)	0.5µl
326 Primer (10pmol/µl)	0.5µl
Enzyme Mix	1.0µl
DW	8.0µl
Sample	2.5µl
<hr/>	
Total	25.0µl/tube

[PCR reaction condition]



b. Agarose cataphoresis and restriction enzyme treatment

For CSFV, about 280 bp (often 284 bp) of PCR products are produced. Products are electrophoresed in 1-2% agarose gel and observed and photographed under UV radiation.

Since about 280 bp of products are produced also for other pestiviruses, such as BVDV, it is impossible to distinguish CSFV from BVDV on agarose gel cataphoresis. While determination of base sequence is necessary for identifying the virus for sure, simple identification is also possible by digesting the PCR product with restriction enzyme and assaying it with agarose cataphoresis (RFLP analysis).

Moreover, with the method written on this manual, it is possible to check the cross-contamination by processing with 2 kinds of restriction enzyme. The restriction enzyme *Bgl*I and *Eco*RV are used and the assay shall be conducted referencing the constitution of the reaction solution shown below.

If it is CSFV (the PCR product before the processing is 284 bp), it is cleaved exclusively by *Bgl*I and the size becomes smaller than before the processing to be 243

bp. (the fragment of about 41 bp is cut out by the restriction enzyme).

Conversely, in BVDV of the positive control sample 1, since it is not cleaved by *Bgl*I or *EcoRV*, it remains 284 bp after the treatment, the same as before.

In addition, since the DNA of the positive control sample 2 is cleaved by both *Bgl*I and *EcoRV*, the size after treatment becomes 144 bp (about 41 and 99 bp are cut out by the restriction enzyme), smaller than that of CSV.

[Constitute of the reaction liquid] Treatment with *Bgl*I and *EcoRV*

PCR reaction liquid	5.0μl
10×high buffer	2.0μl
<i>Bgl</i> I	0.5μl
<i>EcoRV</i>	0.5μl
DW	12.0μl
<hr/>	
Total	20.0μl/tube

[Reaction condition of the restriction enzyme treatment]

37°C 60min

\* According to the optimum temperature of commercially available restriction enzyme

## 7 Real-time RT-PCR

In the implementation of real-time RT-PCR, commercially available kits are convenient. For the reaction condition, refer to the manual included in the kit.

Blood (whole blood or serum) is used as the test specimen, which is prepared according to the manual included in the kit. The blood as fresh, with less hemolysis, and clear as possible should be used. Additionally, if it is difficult to collect blood, organs (tonsil, spleen) can be used. The specimen shall be prepared according to the manual included in the kit.

If the real-time RT-PCR test result is positive, the sample shall be delivered to NIAH and confirmation test shall be done.

## 8 Handling of the test results

If the findings of the frozen section and viral isolation show a positive result is likely, responses should be pursuant to IV-6 of the Guideline.

## II Antibody test

### 1 Policy of testing

In the acute form of CSF, detecting by clinical inspection is important since many pigs die before production of antibody. Conversely, in the chronic form of CSF, no clear

symptoms are shown and it is difficult to detect the disease in a clinical test, but as many affected pigs produce the antibody, this disease can be detected in an antibody test. In addition, unlike the fluorescent antibody method, the antibody test can be implemented as an antemortem test and is useful as one of the monitoring tests for free status confirmation. In outdoor viral infections, pathogens easily spread through horizontal infection. Conducting a test on antibody-positive and epidemiologically related pigs allows the antibody test to be evaluated as a pig group. In addition, pigs vaccinated with this disease as a live vaccine can retain antibodies against CSFV virus for the rest of their lives. When using the vaccine, this should be focused on when making the evaluation.

As a rule, an antibody test should be implemented immediately after collecting samples. If the results trigger suspicions of an outdoor infection, this disease should be immediately reconfirmed (via an antigen test).

## **2 Adjustment of test blood serum**

Isolate the serum from the collected blood as soon as possible and after separating the live serum for antibody tests such as viral isolation, be sure to inactivate serum to be offered to an antibody test, (56°C heat treatment for 30 minutes). Any residue and blood serum not immediately used can be preserved in a frozen state at -20°C. With the possibility of virus infection in mind, place the live serum into a sealing container and preserve it at -80°C.

## **3 Enzyme-linked immunosorbent assay (ELISA) method**

Use a commercially available ELISA kit and determine manipulation and judgment in accordance with the attached manual. As a neutralization test does not use live virus, the test results can be obtained safely and promptly. In future, this method will underpin the antibody test.

## **4 Neutralization test**

Use a vaccine virus GPE strain as an indicator virus for a neutralization test, use +++ as cultured cells and use porcine kidney cell line (CPK-NS cell) suitable for a serum-free medium as cultured cell. By combining this virus and cultured cells, a neutralization antibody can be determined using a cytopathic effect (CPE) as an indicator. The CPK-NS cell is unsuitable for viral isolation and producing indicator viruses given the lack of scope for the CPK-NS cell to proliferate CSFV. In addition, since handling the vaccine virus involves a live virus, thorough management is required, including leakage prevention outside the laboratory and focusing on pollution of the cultured cell and sample.

### **(1) Preparation for serum-free cultured cells**

In a neutralization test, CPK-NS cells, which can be proliferated in serum-free cultures, shall be used. In the subculture of this cell, a new plastic culture flask should be used rather than a recycled flask. Other than the sealing plug (tightening the flask

plug) culture and at least two centrifuge/washing manipulations repeated to remove cell dispersions (trypsin solution) at the time of subculture, there is no difference in normal subculture. Therefore, under normal circumstances, a subculture is conducted every seven days, with a cell surface area ratio three times. In the case of 25cm<sup>2</sup> (75cm<sup>2</sup>), suspend in 15 mL (45 mL) and dispense 5 mL (15 mL) every time for incubation.

[Method to prepare serum-free cultures]

Eagle MEM: 9.4 g (product evaluation value)

TPB (Tryptose Phosphate Broth): 2.95 g

BES (N, N-Bis (2-hydroxyethyl)-2-aminoethanesulfonic acid) : 2.13 g

Bacto Peptone: 5.0 g

Weigh the above-described reagent, dissolve it with 1 liter of pure water or ultrapure water and use an autoclave at 121°C for 20 minutes. After cooling to room temperature, add 10 ml of 3% L-glutamine and 30 mL of 7.5% baking soda and use it as usage liquid.

- a. Remove the medium and wash once with PBS, which is twice or three times as much PBS as a medium.
- b. Digest the cell with trypsin solution (normally, around 10-30 minutes) and add a minor amount of medium. Once the cells are fully dispersed by pipetting, suspend them in ten times the amount of medium to trypsin solution.
- c. Collect the cell suspension liquid in a centrifuge tube to centrifuge (1,000 rpm for 5 minutes). After centrifuging, remove the supernatant, add the medium and suspend the cells.
- d. Centrifuge the cells again (1,000 rpm for five minutes) to remove supernatant.
- e. After refloating the medium again in three times the amount to the original cell surface dispense the cell suspension liquid within the plastic culture flask.
- f. Tighten the plastic culture flask plug, leave it to stand at 37°C. Subculture cells again seven days later or provide a neutralization test. Cells can be subcultured on around the fourth day, but note that the small number of cells means subculturing with three times the amount is not possible.

## (2) Neutralization test

A vaccine (GPE) strain shall be used as an indicator virus in the neutralization test, causing CPE in CPK-NS cells but rarely proliferating. To produce the indicator virus stock for a neutralization test, like viral isolation, use PK cells (note that the cells differ from the CPK-NH cells in II-4). For medium, use one with 5% serum added. Use CPK-NS cells in a serum-free medium to measure the virus or neutralization titer of non-virus stock production.

### a. Virus fluid adjustment method

- (a) Vaccinate sheet CPK cells for about 0.1 multiplicity of infection (M.O.I) and stand it to allow virus absorption for one hour. During this time, tilting should be conducted at 15 - 20-minute intervals.

- (b) Wash the cell surfaces in PBS or medium.
  - (c) Add 5% serum-added cultures and incubate at 37°C.
  - (d) For an open culture, collect the culturing supernatant in a centrifuge tube on the fourth or fifth day after culture. Observe it before collecting with a microscope and a small cytopathic effect (CPE) caused by viral proliferation can be recognized. To check the best time for collecting the virus fluid more effectively, place the cells to be vaccinated against the virus in a coverslip in advance as for viral isolation, collect the coverslip under germ-free conditions and confirm using the fluorescent antibody method that the antigen has spread all over the cell sheet. Centrifuge the collected culturing supernatant (1,000 RPM for five minutes) to remove the supernatant.
  - (e) Further centrifuge the centrifuged supernatant for 15 minutes at 3,000 RPM to remove cell debris and dispense in a small amount of 0.5 ml. The dispensed virus fluid is then preserved at -80°C and the thawed virus titer measured.
- b. Virus titer measurement method
- (a) Digest CPK-NS cells with trypsin, centrifuge twice and adjust the cell suspension liquid. Refloat cells in the same amount of serum-free medium as for normal subculture.
  - (b) Dilute the virus fluid to be measured in a serum-free medium tenfold.
  - (c) Place the diluted virus fluid in a 96-hole microplate, with 100µl per hole.
  - (d) Input 100 µl of the adjusted cell suspension liquid in each hole, culture it for seven days in a carbon dioxide incubator at 37°C
  - (e) Use the CPL observed on the cell surface as an indicator to find the virus titer. (TCID<sub>50</sub>).
- c. Neutralization antibody measurement technique
- (a) Place 50 µl of inactivated test blood in a 96-hole microplate, dilute twofold with 50 µl of serum-free culture and produce two rows of four- tube (2 - 16 folds) dilution rows with 50 µl diluted up to 16fold in each hole. On this occasion, prepare holes for cell control and back titration, which are not vaccinated for the virus. Place 100 µl of serum-free cultures in the cell control hole and 50 µl in the back titration hole.
  - (b) Vaccinate the virus fluid adjusted to 200 TCID<sub>50</sub> per 100 µl in a 96-hole microplate, into the serum-diluted row by 50 µl. At the same time, vaccinate 50 µl of 10-fold diluted and adjusted virus fluid per hole with 50 µl serum-free cultures and perform back titration.
  - (c) After agitating the plate, sensitize it in a carbon dioxide incubator at 37°C for 1 hour.
  - (d) During the sensitization, digest CPK-NS cells with trypsin solution, centrifuge twice and adjust the cell suspension liquid. Refloat cells in the same amount of cultures as used for normal subculture.
  - (e) Input 100 µl of the cell suspension liquid in each hole, culture it for seven days in a carbon dioxide incubator at 37°C.

(f) Use CPE recognized in the cell surface as an indicator to find the neutralization antibody.

## **5 Handling of the test results**

If positive or pseudo-positive findings emerge in the enzyme immunoassay method or a neutralization test, pursuant to IV-6 of the Guideline.

### **B Wild boar**

This manual is also applied to test wild boars.

Since care must be taken with wild boar samples to avoid contamination with those from domestic pigs, Conventional RT-PCR specified in item 2 of A, restriction enzyme treatment, and electrophoresis after PCR reaction are unnecessary. Commercially available real-time RT-PCR, which is also fitted for multi-sample treatment, shall be considered. In the implementation of real-time PCR tests, commercially available test kits are convenient. As for the reaction condition, refer to the manual included in the kit.

For the test sample, blood (whole blood or serum) shall be used. The specimen shall be prepared according to the manual included in the kit. The blood should be as fresh (with little hemolysis) and clear as possible. Additionally, in cases where it is difficult to collect blood, organs (tonsils, spleens) can be also used. Test specimens shall be prepared according to the manual included in the kit.

If infection is confirmed in the real-time RT-PCR in the initial case of wild boars, samples shall be delivered to NIAH and confirmation tests shall be done.

## Calculation Method of Appraised Value of Pigs

### 1 Fattening pigs

(1) Basic method of calculating appraised value

Introduction price of original livestock + fattening cost (production cost per day x rearing days)

(2) Calculation method of introductory price of original livestock and fattening cost

(i) The introductory price is the cost required for introducing original livestock and is confirmed by a purchase slip

(ii) When an original livestock is born at the farm or the introductory price cannot be confirmed, a delivered price shall be used and the introductory price shall be calculated by multiplying the production cost of the fattening pig in the livestock product production cost in the latest year by 9/100.

(iii) As for the production cost per day, subtract the total production cost by the childbirth price and divide it by the fattening period (average sales month age), then multiply the cost by 50/100 to calculate the production cost per day in the previous period (from birthdate to 70 days) and calculate the production cost per day in the latter period (from 71 days to the time of shipment) by multiplying 130/100.

(iv) The rearing days marks the number of days from the introduction of original livestock in case of the introduction of livestock and from the birthdate in case of the original breeding/fattening consistent management, to the day on which affected animals or suspected affected animals are determined.

[Reference] Production cost per day (2011 Livestock Product Production Cost Survey)

- Delivered price (national average)

Total production cost 31,903 yen x the percentage of cost required for producing a piglet to the total pork production cost: 9% = 2,871 yen

- Production cost of fattening pig per day (national)

(whole production cost 31,903 yen - childbirth price 2,871 yen)/fattening period 6.4 months x 30.4 day)

= 149 yen

- production cost per day (0-2.3 month old) : 50% of production cost per day = 75 yen

- late production cost per day (2.3-6.4 month old) : 130% of production cost per day = 194 yen

[E.g.] Evaluation at the shipment of fattening pigs (6.4 month old)

[If introducing 100-day-old piglets]

Introductory price\*: production cost per day x rearing days

15,220 yen + (194 yen x (6.4 - 3.3 months) x 30.4 days) = 33,503 yen

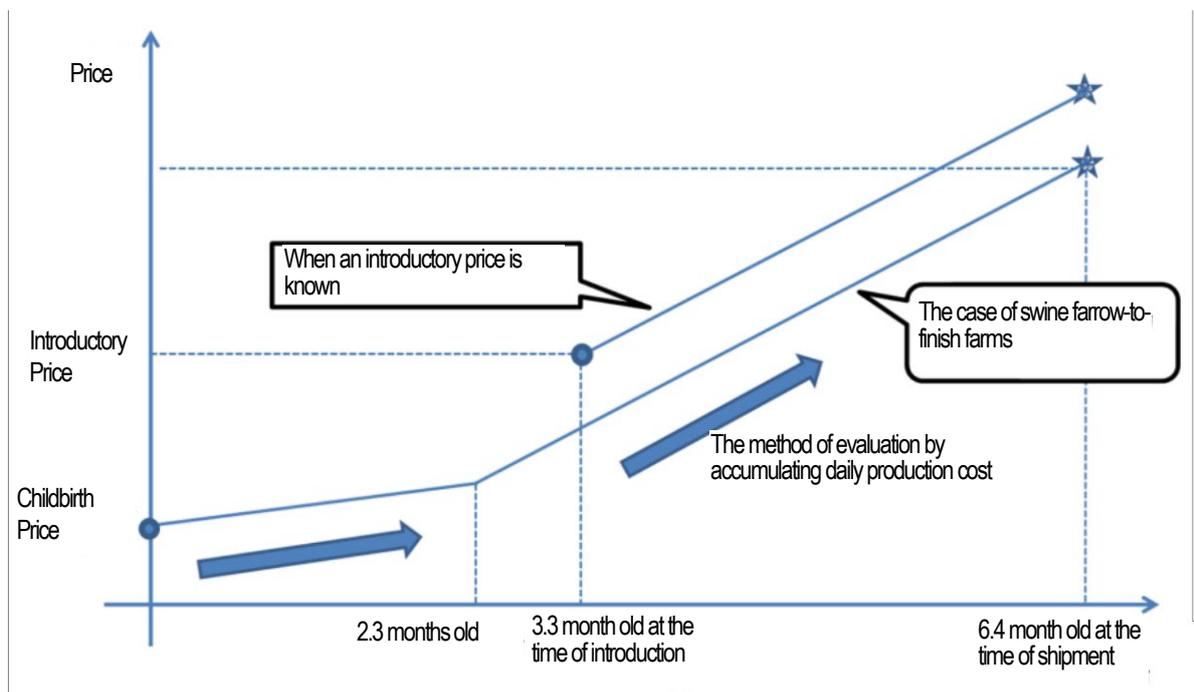
\* In this calculation example, the introductory price is set using agricultural price statistics.

\* [In case where there is no introductory price due to breeding/fattening consistent management]

Birth price: production cost per day x rearing days

$$2,871 \text{ yen} + ((75 \text{ yen} \times 2.3 \text{ months}) + (194 \text{ yen} \times 4.1 \text{ months})) \times 30.4 \text{ days} = \boxed{32,295 \text{ yen}}$$

## Fattening pigs



## 2 Breeding sows

[Breeding sows (nulliparous)]

(1) Basic method of calculating appraised value

Introductory price of original livestock + rearing cost (production cost per day x rearing days) + price for conception

(2) Introductory price of original livestock and rearing cost

(i) The introductory price is the cost required for introducing original livestock and confirmed by a purchase slip

(ii) When the introductory price cannot be confirmed or when the original livestock is born at the farm, it shall be the average trading price (during the most recent one-year period) of a pig equivalent to the original livestock (pig with similar race, usage (suitable for breeding)) at a domestic animal market normally used by the domestic animal owner.

(iii) The production cost per day shall be that of a fattening pig in the production cost

survey.

- (iv) The rearing days marks the number of days from the introduction of original livestock to the date of determining affected animals or suspected affected animals.
- (v) In case of conception, around 20% of the value of the mother pig should be added (limited to cases where a veterinarian can confirm the conception in a pregnancy test).

[Breeding sows (para)]

(1) Basic method of calculating appraised value

Standard price at the first childbirth x estimated index/100 + price for conception

(2) Standard price at the first childbirth and the method of calculating estimated index

(i) The standard price at the first childbirth shall be calculated via the following formula:

Introductory price of original livestock + average rearing cost to the first childbirth month  
age (production cost per day x rearing days)

Besides, the introductory price of original livestock and rearing cost shall be calculated using the same method as for a breeding sow (nulliparous).

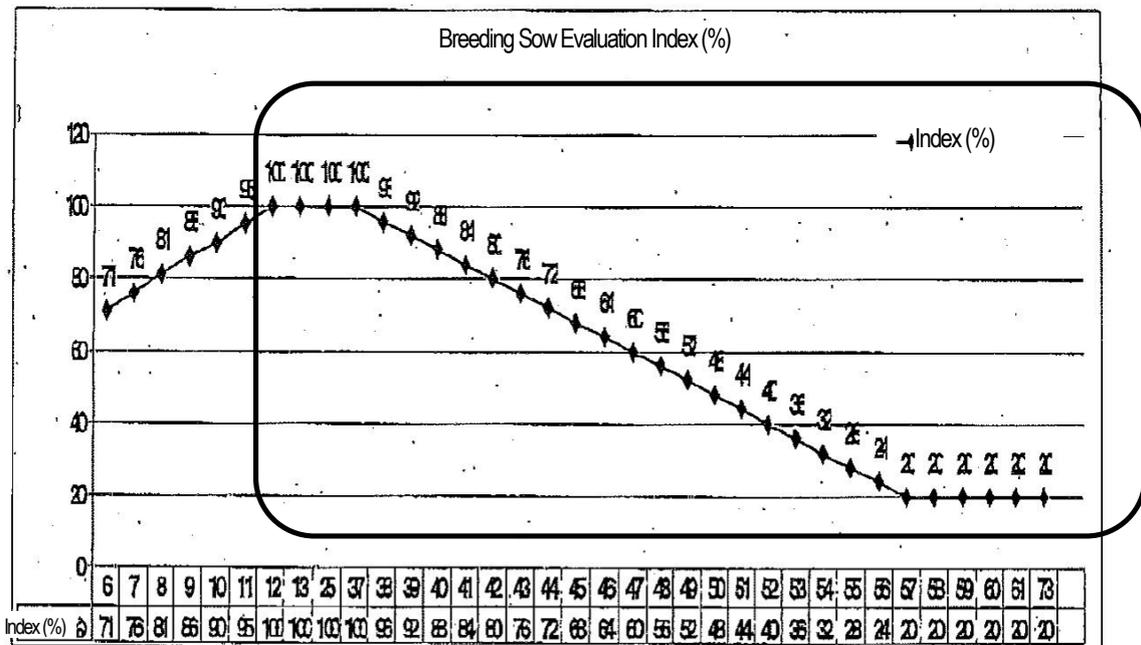
(ii) The estimated index is the index of decrease in value due to deterioration over time, assuming a value of 100 at the first childbirth and applying the prefectural livestock mutual aid payment system for calculation.

(iii) The production cost per day shall be that of a fattening pig in the production cost survey.

(iv) In case of conception, around 20% of the value of the mother pig should be added (limited to cases where a veterinarian can confirm the conception in a pregnancy test).

[Reference] Estimated index used by Miyazaki Pref. at the Time of Outbreak of Foot-and-Mouth Disease (Breeding Sow)

Every prefecture has its own similar index.



[e.g.] Evaluation of breeding sows at the time of first childbirth (about 12 months old)  
 Introductory price: (production cost per day x rearing days) Addition for conception  
 {55,280 yen (the average purchase price of breeding sow (hybrid)) + 194 yen x (12 -  
 3.3 months) x 30.4 days} x 1.2 = 127,779 yen

(Note)

Appended Form 1 – Appended Form 12 (refer to the attachment)