**Japanese Encephalitis Vaccine, Live, Seed**

1 Definition

Japanese Encephalitis Vaccine, Live, Seed is a freeze-dried vaccine of virus suspension obtained by propagating attenuated Japanese encephalitis virus that meets the Seed Lot Specifications in cell cultures that meet the said specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Attenuated Japanese encephalitis virus at strain or strain approved as equivalent thereof

2.1.2 Properties

Viremia does not occur even when this virus strain is inoculated to swine aged one month. The strain does not infect the fetus even when inoculated into swine one month before and after pregnancy. The infection rate of the virus to *Culex tritaeniorhynchus* has been significantly reduced. The strain can be propagated in the brains of suckling mice or in primary cultured cells of swine kidney or swine testis.

2.1.3 Master seed virus

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in primary cultured cells of hamster kidney that meet 2.14 in the SPF Animal Specifications or cultured cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than production of vaccines. The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4 Working seed virus

2.1.4.1 Propagation, passage and storage

The working seed virus shall be propagated and passaged in primary cultured cells of hamster kidney that meet 2.14 in the SPF Animal Specifications or cultured cells approved as suitable.

The working seed virus shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.5 Production seed virus

2.1.5.1 Propagation and storage

The production seed virus shall be propagated in primary cultured cells of hamster kidney that meet 2.14 in the SPF Animal Specifications or cultured cells approved as suitable.

The production seed virus shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 When using primary cultured cells

2.2.1.1 Primary cultured cells

The primary cultured cells of hamster kidney that meet 2.14 in the SPF Animal Specifications or primary cultured cells approved as suitable for production shall be used.

2.2.1.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.1.3 Master primary cell seeds (production primary cell seeds)

2.2.1.3.1 Propagation, passage, and storage

The master primary cell seeds (production primary cell seeds) shall be propagated using culture medium in 2.2.1.2 and will not be passaged or stored.

Master primary cell seeds (production primary cell seeds) shall be tested as specified in 3.2.1.

2.2.2 When using cell line

2.2.2.1 Cell line

HmLu-1 cells or cell lines approved as suitable for production shall be used.

2.2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.2.3 Master cell seeds

2.2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower.

The master cell seeds shall be tested as specified in 3.3.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.2.4 Working cell seed

2.2.2.4.1 Propagation, passage and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.2.

The working cell seeds shall be stored frozen at -70°C or lower.

The working cell seeds shall be tested as specified in 3.3.2.

2.2.2.5 Production cell seed

2.2.2.5.1 Propagation and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.3.3 if it needs to be stored.

2.3 Bulk material

2.3.1 Culture of cultured cells

2.3.1.1 When using master primary cell seeds (production primary cell seeds)

The cultured cells obtained in one session shall be regarded as an individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

The individual cultured cells shall be subjected to the test given in 3.4.

2.3.1.2 When using production cell seeds

The cultured cells obtained in one session shall be regarded as an individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

2.3.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.1. The filtrate or supernatant after centrifugation of culture medium collected from individual cultured cells at the peak of virus propagation shall be mixed, and the mixture shall serve as the bulk material.

The bulk material shall be tested as specified in 3.5.

2.4 Final bulk

A diluted solution and a stabilizer approved as suitable shall be added to the bulk material and mixed to serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product.

The final product shall be tested as specified in 3.6.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The tests given in 1.4.2.1.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

For Porcine reproductive and respiratory syndrome virus and Lymphocytic choriomeningitis, the tests given in 1.1 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For Classical swine fever virus, Porcine circovirus, Bovine viral diarrhea virus, Rotavirus and Rabies virus, the tests given in 1.1, 3.2.3, 3.2.4, 3.2.5, 3.2.7 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.7 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.1.8 Marker test

This test may be omitted if it is conducted for working seed virus or bulk material.

3.1.1.8.1 Materials

3.1.1.8.1.1 Injection materials

The test article and the Nakayama-Yakken strain for control shall be used and adjusted with a diluent approved as suitable to ensure that the dilution contains the strain at a virus content of 107.0 TCID50 per mL or 107.0 LD50 per mL or higher, and this shall serve as the injection material.

3.1.1.8.1.2 Test animals

Mice aged 3 weeks shall be used.

3.1.1.8.2 Test procedure

A dose of 0.3 mL of the injection material shall be injected intraperitoneally to at least ten test animals and observed for 14 days.

3.1.1.8.3 Judgment

The mortality rate of mice injected with the test article shall not be more than 20%. In this case, the mortality rate of the mice injected with the control shall be 80% or higher.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.2.3 Marker test

The test given in 3.1.1.8 shall apply. This test may be omitted if it is conducted for master seed virus or bulk material.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on primary cells

3.2.1 Tests on master primary cell seeds (production primary cell seeds)

3.2.1.1 Test for confirmation of cell properties

The test given in 2.2.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.2.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on cell line

3.3.1 Tests on master cell seeds

3.3.1.1 Test for confirmation of cell properties

The test given in 2.1.4.2.1.1 of the Seed Lot Specifications shall apply.

3.3.1.2 Test for identification of the animal species of the cell

The test given in 2.1.4.2.1.2 of the Seed Lot Specifications shall apply.

3.3.1.3 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.3.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3.1.5 Test for freedom from extraneous viruses

3.3.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.3.1.5.2 Test for freedom from specific viruses

3.3.1.5.2.1 General test for freedom from specific viruses

For Porcine reproductive and respiratory syndrome virus and Lymphocytic choriomeningitis, the tests given in 1.2 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.3.1.5.2.2 Individual test for freedom from specific viruses

For Classical swine fever virus, Porcine circovirus, Bovine viral diarrhea virus, Rotavirus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.2, 3.2.3, 3.2.4, 3.2.5, 3.2.7 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.3.1.6 Test for karyological (chromosomal) characterization

The test given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.3.1.7 Test for freedom from tumorigenicity

The test given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.3.2 Tests on working cell seeds

3.3.2.1 Test for confirmation of cell properties

The test given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.3.2.2 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.3.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3.3 Tests on production cell seeds

3.3.3.1 Test for confirmation of cell properties

The test given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.3.3.2 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.3.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.4 Tests on individual cultured cells

A quantity of 1% or more of individual cultured cells shall serve as control cultured cell and shall be subjected to the tests given below.

3.4.1 Culture observation

The control cultured cells shall be incubated without virus-inoculation under the same condition.

Control cultured cells shall be pooled and passaged into at least four culturing bottles and at least four Petri dishes with a cover glass placed in each. After culturing for seven days, no CPE shall be detected upon observation.

3.4.2 Hemadsorption test

On the final test day in 3.4.1, the culture medium shall be removed from the culturing bottle, and after washing the cell surface twice with phosphate-buffered saline, the bottles shall be divided into three groups. A 0.1 vol% suspension of guinea pig red blood cells, goose red blood cells, and chicken blood cells not older than 7 days, shall be overlaid. After allowing to stand for 60 minutes, upon observation for hemadsorption, no hemadsorption shall be observed in the cultured cells.

3.4.3 Inclusion body staining test

On the final day of the test in 3.4.1, the cultured cover glasses shall be washed with phosphate-buffered saline. Following immobilization, Giemsa staining shall be performed. Upon observation for inclusion bodies, no inclusion bodies shall be observed in the cultured cells.

3.4.4 Test for Freedom from extraneous viruses

The culture obtained on the final day of the test in 3.4.1 shall be used as the test material, and the tests given in 2.3.1, 2.3.2 and 2.4.2 in. the Test for Freedom from Extraneous Viruses for Live Vaccines and Sera of the General Tests shall apply.

3.5 Tests on bulk material

3.5.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.5.2 Test for virus content

The test shall be performed through the inoculation test in mice or inoculation test in cultured cells.

3.5.2.1 Inoculation test in mice

3.5.2.1.1 Materials

3.5.2.1.1.1 Test materials

The test article shall be serially diluted ten-fold with phosphate-buffered saline or a diluent approved as suitable and each level dilution shall serve as a test material.

3.5.2.1.1.2 Test animals

Suckling mice aged not older than 2 days shall be used.

3.5.2.1.2 Test procedures

A dose of 0.02 mL of the test material shall be injected intracerebrally at least four test animals and observed for 14 days.

3.5.2.1.3 Judgment

Mice that die after showing encephalopathic symptoms shall be considered infected and the LD50 shall be calculated.

The virus content of the test article shall be 107.0LD50 or higher per mL.

3.5.2.2 Inoculation test in cultured cells

3.5.2.2.1 Materials

3.5.2.2.1.1 Test materials

The test article shall be diluted ten-fold with virus growth medium (Note 1) and each level dilution shall serve as a test material.

3.5.2.2.1.2 Cultured cells

Vero cells, ESK cells or cells approved as suitable shall be cultured, and the resulting monolayer cells shall be used.

3.5.2.2.2 Test procedure

A 0.1 mL portion of the test material shall be inoculated into at least four tubes of cultured cells and allowed to stand for adsorption at 37°C for 60 minutes. A 0.5 mL of virus growth medium shall be added, cultured at 37°C for seven days and observed.

3.5.2.2.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate TCID50.

The virus content of the test article shall be 107.0TCID50 or higher per mL.

3.5.3 Marker test

The test given in 3.1.1.8 shall apply. This test may be omitted if it is conducted for master or working seed virus.

3.6 Tests on final product

3.6.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a liquid or homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.6.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.6.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.6.4 Sterility test

The test given in the Sterility Test given of the General Tests shall apply.

3.6.5 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

However, this test may be omitted if it is conducted during the intermediate process including the bulk material.

3.6.6 Test for virus content

When the test is performed as specified in 3.5.2, the virus content of the test sample shall be 105.0 LD50 or 105.0 TCID50 or higher per animal.

3.6.7 Safety test

3.6.7.1 Materials

3.6.7.1.1 Injection materials

The test sample shall be used as an injection material.

3.6.7.1.2 Test animals

Swine aged about 1 month shall be used.

3.6.7.2 Test procedures

A group of three test animals shall be used as the test group and one test animal as the control group. A dose of injection material for one animal shall be injected in accordance with the administration in the test group and both the test and control groups shall be observed for three weeks.

3.6.7.3 Judgment

No abnormalities shall be observed in the test or control group during the observation period.

3.6.8 Potency test

Hemagglutination inhibition test or neutralization test shall be conducted.

3.6.8.1 Hemagglutination inhibition test

3.6.8.1.1 Materials

3.6.8.1.1.1 Test animals

The animals used in the test specified in 3.6.7 shall be used.

3.6.8.1.1.2 Hemagglutination antigen

Hemagglutination antigen (Note 2) prepared using Japanese encephalitis virus Nakayama-Yakken strain or strain approved as suitable shall be used.

3.6.8.1.2 Test procedures

Hemagglutination inhibition test shall be performed on sera obtained from the test and control groups on the final day of the test specified in 3.6.7. The sera from the test group shall be pooled at equal volumes.

The test sera shall be treated with a 25 w/v% kaolin solution or acetone, further processed with red blood cells from either chicken not older than 7 days or a goose, and then heat-inactivated. Each treated serum shall be serially diluted two-fold with borate-buffered saline containing bovine serum albumin (Note 3) or a diluent approved as suitable. Each diluted serum shall be added with 8 units of hemagglutination antigen and reacted over night at 4°C. Subsequently, a 0.33 vol% suspension of red blood cells from chicken not older than 7 days or a goose adjusted with VAD solution (Note 4) shall be added, allowed to stand at 37°C for 60 minutes and then observed for hemagglutination.

3.6.8.1.3 Judgment

The maximum dilution factor of the serum that results in inhibition of hemagglutination shall be defined as the hemagglutination inhibition antibody titer.

The hemagglutination inhibition antibody titer in the test group shall be 20-fold or higher. In this case, the titer must be less than ten-fold in the control group.

3.6.8.2 Neutralization test

3.6.8.2.1 Materials

3.6.8.2.1.1 Test animals

The animals used in the test specified in 3.6.7 shall be used.

3.6.8.2.1.2 Virus for neutralization test

Japanese encephalitis virus Nakayama-Yakken strain, JaGAr-01 strain or a strain approved as suitable shall be used.

3.6.8.2.1.3 Cultured cells

Primary cultured cells of chicken embryos specified in 2.1.1 in the Materials for Live Vaccine Production shall be cultured in Petri dishes, and the resulting monolayer cells shall be used.

3.6.8.2.2 Test procedures

Neutralization test shall be performed on sera obtained from each animal in the test and control groups on the final day of the test specified in 3.6.7. The sera from the test group shall be pooled at equal volumes.

The test sera shall be heat-inactivated, and then shall be diluted five-fold with virus growth medium and further diluted serially two-fold. Each diluted serum shall be mixed in equal volumes with virus suspension for neutralization test containing approximately 200 PFU per 0.4 mL and the mixtures shall be reacted at 37°C for 90 minutes.

A 0.4 mL of each mixture shall be inoculated onto four separate cultured cell plates. After the plates shall be allowed to stand for adsorption at 37°C for 60 minutes, the mixture shall be discarded, the first overlaying agar medium (Note 5) shall be added and incubated at 37°C for three days. Subsequently, the second overlaying agar medium (Note 6) shall be applied and incubated at 37°C for one day, and then plaque count shall be calculated. Additionally, 0.4 mL of the virus suspension for the neutralization test shall be inoculated onto each cultured cell plate and treated in the same manner (referred to as “virus control” within this section), and then the plaque count shall be calculated.

3.6.8.2.3 Judgment

The maximum dilution factor of the serum that results in a reduction of the plaque count to 50% or less, compared to the virus control shall be defined as the neutralizing antibody titer.

The neutralizing antibody titer in test group shall be at least 20-fold. In this case, the titer must be less than ten-fold in the control group.

4 Storage and expiry date

The expiry date shall be two years after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Virus growth medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Bovine serum | 20-50 mL | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 7.0 - 7.4.

Minimal amounts of antibiotics may be added.

Note 2 Hemagglutination antigen

Hemagglutination antigen, prepared using the Japanese encephalitis virus Nakayama-Yakken strain or a strain approved as suitable, shall have a hemagglutination titer at least 64-fold.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 3 | Borate-buffered saline containing bovine serum albumin | | |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 10.52 g | |
|  | Boric acid | 3.09 g | |
|  | Sodium hydroxide | 0.96 g | |
|  | Water | Residual quantity | |

After adding bovine serum albumin to achieve a concentration of 0.2 w/v%, adjust the pH to 9.0.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 4 | VAD solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.77 g | |
|  | Disodium hydrogen phosphate dodecahydrate | 5.68 g | |
|  | Sodium dihydrogen phosphate dihydrate | 40.56 g | |
|  | Water | Residual quantity | |

Mix with borate-buffered saline containing bovine serum albumin in equal volumes and adjust the pH to 6.0.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 5 | First overlaying agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Bovine serum | 20-50 mL | |
|  | Agar | 10 g | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 7.0 - 7.4.

Minimal amounts of antibiotics may be added.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 6 | Second overlaying agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Neutral red | 0.5 g | |
|  | Agar | 10 g | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 7.0 - 7.4.

Minimal amounts of antibiotics may be added.

**Transmissible Gastroenteritis, Porcine Epidemic Diarrhea Vaccine, Live, Seed**

1 Definition

This Vaccine, Live, Seed is a freeze-dried vaccine of mixed virus suspensions obtained by propagating attenuated Transmissible gastroenteritis virus and attenuated Porcine epidemic diarrhea virus that meet the Seed Lot Specifications in cell lines that meet the said specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Transmissible gastroenteritis virus

2.1.1.1 Name

Attenuated Transmissible gastroenteritis virus h-5 strain or strain approved as equivalent thereof

2.1.1.2 Properties

This virus strain shall be propagated with CPE in the following cells : primary or passaged cultured cells of swine kidney, or primary cultured cells of swine testis.

2.1.1.3 Master seed virus

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in MPK-IIIa cells or cultured cells approved as suitable, prepared in consecutive processes, and stored in storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than production of vaccines. The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.1.4 Working seed virus

2.1.1.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in MPK-IIIa cells or cultured cells approved as suitable.

The working seed virus shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.1.5 Production seed virus

2.1.1.5.1 Propagation, passage, and storage

The production seed virus shall be propagated in MPK-IIIa cells or cultured cells approved as suitable.

The production seed virus shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 Porcine epidemic diarrhea virus

2.1.2.1 Name

Attenuated Porcine epidemic diarrhea virus P-5V strain or strain approved as equivalent thereof

2.1.2.2 Properties

This virus strain shall be propagated with CPE in Vero cells without addition of trypsin.

2.1.2.3 Master seed virus

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in Vero cells or cultured cells approved as suitable, prepared in consecutive processes, and stored in storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than production of vaccines. The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.2.4 Working seed virus

2.1.2.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in Vero cells or cultured cells approved as suitable.

The working seed virus shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.2.5 Production seed virus

2.1.2.5.1 Propagation, passage, and storage

The production seed virus shall be propagated in Vero cells or cultured cells approved as suitable.

The production seed virus shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Transmissible gastroenteritis virus

2.2.1.1 Cultured cells

MPK-IIIa cells or cell lines approved as suitable for production shall be used.

2.2.1.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.1.3 Master cell seed

2.2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.1.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower.

The master cell seeds shall be tested as specified in 3.2.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.1.4 Working cell seed

2.2.1.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.1.2.

The working cell seeds shall be stored frozen at -70°C or lower.

The working cell seeds shall be tested as specified in 3.2.2.

2.2.1.5 Production cell seed

2.2.1.5.1 Propagation, passage, and storage

The production cell seeds shall be propagated using culture medium in 2.2.1.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3 if it needs to be stored.

2.2.2 Porcine epidemic diarrhea virus

2.2.2.1 Cultured cells

Vero cells or cell lines approved as suitable for production shall be used.

2.2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.2.3 Master cell seed

2.2.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining production cell seeds

The master cell seeds shall be propagated in a culture medium in 2.2.2.2, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master cell seeds shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower.

The master cell seeds shall be tested as specified in 3.2.1.

The master cell seeds shall not be passaged for purposes other than production or testing of vaccines. The master cell seeds shall not be passaged more than 20 times to obtain the production cell seeds.

2.2.4 Working cell seeds

2.2.4.1 Propagation, passage, and storage

The working cell seeds shall be propagated and passaged using culture medium in 2.2.2.2.

The working cell seeds shall be stored frozen at -70°C or lower.

The working cell seeds shall be tested as specified in 3.2.2.

2.2.5 Production cell seeds

2.2.5.1 Propagation, passage, and storage

The production cell seeds shall be propagated using culture medium in 2.2.2.2.

The production cell seeds shall be stored frozen at -70°C or lower when it needs to be stored.

The production cell seeds shall be tested as specified in 3.2.3 if it needs to be stored.

2.3 Bulk material

2.3.1 Transmissible gastroenteritis virus bulk material

2.3.1.1 Production cell seed cultivation

The cultured cells obtained in one session shall be regarded as an individual cultured cells. No abnormalities shall be detected prior to the inoculation of virus.

2.3.1.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.1.1. The filtrate of culture medium collected from individual cultured cells at the peak of virus propagation or supernatant after centrifugation shall serve as the bulk material.

The bulk material shall be tested as specified in 3.3.1 and 3.3.2.1.

2.3.2 Porcine epidemic diarrhea virus bulk material

2.3.2.1 Production cell seed cultivation

The cultured cells obtained in one session shall be regarded as an individual cultured cells. No abnormalities shall be detected prior to the inoculation of virus.

2.3.2.2 Virus cultivation

The production seed virus shall be cultivated in the cells in 2.3.2.1. The filtrate of culture medium collected from individual cultured cells at the peak of virus propagation or supernatant after centrifugation shall serve as the bulk material.

The bulk material shall be tested as specified in 3.3.1 and 3.3.2.2.

2.4 Final bulk

The bulk materials shall be mixed and adjusted by adding a stabilizer approved as appropriate and the mixture shall serve as the final bulk. A diluent approved as suitable can be added as needed.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product.

The final product shall be tested as specified in 3.4.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The test given in 1.4.2.1.1.1.2 of the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

When using swine-derived cells, the tests given in 1.1 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply to test Porcine reproductive and respiratory syndrome virus.

When using monkey-derived cells, the tests given in 1.1 and 3.1.1 of the Test for Freedom from Extraneous Viruses of the General Tests shall apply for Porcine reproductive and respiratory syndrome virus and Endogenous retrovirus (type C and D particles).

3.1.1.4.2.2 Individual test for freedom from specific viruses

For Classical swine fever virus, Porcine circovirus, Bovine viral diarrhea virus, Rotavirus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.1, 3.2.3, 3.2.4, 3.2.5, 3.2.7 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.7 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.1.8 Marker test

For Porcine epidemic diarrhea virus, if a marker is present in the production strain, the test shall proceed as follows:

3.1.1.8.1 Materials

3.1.1.8.1.1 Test materials

The test article shall be diluted ten-fold with a diluent (Note 1) and each level dilution shall serve as a test material.

3.1.1.8.1.2 Cultured cells

Vero cells or cells approved as suitable shall be cultured and the resulting monolayer cells shall be used.

3.1.1.8.1.3 Culture medium

Virus growth medium (Note 2) and trypsin-supplemented medium, which is prepared by adding crystalline trypsin at 2 μg per mL to the diluent, shall be used.

3.1.1.8.2 Test procedure

A 0.1 mL portion of each test material shall be inoculated into eight tubes (wells) of cultured cell that have been washed twice with diluent and the wells are divided into two groups. A 0.5 mL portion of virus growth medium or trypsin-supplemented culture medium shall be added to each tubes (well) of cell culture. Then the cultures shall be incubated under 5 vol% carbon dioxide gas at 37°C for seven days and observed.

3.1.1.8.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate TCID50.

The virus content measured in the virus growth medium shall be at least 100 times higher than the content measured in the trypsin-supplemented medium.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Tests on cell line

3.2.1 Tests on master cell seeds

3.2.1.1 Test for confirmation of cell properties

The test given in 2.1.4.2.1.1 in the Seed Lot Specifications shall apply.

3.2.1.2 Test for identification of the animal species of the cell

The test given in 2.1.4.2.1.2 in the Seed Lot Specifications shall apply.

3.2.1.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.1.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.1.5 Test for freedom from extraneous viruses

3.2.1.5.1 Common test for freedom from extraneous viruses

The tests given in 1.2, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.5.2 Test for freedom from specific viruses

3.2.1.5.2.1 General test for freedom from specific viruses

When using swine-derived cells, the tests given in 1.2 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply for Porcine reproductive and respiratory syndrome virus.

When using monkey-derived cells, the tests given in 1.2 and 3.1.1 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply for Porcine reproductive and respiratory syndrome virus and Endogenous retrovirus (type C and D particles).

3.2.1.5.2.2 Individual test for freedom from specific viruses

For Classical swine fever virus, Porcine circovirus, Bovine viral diarrhea virus, Rotavirus, Japanese encephalitis virus, and Rabies virus, the tests given in 1.2, 3.2.3, 3.2.4, 3.2.5, 3.2.7 and 3.2.9 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.2.1.6 Test for karyological (chromosomal) characterization

The test given in 2.1.4.2.1.6 in the Seed Lot Specifications shall apply.

3.2.1.7 Test for freedom from tumorigenicity

The test given in 2.1.4.2.1.7 in the Seed Lot Specifications shall apply.

3.2.2 Tests on working cell seeds

3.2.2.1 Test for confirmation of cell properties

The test given in 2.1.4.2.2.1 in the Seed Lot Specifications shall apply.

3.2.2.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.2.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2.3 Tests on production cell seeds

3.2.3.1 Test for confirmation of cell properties

The test given in 2.1.4.2.3.1 in the Seed Lot Specifications shall apply.

3.2.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.2.3.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.3 Tests on bulk material

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 Test for virus content

3.3.2.1 Swine transmissible gastroenteritis virus

3.3.2.1.1 Materials

3.3.2.1.1.1 Test materials

The test article shall be diluted ten-fold with virus growth medium (Note 1) or a diluent approved as suitable, and each level dilution shall serve as a test material.

3.3.2.1.1.2 Cultured cells

Primary cultured cells of swine testis or passaged cells shall be cultivated, and the resulting monolayer cells shall be used.

3.3.2.1.2 Test procedures

A 0.1 mL portion of the test material shall be inoculated into at least four tubes (wells) of cultured cells and allowed the adsorption at 37°C for 60 minutes. A 0.5 mL of virus growth medium shall be added, cultured at 37°C for five to seven days and observed.

3.3.2.1.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate TCID50.

The virus content of the test article shall be 107.5TCID50 or higher per mL.

3.3.2.2 Porcine epidemic diarrhea virus

3.3.2.2.1 Materials

3.3.2.2.1.1 Test materials

The test article shall be diluted ten-fold with virus growth medium or a diluent approved as suitable, and each level dilution shall serve as a test material.

3.3.2.2.1.2 Cultured cells

Vero cells shall be cultured, and the resulting monolayer cells shall be used.

3.3.2.2.2 Test procedure

A 0.1 mL portion of the test material shall be inoculated into at least four tubes (wells) of cultured cells and allowed the adsorption at 37°C for 60 minutes. A 0.5 mL portion of virus growth medium shall be added, cultured at 37°C for seven days and observed.

3.3.2.2.3 Judgment

The cultured cells in which CPE is observed shall be regarded as infected to calculate TCID50.

The virus content of the test article shall be 106.6TCID50 or higher per mL.

3.4 Tests on final product

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a liquid with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.4.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.4.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.4.4 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.5 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

However, this test may be omitted if it is conducted during the intermediate process including the bulk material.

3.4.6 Test for virus content

3.4.6.1 Transmissible gastroenteritis virus

When the test is performed as specified in 3.3.2.1, the virus content of the test sample shall be 105.5 TCID50 or higher per animal.

3.4.6.2 Porcine epidemic diarrhea virus

When the test is performed as specified in 3.3.2.2, the virus content of the test sample shall be 104.5 TCID50 or higher per animal.

3.4.7 Safety test

3.4.7.1 Materials

3.4.7.1.1 Injection materials

The test sample shall be used as an injection material.

3.4.7.1.2 Test animals

Swine aged about 2 to 3 months shall be used.

3.4.7.2 Test procedures

A group of four test animals shall be used as the test group and one test animal as the control group. The injection materials for ten doses and another for one dose shall be injected intramuscularly to two test animals each, and both the test and control groups shall then be bred together and observed for 14 days.

3.4.7.3 Judgment

No abnormalities shall be observed in the test or control group during the observation period.

3.4.8 Potency test

3.4.8.1 Materials

3.4.8.1.1 Test animals

Among the animals used in the test specified in 3.4.7, the swine that were injected with the dose intended for one animal of the injection material and the control swine shall be used.

3.4.8.1.2 Virus for neutralization test

Production strains shall be used.

3.4.8.1.3 Cultured cells

3.4.8.1.3.1 Transmissible gastroenteritis virus

Primary cultured cells of swine testis or passaged cells shall be cultivated, and the resulting monolayer cells shall be used.

3.4.8.1.3.2 Porcine epidemic diarrhea virus

Vero cells suspended in cell growth medium (Note 3) to achieve a concentration of 3 × 105.0 cells per mL (hereafter ‘Vero cell suspension’ within this section) shall be used.

3.4.8.2 Test procedures

On seven days after completing the test in 3.4.7, the swine that were injected with the injection material at the dose intended for one animal shall be further injected intramuscularly with another dose intended for one animal.

Sera obtained from each animal in the test and control groups seven days after the additional injection shall be used for the neutralization test.

3.4.8.2.1 Neutralization test for Swine transmissible gastroenteritis virus

The serum shall be heat-inactivated and then shall be diluted two-fold with virus growth medium. With 0.5 mL of each diluted serum, 0.5 mL of the virus suspension for the neutralization test with approximately 200 TCID50 per 0.1 mL shall be mixed and reacted at 37°C for 90 minutes.

A 0.1 mL of the mixture shall be inoculated into each of four tubes (wells) of cultured cells, allowed to stand for adsorption at 37°C for 90 minutes, and then 0.5 mL of virus growth medium shall be added. The cultured cells shall be incubated at 37°C for seven days and observed.

3.4.8.2.2 Neutralization test for Porcine epidemic diarrhea virus

The serum shall be heat-inactivated and then shall be diluted two-fold with virus growth medium. With 0.5 mL of each diluted serum, 0.5 mL of the virus suspension for the neutralization test with approximately 200 TCID50 per 0.1 mL shall be mixed and reacted at 37°C for 90 minutes.

A 0.1 mL portion of the mixture shall be dispensed into each of four tubes(wells) and 0.5 mL portion of Vero cell suspension shall be added. The cultured cells shall be incubated at 37°C for seven days and observed.

3.4.8.3 Judgment

The maximum dilution factor of serum at which CPE is observed in at least two tubes (wells) of cultured cells shall be defined as the neutralizing antibody titer.

In the test group, the neutralizing antibody titers shall be at least 128-fold against Transmissible gastroenteritis virus and at least 16-fold against Porcine epidemic diarrhea virus. In this case, the antibody titer in the control group must be less than two-fold against the both viruses.

4 Storage and expiry date

The expiry date shall be two years after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Diluent |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 7.2 - 7.6.

Minimal amounts of antibiotics may be added.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 2 | Virus growth medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Bovine or goat serum | 20-50 mL | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 7.0 -7.6.

Bovine or goat serum to be used shall be negative for neutralizing antibodies against Transmissible gastroenteritis virus and Porcine epidemic diarrhea virus.

Minimal amounts of antibiotics may be added.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 3 | Cell growth medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Bovine or goat serum | 50-150 mL | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 7.0 -7.6.

Bovine or goat serum to be used shall be negative for neutralizing antibodies against Transmissible gastroenteritis virus and Porcine epidemic diarrhea virus.

Minimal amounts of antibiotics may be added.

**Swine Erysipelas Vaccine (adjuvant), Inactivated, Seed**

1 Definition

Swine Erysipelas Vaccine (adjuvant), Inactivated, Seed is a vaccine of inactivated culture bacteria medium of *Erysipelothrix rhusiopathiae* that meets the Seed Lot Specifications, by adding an aluminum gel adjuvant.

2 Production methods

2.1 Bacterial strain used for production

2.1.1 Name

*Erysipelothrix rhusiopathiae* Tama-96 strain (serotype 2) or strain approved as equivalent thereof

2.1.2 Properties

When inoculated into susceptible swine, the bacterial strain can induce swine erysipelas.

2.1.3 Master seed bacteria

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a plate medium (Note 1) or a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.4 Working seed bacteria

2.1.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a plate medium or a medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.5 Production seed bacteria

2.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a plate medium or a medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when they need to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if they need to be stored.

2.2 Materials for production

2.2.1 Medium

A medium approved as suitable for production shall be used.

2.3 Bulk material

2.3.1 Cultivation

The production seed bacteria shall be inoculated into a broth medium (Note 2) or a medium approved as suitable and the culture, and the culture shall be further inoculated into broth medium 2 (Note 3) or medium approved as suitable, and the resulting broth shall serve as cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.2 Bulk material preparation

The cultured bacterial medium shall be adjusted for its pH level and inactivated by addition of formalin, or the broth shall be inactivated by addition of formalin and enriched by a method approved as suitable and then adjusted for its concentration. The resulting medium shall serve as bulk material.

The bulk material shall be tested as specified in 3.3.

2.4 Final bulk

The bulk material shall be mixed with an aluminum gel adjuvant or the mixture shall be adjusted for its pH level and concentration, and this shall serve as the final bulk.

In this case, preservatives approved as suitable may be added.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.4.

3 Test methods

3.1 Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

The tests given in 1.4.2.4.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

3.1.1.2.1 Culturing method using broth medium

The test given in the Sterility Test of the General Tests must not show any bacterial growths other than *Erysipelothrix rhusiopathiae*.

3.1.1.2.2 Culturing method using nutrient agar slant medium

3.1.1.2.2.1 Medium

A nutrient agar slant medium shall be used.

3.1.1.2.2.2 Test procedures

A 0.5 mL portion each of the test article shall be inoculated into four tubes of nutrient agar slant medium and cultured at 37°C for seven days.

3.1.1.2.2.3 Judgment

No bacterial growth other than *Erysipelothrix rhusiopathiae* must be observed.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from Contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.1.3 Tests on production seed bacteria

3.1.3.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.2 Tests on cultured bacterial medium

The test given in both 3.2.1 and 3.2.3.1 or in both 3.2.2 and 3.2.3.2 shall be conducted.

3.2.1 Test for freedom from contaminant microorganisms

3.2.1.1 Culturing method using broth medium

The test given in the Sterility Test 1 of the General Tests must not show any bacterial growth other than *Erysipelothrix rhusiopathiae*.

3.2.1.2 Culturing method using nutrient agar slant medium

The test given in 3.1.1.2.2 shall apply.

3.2.2 Staining test

3.2.2.1 Test procedures

An appropriate amount of the test article shall be smeared onto a slide glass, allowed to dry, heat-fixed by passing through a flame, and then stained using Gram staining to prepare the specimen. Note that the specimen shall be prepared in such a manner as to show an appropriate distribution of bacteria suitable for observation.

3.2.2.2 Judgment

When observing under a microscope magnifying at least 100 times, no bacterial growth other than *Erysipelothrix rhusiopathiae* must be observed.

3.2.3 Viable count test

3.2.3.1 Viable count test 1

3.2.3.1.1 Materials

3.2.3.1.1.1 Test materials

The test article shall be serially diluted ten-fold with nutrient broth, and each level dilution shall serve as a test material.

3.2.3.1.1.2 Medium

A nutrient agar medium shall be used.

3.2.3.1.2 Test procedures

A 1 mL portion of the test material shall be dispensed onto each of two Petri dishes, cultured using the mixed dilution pour plate culture method at 37°C for 48 hours, and the resulting number of *Erysipelothrix rhusiopathiae* colonies shall be counted.

3.2.3.1.3 Judgment

The viable count shall be calculated from the mean colony counts in dilutions at each level, dilution factor and quantity of inoculum per medium. The viable count in the test article shall be 1.5 × 109 or higher per mL.

3.2.3.2 Viable count test 2

3.2.3.2.1 Materials

The test article shall be used.

3.2.3.2.2 Test procedures

At least 2 mL of the test article shall be measured for absorbance at a wavelength of 530 nm as the bacterial amount of *Erysipelothrix rhusiopathiae* contained in the test article.

3.2.3.2.3 Judgment

The absorbance in the test article shall be at least 0.25.

3.3 Tests on bulk material

The test given in both 3.3.1.1 and 3.3.2 or in 3.3.1.2 shall be conducted.

3.3.1 Inactivation test

3.3.1.1 Inactivation test 1

The test given in the Sterility Test 1 of the General Tests shall apply.

Provided, the judgment shall be made three days after the inoculation.

3.3.1.2 Inactivation test 2

3.3.1.2.1 Materials

3.3.1.2.1.1 Test materials

The test article shall be used as the inoculation material.

3.3.1.2.1.2 Medium

A blood agar medium (Note 4) or a plate medium approved as suitable shall be used.

3.3.1.2.2 Test procedures

A 1.0 mL portion of the inoculation material shall be mixed with blood agar medium using the mixed dilution pour plate culture method, or a 0.1 mL portion of the inoculation material shall be spread over a blood agar medium and then cultured at 35ºC to 39°C for 18 to 24 hours. As a positive control, a medium containing the strain used for production of *Erysipelothrix rhusiopathiae* shall be tested in the same procedure.

3.3.1.2.3 Judgment

No bacterial growth shall be observed on the medium inoculated with the test material, and a bacterial growth of *Erysipelothrix rhusiopathiae* shall be observed on medium inoculated with a medium containing the strain used for production of *Erysipelothrix rhusiopathiae*.

3.3.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4 Tests on final product

If the test given in 3.4.7 does not apply, the test given in 3.4.8 shall apply.

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be liquid with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.4.2 Test for pH

For conducting test for pH, when the test given in the Test for pH of the General Tests shall apply, the pH shall have an intrinsic value.

3.4.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.4 Test for thimerosal content

For products added with thimerosal, the test given in the Test for Thimerosal Content of the General Tests shall apply.

3.4.5 Test for formalin content

For products added with formalin, when the test given in the Test for Formalin Content of the General Tests apply, the formalin content must be not more than 0.2 vol% unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.4.6 Test for aluminum content

When the test given in the Test for Aluminum Content of the General Tests shall apply, the aluminum content shall be not more than 2.5 mg per mL unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.4.7 Test for freedom from abnormal toxicity

The test given in the Test for Freedom from Abnormal Toxicity of the General Tests shall apply.

3.4.8 Toxicity Limit Test

The test given in the Toxicity Limit Test 1 of the General Tests shall apply. However, the injection volume of the test sample shall be 0.3 mL, and the weight shall be measured on four days post-injection.

3.4.9 Potency test

The test specified in 3.4.9.1 or 3.4.9.2 shall be conducted.

3.4.9.1 Two times immunization

3.4.9.1.1 Materials

3.4.9.1.1.1 Injection materials

The test sample shall be used as an injection material.

3.4.9.1.1.2 Test animals

Mice aged 5 weeks shall be used.

3.4.9.1.1.3 Challenge medium

Freeze-dried *Erysipelothrix rhusiopathiae* Fujisawa strain, or any strain with equivalent toxicity, shall be inoculated into a broth medium and cultured at 37°C for 14 to 20 hours. The resulting medium shall be diluted with nutrient broth to achieve a viable count of 103 per mL, and this diluted broth shall serve as the challenge medium.

3.4.9.1.2 Test procedures

A group of ten test animals shall be used as the test group and another ten test animals as the control group.

A dose of 0.5 mL of the injection material shall be injected subcutaneously into the inner thigh of the animals in the test group twice at intervals of two weeks. On two weeks after the second injection, a dose of 0.1 mL of the challenge medium shall be injected subcutaneously into the inner thigh of the animals in the test and control groups for challenge and the animals shall be observed for seven days.

3.4.9.1.3 Judgment

The survival rate of the test group must be 70% or higher. In this case, the mortality rate of the control group must be 90% or higher.

3.4.9.2 One time immunization

3.4.9.2.1 Materials

3.4.9.2.1.1 Injection materials

The test sample shall be diluted three-fold with phosphate-buffered saline to serve as the injection material.

3.4.9.2.1.2 Test animals

Mice aged 4 weeks shall be used.

3.4.9.2.1.3 Challenge medium

Freeze-dried *Erysipelothrix rhusiopathiae* Fujisawa strain, or any strain with equivalent toxicity, shall be inoculated into a challenge medium and cultured at 37°C for 14 to 20 hours. The resulting medium shall be diluted with nutrient broth to achieve a viable count of 104 per mL, and this diluted broth shall serve as the challenge medium.

3.4.9.2.2 Test procedures

A group of ten test animals shall be used as the test group and another ten test animals as the control group.

A dose of 0.1 mL of the injection material shall be injected subcutaneously into the inner thigh of the animals in the test group. On the 10th day of the injection, a dose of 0.1 mL of the challenge medium shall be injected subcutaneously into the inner thigh of the animals in the test and control groups for challenge and the animals shall be observed for ten days.

3.4.9.2.3 Judgment

The survival rate of the test group must be 70% or higher. In this case, the mortality rate of the control group must be 90% or higher.

4 Storage and expiry date

The expiry date shall be three years and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Plate medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 30 g | |
|  | Proteose peptone No. 3 | 10 g | |
|  | Polysorbate 80 | 1 mL | |
|  | Agar | 10 g | |
|  | Water | Residual quantity | |

Adjust the pH to 7.4 - 7.8, sterilize by autoclaving at 121°C for 15 minutes.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 2 | Broth medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 30 g | |
|  | Proteose peptone No. 3 | 10 g | |
|  | Polysorbate 80 | 1 mL | |
|  | Water | Residual quantity | |

Adjust the pH to 7.4 - 7.8, sterilize by autoclaving at 121°C for 15 minutes.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 3 | Broth medium 2 |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 30 g | |
|  | Polysorbate 80 | 1 mL | |
|  | Water | Residual quantity | |

Adjust the pH to 8.0 and autoclave at 121°C for 15 minutes. After cooling, add 10 to 50 mL of bovine serum sterilized by filtration.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 4 | Blood agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Casein peptone | 15 g | |
|  | Soybean peptone | 5 g | |
|  | Sodium chloride | 5 g | |
|  | Agar | 15 g | |
|  | Water | Residual quantity | |

Adjust the pH to 7.1 - 7.5, sterilize by autoclaving at 121°C for 15 minutes. After cooling to about 50°C, add sheep blood to achieve a concentration of 5 vol%.

**Porcine Actinobacillus pleuropneumoniae (Serotype 1, 2, 5・ recombinant toxin) Infection Vaccine (adjuvant), Inactivated, Seed**

1 Definition

This Vaccine (adjuvant), Inactivated, Seed is a vaccine, made by mixing the inactivated culture bacteria medium of *Actinobacillus pleuropneumoniae* (hereafter “App”) serotypes 1, 2, and 5 that meets the Seed Lot Specifications and nontoxic mutant App toxins (rApx I, rApx II and rApx III) produced in recombinant *Escherichia coli* that meets the specifications, and by adding an aluminum gel adjuvant.

2 Production methods

2.1 Bacterial strain used for production

2.1.1 App1 serotype

2.1.1.1 Name

App 41-1 strain (serotype 1) or strain approved as equivalent thereof

2.1.1.2 Properties

This bacterial serotype produces cytotoxins Apx I and Apx II. When intratracheally inoculated into susceptible swine, the strain can induce pleuropneumonia.

2.1.1.3 Master seed bacteria

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on chocolate agar medium (Note 1) or medium approved as suitable, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

2.1.1.4 Working seed bacteria

2.1.1.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a medium approved as suitable.

The working seed bacteria shall be stored frozen at -60°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.1.5 Production seed bacteria

2.1.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable.

The production seed bacteria shall be stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 App2 serotype

2.1.2.1 Name

App SHP-1 strain (serotype 2) or strain approved as equivalent thereof

2.1.2.2 Properties

This bacterial serotype produces cytotoxins Apx II and Apx III. When intratracheally inoculated into susceptible swine, the strain can induce pleuropneumonia.

2.1.2.3 Master seed bacteria

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on chocolate agar medium or medium approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

2.1.2.4 Working seed bacteria

2.1.2.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a medium approved as suitable.

The working seed bacteria shall be stored frozen at -60°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.2.5 Production seed bacteria

2.1.2.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable.

The production seed bacteria shall be stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.3 App5 serotype

2.1.3.1 Name

App Ng-2 strain (serotype 5) or strain approved as equivalent thereof

2.1.3.2 Properties

This bacterial serotype produces cytotoxins Apx I and Apx II. When intratracheally inoculated into susceptible swine, the strain can induce pleuropneumonia.

2.1.3.3 Master seed bacteria

2.1.3.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on chocolate agar medium or medium approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

2.1.3.4 Working seed bacteria

2.1.3.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a medium approved as suitable.

The working seed bacteria shall be stored frozen at -60°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.3.5 Production seed bacteria

2.1.3.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable.

The production seed bacteria shall be stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.4 rApx I-producing recombinant *Escherichia coli*

2.1.4.1 Name

rApx I-producing recombinant *Escherichia coli* ESN1113 strain or strain approved as equivalent thereof

2.1.4.2 Properties

This bacterial strain shall be resistant to ampicillin and tetracycline and contain a plasmid pSN110 with the *apxIA* gene derived from the chromosomal DNA of the App 41-1 strain. It has been identified by SDS-polyacrylamide gel electrophoresis that when cultured in a medium containing isopropyl thiogalactoside (hereafter “IPTG”), the strain produces rApx I proteins.

2.1.4.3 Master seed bacteria

2.1.4.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on an LB-Amp agar medium (Note 2) or medium approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

2.1.4.4 Working seed bacteria

2.1.4.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in an LB-Amp agar medium or a medium approved as suitable.

The working seed bacteria shall be stored frozen at -60°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.4.5 Production seed bacteria

2.1.4.5.1 Propagation and storage

The production seed bacteria shall be propagated in an LB-Amp agar medium approved as suitable.

The production seed bacteria shall be stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.5 rApx II-producing recombinant *Escherichia coli*

2.1.5.1 Name

rApx II-producing recombinant *Escherichia coli* ESN1074 strain or strain approved as equivalent thereof

2.1.5.2 Properties

This bacterial strain shall be resistant to ampicillin and tetracycline and contain a plasmid pSN63 with the *apxIIA* gene derived from the chromosomal DNA of the App Ng-2 strain. It has been identified by SDS-polyacrylamide gel electrophoresis that when cultured in a medium containing IPTG, the strain produces rApx II proteins.

2.1.5.3 Master seed bacteria

2.1.5.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on an LB-Amp agar medium or medium approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

2.1.5.4 Working seed bacteria

2.1.5.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in an LB-Amp agar medium or a medium approved as suitable.

The working seed bacteria shall be stored frozen at -60°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.5.5 Production seed bacteria

2.1.5.5.1 Propagation and storage

The production seed bacteria shall be propagated in an LB-Amp agar medium approved as suitable.

The production seed bacteria shall be stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.6 rApx III-producing recombinant *Escherichia coli*

2.1.6.1 Name

rApx III-producing recombinant *Escherichia coli* ESN1166 strain or strain approved as equivalent thereof

2.1.6.2 Properties

This bacterial strain shall be resistant to ampicillin and tetracycline and contain a plasmid pSN148 with the *apxIIIA* gene derived from the chromosomal DNA of the App SHP-1 strain. It has been identified by SDS-polyacrylamide gel electrophoresis that when cultured in a medium containing IPTG, the strain produces rApx III proteins.

2.1.6.3 Master seed bacteria

2.1.6.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on an LB-Amp agar medium or medium approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

2.1.6.4 Working seed bacteria

2.1.6.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in an LB-Amp agar medium or a medium approved as suitable.

The working seed bacteria shall be stored frozen at -60°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.6.5 Production seed bacteria

2.1.6.5.1 Propagation and storage

The production seed bacteria shall be propagated in an LB-Amp agar medium approved as suitable.

The production seed bacteria shall be stored frozen at -60°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Medium

2.2.1.1 App serotypes 1, 2 and 5

Agar and broth medium approved as suitable for production shall be used.

2.2.1.2 Recombinant *Escherichia coli*

Agar and broth medium approved as suitable for production shall be used.

2.3 Bulk material

2.3.1 App1 serotypes 1, 2 and 5

2.3.1.1 Cultivation

The production seed bacteria of each App strain shall be inoculated onto an agar medium and cultured. The cultured bacteria shall then be inoculated into a broth medium for further culturing. This cultured broth shall be further inoculated into a broth medium, and the resulting broth shall serve as the cultured bacterial medium of each strain.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.1.2 Inactivation and bacterial harvesting

The cultured bacterial medium for each strain shall be inactivated with formalin or an inactivator approved as suitable, bacterial cells obtained from centrifugation shall be homogeneously suspended in phosphate-buffered saline, and thimerosal or a preservative approved as suitable shall be added. The resulting mixture shall serve as inactivated bacterial solution of each strain.

The inactivated bacterial solution shall be tested as specified in 3.3.

2.3.1.3 Concentration adjustment

The inactivated bacterial solution for each strain shall be diluted with phosphate-buffered saline to contain the required amount of the total bacterial count, and thimerosal or a preservative approved as suitable shall be added. This mixture shall serve as bulk material of each strain.

The bulk material shall be tested as specified in 3.6.1.

2.3.2 rApx I, II and III protein

2.3.2.1 Cultivation

The production seed bacteria of each recombinant *Escherichia coli* strain shall be inoculated onto an agar medium and cultured. The cultured bacteria shall then be inoculated into a broth medium for further culturing. This cultured broth shall be further inoculated into a broth medium, and the resulting broth shall serve as the cultured bacterial medium of each strain.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.2.2 Protein expression culture

The bacterial medium, obtained by culturing the broth medium of each strain with the addition of IPTG, shall serve as the cultured expression medium for each strain. The cultured expression medium shall be tested as specified in 3.4.

2.3.2.3 Bacterial harvesting and homogenization

The cultured expression medium for each strain shall be centrifuged, the bacterial cells shall be resuspended in a buffer solution approved as suitable, at approximately 1/100 of the volume of the expression medium, lysozyme shall be added, and the mixture shall be agitated. To this, a buffer approved as suitable shall be added, the mixture shall be processed with a high-pressure homogenizer and the resulting medium shall serve as homogenized bacterial medium of each strain.

The homogenized bacterial medium shall be tested as specified in 3.5.

2.3.2.4 rApx protein recovery and solubilization

Homogenized bacterial medium shall be centrifuged and each rApx protein shall be resuspended in sterilized distill water about 1/250 volume of cultured expression medium. To this mixture, a solubilizer approved as suitable shall be added to allow solubilization and the mixture shall be centrifuged. The obtained supernatant shall serve as bulk material of each rApx protein.

The bulk material shall be tested as specified in 3.6.

2.4 Final bulk

2.4.1 App bulk

The bulk material of each App strain shall be diluted with phosphate-buffered saline and aluminum hydroxide gel to adjust the total bacterial count to the required concentration. To this, formalin and either thimerosal or a preservative approved as suitable shall be added, and this mixture shall serve as App bulk of each strain.

2.4.2 rApx bulk

After adjusting the protein concentrations, the bulk materials of rApx I, II, and III proteins shall be mixed, phosphate-buffered saline and aluminum hydroxide gel shall be added to sensitize the mixture, allowing each rApx protein to adsorb onto the aluminum hydroxide gel. The aluminum hydroxide gel adsorbed with rApx protein shall be recovered by centrifugation and the pellet shall be resuspended in phosphate-buffered saline in a volume equal to the initial volume of the bulk material. To this, formalin and either thimerosal or a preservative approved as suitable shall be added, and this mixture shall serve as rApx bulk.

2.4.3 Final bulk

A mixture of rApx bulk and App bulks of three strains shall serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product. The final product shall be tested as specified in 3.7.

3 Test methods

3.1 Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

3.1.1.1.1 Identification test for App strains

The tests given in 1.4.2.4.1.1 in the Seed Lot Specifications shall apply.

3.1.1.1.2 Identification test for recombinant *Escherichia coli* strains

The test given in 1.4.2.5.2.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

3.1.1.2.1 Test for freedom from contaminant microorganisms for App strains

The test given in the Sterility Test 1 of the General Tests shall apply.

3.1.1.2.2 Test for freedom from contaminant microorganisms for recombinant *Escherichia coli* strains

The test given in the Sterility Test 1 of the General Tests must not show any bacterial growths other than recombinant *Escherichia coli*.

3.1.1.3 Test for Stability Confirmation of Recombinant Gene

3.1.1.3.1 Test for Stability Confirmation of Recombinant Gene for recombinant *Escherichia coli*

The test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.1.3 Tests on production seed bacteria

The bacteria to be stored shall be tested as specified below.

3.1.3.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.2 Tests on cultured bacterial medium

3.2.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.3 Tests on inactivated bacterial solution

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.2 Inactivation test

3.3.2.1 Materials

The test article and a medium approved as suitable for production shall be used.

3.3.2.2 Test procedures

A 0.5 mL portion each of the test article shall be inoculated into 2 tubes or more of 20 mL medium and cultured at 37°C for two days.

3.3.2.3 Judgment

No bacterial growth must be observed.

3.3.3 Total count test

3.3.3.1 Materials

The test article shall be diluted appropriately with phosphate-buffered saline to serve as the test material.

3.3.3.2 Test procedures

The absorbance of the test material shall be measured using a spectrophotometer.

3.3.3.3 Judgment

The total count shall be calculated from the standard curve, the measured absorbance and the dilution factor of the test article.

The total count in the test article shall be 3 × 1010 or higher per mL.

3.4 Tests on cultured expression medium

3.4.1 Identification test on expressed proteins

3.4.1.1 Materials

The test material shall be prepared by adding an equal volume of sample buffer (Note 3) to the expression medium of each *Escherichia coli* strain and boiling the mixture.

3.4.1.2 Test procedures

After loading 10 μL of the test material onto a 10 w/v% SDS-polyacrylamide gel for electrophoresis, Coomassie blue staining shall be performed to observe the electrophoresis image.

3.4.1.3 Judgment

A clear band shall be observed at a molecular weight of approximately 105 kDa for the test article of ESN1113 and ESN1074 strains and at a molecular weight of approximately 120 kDa for the test article of ESN1166.

3.5 Tests on the homogenized bacterial medium

3.5.1 Confirmation test on homogenization

3.5.1.1 Materials

The homogenized bacterial medium shall serve as the test material.

3.5.1.2 Test procedures

A 0.01 mL portion of the test article shall be smeared within a 1 cm2 area on a slide glass, allowed to dry, heat-fixed by passing through a flame, and then stained using Giemsa or Gram staining.

3.5.1.3 Judgment

Almost all bacteria shall be observed microscopically showing disruption image.

3.6 Tests on bulk material

3.6.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6.2 Identification test

3.6.2.1 Materials

The bulk material of each rApx protein shall be diluted with either distilled water or 2 to 4 mol/L urea solution to achieve a protein concentration of 300 μg per mL, and the mixture shall serve as the test material.

3.6.2.2 Test procedures

3.4.1.2 shall apply to the test procedure.

3.6.2.3 Judgment

A clear band shall be observed at a molecular weight 105 kDa for the test material of rApx I and rApx II proteins and at a molecular weight 120 kDa for the test material of rApx III protein.

3.6.3 Test for protein content

3.6.3.1 Materials

The bulk material of each rApx protein shall be serially diluted two-fold to achieve a protein concentration of 20 to 200 μg per mL and the dilution shall serve as the test material.

3.6.3.2 Test procedures

Lowry method shall be used for measurement, and the protein content in 1 mL of the bulk material shall be calculated.

3.6.3.3 Judgment

The protein content in the bulk material for each rApx protein shall be at least 4 mg per mL.

3.7 Tests on final product

3.7.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be liquid with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.7.2 Test for pH

When the test given in the Test for pH of the General Tests shall apply, the pH shall have an intrinsic value.

3.7.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.7.4 Test for thimerosal content

The test given in the Test for Thimerosal Content of the General Tests shall apply.

3.7.5 Test for formalin content

When the test given in the Test for Formalin Content of the General Tests applies, the formalin content must be not more than 0.25 vol%.

3.7.6 Test for aluminum content

When the test given in the Test for Aluminum Content of the General Tests shall apply, the aluminum content shall be not more than 3.6 mg per mL.

3.7.7 Toxicity limit test

The test given in the Toxicity Limit Test 1 of the General Tests shall apply. However, the weight shall be measured on four days post-injection.

3.7.8 Potency test

3.7.8.1 Materials

3.7.8.1.1 Injection materials

The test sample shall be diluted 20-fold with phosphate-buffered saline, or a dilute solution approved as suitable to serve as the injection material.

3.7.8.1.2 Test animals

Mice aged about 3 weeks shall be used.

3.7.8.1.3 Challenge medium

App1 serotype AH-1 strain, App2 serotype SHP-1 strain, App5 serotype Ng-2 strain, or an alternative strain approved as suitable for each, shall each be transferred into a separate testing medium 1 (Note 4) and cultured at 37°C for 16 hours. Colonies shall be picked and inoculated into testing medium 2 (Note 5), cultured with shaking at 37°C for 3 to 6 hours and then shall serve as challenge medium for each strain.

3.7.8.2 Test procedures

A group of at least 70 test animals shall be used as the test group and at least another 70 test animals as the control group.

A dose of 0.5 mL of the injection material shall be injected intraperitoneally into the test animals in the test group.

On the 14th day of the injection, both the test and control groups shall be divided into seven groups of at least 10 animals to form a total of 14 groups. The challenge media for serotypes 1 and 2 shall be diluted in a two-step process with heart infusion medium and the serotype 5 challenge medium in a three-step process with heart infusion medium. These diluted bacterial broths shall then be further diluted ten-fold with a 10 w/v% mucin solution, 0.5 mL of each shall be injected intraperitoneally into the test and control groups for challenge and observed clinically for seven days.

3.7.8.3 Judgment

For the challenge bacterial load that resulted in at least 80% mortality in the control group for each challenge strain, the tolerability and survival of the test group must be 80% or higher.

4 Storage and expiry date

The expiry date shall be three years and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Chocolate agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Heart infusion agar | 40 g | |
|  | Water | Residual quantity | |

After dissolving the medium with aid of heat, sterilize by autoclaving at 121°C for 15 minutes. After cooling to approximately 80°C, add horse defibrinated blood to achieve the concentration of 10 vol%.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 2 | LB-Amp agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Casein peptone | 10 g | |
|  | Yeast extract | 5 g | |
|  | Sodium chloride | 5 g | |
|  | Water | Residual quantity | |

After dissolving the medium with aid of heat, adjust the pH to 7.4 - 7.6, sterilize by autoclaving at 121°C for 15 minutes. After cooling to 56°C, add ampicillin so that the final potency is 250 μg per mL.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 3 | Sample buffer |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | 0.25 mol/L Tris HCl buffer, pH 6.8 | 500 mL | |
|  | 20 w/v% sodium lauryl sulfate | 260 mL | |
|  | Glycerin | 200 mL | |
|  | Dithiothreitol | 1.54 g | |
|  | Bromophenol blue | 1.00 g | |
|  | Water | Residual quantity | |

Note 4 Testing medium 1

This is prepared by autoclaving heart infusion agar at 121°C for 15 minutes, allowing it to cool and then adding 5 vol% of filtered-sterilized inactivated poultry serum and 1 vol% of a 0.1 w/v% β-nicotinamide adenine dinucleotide (β-NAD) solution.

Note 5 Testing medium 2

This is prepared by autoclaving heart infusion medium at 121°C for 15 minutes, allowing it to cool and then adding 5 vol% of filtered-sterilized inactivated poultry serum and 1 vol% of a 0.1 w/v% β-NAD solution.

**Mycoplasma hyopneumoniae Infection Vaccine (adjuvant), Inactivated, Seed**

1 Definition

Mycoplasma hyopneumoniae Infection Vaccine (adjuvant), Inactivated, Seed is a vaccine of inactivated culture bacteria medium of *Mycoplasma hyopneumoniae* that meets the Seed Lot Specifications, or its concentrated solution, by adding an aluminum gel adjuvant.

2 Production methods

2.1 Bacterial strain used for production

2.1.1 Name

*Mycoplasma hyopneumoniae* BQ14 strain or strain approved as equivalent thereof

2.1.2 Properties

The biological properties shall conform to the *Mycoplasma hyopneumoniae* standard strain.

2.1.3 Master seed bacteria

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a broth medium approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

2.1.4 Working seed bacteria

2.1.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a broth medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.5 Production seed bacteria

2.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a broth medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Medium

A broth medium approved as suitable for production shall be used.

2.3 Bulk material

2.3.1 Cultivation

Either the working seed bacteria or the production seed bacteria shall be inoculated in a medium, cultured and passaged, then further inoculated to a medium. The resulting medium shall serve as cultured bacterial medium. The cultured bacterial medium may be semi-filtered.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.2 Bulk material preparation

The cultured bacterial medium shall be inactivated by an addition of formalin or an inactivator approved as appropriate and shall serve as the bulk material. In this case, the bulk material may be inactivated and then concentrated, or preservatives approved as suitable may be added.

The bulk material shall be tested as specified in 3.3.

2.4 Final bulk

The bulk material shall be mixed with an adequate amount of an adjuvant, and this shall serve as the final bulk. In this case, the pH value may be adjusted or a preservative approved as suitable may be added.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.4.

3 Test methods

3.1. Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

The tests given in 1.4.2.4.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.1.3 Tests on production seed bacteria

3.1.3.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.2 Tests on cultured bacterial medium

The test given in all of 3.2.1, 3.2.2 and 3.2.3, both 3.2.1 and 3.2.4, or both 3.2.1 and 3.2.2 shall be conducted.

3.2.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.2.2 Identification test

The test given in the tests below shall apply unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.2.2.1 Materials

The test article or the test article serially diluted ten-fold with tryptose phosphate broth shall serve as the test material.

3.2.2.2 Test procedures

Using agar plates (Note 1) and filter paper disks impregnated with rabbit anti-*Mycoplasma hyopneumoniae* serum(Note 2), mycoplasma growth inhibition test (Note 3) shall be conducted on the test material. The medium inoculated with the test material shall be cultured microaerophilically at 37°C for 14 days.

3.2.2.3 Judgment

Clear inhibition zones must be observed around the filter paper disks in either of the plates with the test material.

3.2.3 Total count test

3.2.3.1 Materials

The test article shall be centrifuged, the resulting sediment shall be suspended in an adequate amount of phosphate-buffered saline (Note 4), and the suspension shall serve as the test material.

3.2.3.2 Test procedures

The absorbance of the test material shall be measured using a spectrophotometer.

3.2.3.3 Judgment

The total count in the test article calculated using the standard curve and the absorbance shall be 1.4 × 108 or higher per mL.

3.2.4 Viable count test

3.2.4.1 Materials

The semi-filtrated medium shall serve as the inoculation material.

3.2.4.2 Test procedures

The inoculation material shall be serially diluted ten-fold with BHL medium (Note 5) dispensed at 1.8 mL each into test tubes, cultured at 37°C for 14 days and observed.

3.2.4.3 Judgment

Any medium that turns yellow shall be considered Mycoplasma-positive and the CCU shall be calculated. The viable count in the test article shall be 5× 106 CCU or higher per mL.

3.3 Tests on bulk material

The test given in both 3.3.1 and 3.3.2 or all of 3.3.1, 3.3.2, 3.3.3 and 3.3.4 shall be conducted.

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.3.2 Inactivation test

The test given in 3.3.2.1, 3.3.2.2 or 3.3.2.3 shall be conducted.

3.3.2.1 Inactivation test 1

3.3.2.1.1 Test materials

The test article shall be used.

3.3.2.1.2 Test procedures

The test material shall be inoculated into a broth medium (Note 6) and cultured at 37°C for 14 days. If a color change to yellow is observed in a medium, the medium shall be smeared onto an agar plate, cultured microaerophilically at 37°C for 14 days, and observed for the presence of *Mycoplasma hyopneumoniae* growth.

3.3.2.1.3 Judgment

No growth of *Mycoplasma hyopneumoniae* must be observed.

3.3.2.2 Inactivation test 2

3.3.2.2.1 Test materials

The test article shall be used.

3.3.2.2.2 Test procedures

The test article shall be inoculated to a BHL medium, mixed thoroughly, and cultured for 14 days. In this case, a BHL medium inoculated with BHL medium and one inoculated with cultured bacterial medium before inactivation shall be observed as the control in a similar manner.

3.3.2.2.3 Judgment

No growth of *Mycoplasma hyopneumoniae* must be observed in the media inoculated with the test article and BHL medium. Growth of *Mycoplasma hyopneumoniae* must be observed in the media inoculated with cultured bacterial medium before inactivation.

3.3.2.3 Inactivation test 3

3.3.2.3.1 Materials

3.3.2.3.1.1 Test materials

The test article shall be centrifuged, and the sediment shall serve as the test material.

3.3.2.3.1.2 Medium

A broth medium approved as suitable for growth of *Mycoplasma hyopneumoniae* shall be used.

3.3.2.3.2 Test procedures

The test material shall be inoculated to broth medium and cultured at 36°C for 14 days. On Day 7 of inoculation, the medium shall be subcultured at 36°C for 14 days to observe any medium color changes.

3.3.2.3.3 Judgment

No color change to yellow must be observed in the medium.

3.3.3 Identification test

3.3.3.1 Materials

3.3.3.1.1 Test materials

The test materials shall be the bulk material diluted 50-fold, positive control and molecular weight marker approved as suitable.

3.3.3.2 Test procedures

Electrophoresis shall be performed for each test material using a 5 to 10 w/v% polyacrylamide gel, followed by transfer onto a membrane. After blocking the transferred membrane, the membrane shall be allowed for reaction with anti-*Mycoplasma hyopneumoniae* polyclonal antibody solution. After washing the membrane with a buffer solution and blocking, it shall be allowed to react with labeled antibody solution. After washing the membrane, it shall be allowed to react with the substrate solution, the reaction shall be stopped once the sufficient level of color development is confirmed, and the membrane shall be dried.

3.3.3.3 Judgment

When compared with the positive control and molecular weight marker, a band specific to *Mycoplasma hyopneumoniae* must be observed in the bulk material.

3.3.4 Test for antigen content

3.3.4.1 Materials

3.3.4.1.1 Test materials

The reference bulk material approved as suitable and bulk material shall be solubilized and used as test materials.

3.3.4.2 Test procedures

Rabbit anti-*Mycoplasma hyopneumoniae* antibodies approved as suitable shall be diluted and coated in each well of an ELISA plate. After washing the plate, the test material serially diluted two-fold shall be added to each well for reaction at 37°C for 1 hour. The plate shall be washed, peroxidase-labelled rabbit anti-*Mycoplasma hyopneumoniae* antibodies shall be added for a reaction at 37°C for 1 hour. After washing the plate, tetramethylbenzidine substrate solution shall be added for a reaction for 30 minutes, and the reaction shall be quenched with sulfuric acid, and absorbance shall be measured. The content shall be calculated using a parallel line assay in comparison to the reference bulk material.

3.3.4.3 Judgment

The antigen content in the test article shall fall within 500 to 5,000 antigen units per mL.

3.4 Tests on final product

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be liquid with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.4.2 Test for pH

When the test given in the Test for pH of the General Tests shall apply, the pH shall have an intrinsic value.

3.4.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4.4 Test for thimerosal content

For products added with thimerosal, the test given in the Test for Thimerosal Content of the General Tests shall apply.

3.4.5 Test for formalin content

For products added with formalin, when the test given in the Test for Formalin Content of the General Tests applies, the formalin content must be not more than 0.2 vol%.

3.4.6 Test for aluminum content

When the test given in the Test for Aluminum Content of the General Tests shall apply, the aluminum content shall fall within 0.6 to 0.9 mg per mL unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.4.7 Test for freedom from abnormal toxicity

The test given in the Test for Freedom from Abnormal Toxicity of the General Tests shall apply.

3.4.8 Potency test

The test specified in 3.4.8.1 or 3.4.8.2 shall be conducted.

3.4.8.1 Test using double‐antibody sandwich enzyme-linked immunosorbent assay (hereafter ‘double‐antibody sandwich ELISA’)

3.4.8.1.1 Materials

3.4.8.1.1.1 Injection materials

The test sample shall be used as an injection material.

3.4.8.1.1.2 Test animals

Mice aged 6 to 7 weeks shall be used.

3.4.8.1.1.3 Antigen for double‐antibody sandwich ELISA

Polysorbate 20-extracted antigen (Note 7) shall be used.

3.4.8.1.2 Test procedures

A group of 20 test animals shall be used as the test group and five test animals as the control group.

A dose of 1.0 mL of the injection material shall be injected intraperitoneally into the test animals. Sera obtained from each animal in the test and control groups four weeks after injection shall be used for the double-antibody sandwich ELISA.

Sera from the test and control groups and reference positive serum 1 (Note 8) shall be diluted tenfold with a diluent (Note 9) and then serially diluted twofold with the same diluent. A 100 μL of each of these serum dilutions shall be added to each well of an immunosorbent plate 1 (Note 10) and the diluent alone shall be added to separate wells to serve as the serum control. After incubating at 4°C for 18 hours, the plate shall be washed three times with the washing solution (Note 11). Next, 100 μL of labeled antibody 1 (Note 12) shall be added to each well, followed by incubation at 37°C for 90 minutes. The plate shall then be washed three times with the washing solution. Subsequently, 100 μL of the substrate solution 1 (Note 13) shall be added to each well, followed by incubation at 37°C for 30 minutes. Then, 50 μL of 3 mol/L sodium hydroxide solution shall be added to each well to quench the reaction and the absorbance of each well shall be measured at a wavelength of 405 nm.

3.4.8.1.3 Judgment

The maximum dilution factor of serum that exhibits the absorbance equal to or higher than the mean absorbance in each well of the serum control + 0.5 shall be defined as the antibody titer.

The antibody titer in at least 70% of the test animals shall be at least 2,560-fold or higher. In this case, the antibody titer must be 320-fold or lower in all control animals. Furthermore, the antibody titer in reference positive serum 1 must be between 2,560 and 5,120-fold.

3.4.8.2 Test using indirect ELISA

3.4.8.2.1 Materials

3.4.8.2.1.1 Injection materials

The test sample shall be used as an injection material.

3.4.8.2.1.2 Test animals

Mice aged 6 to 7 weeks shall be used.

3.4.8.2.1.3 Antigen for indirect ELISA

Polysorbate 20-extracted antigens shall be used.

3.4.8.2.2 Test procedures

A group of 20 test animals shall be used as the test group and five test animals as the control group.

A dose of 1.0 mL of the injection material shall be injected intraperitoneally into the test animals. Sera obtained from each animal in the test and control groups four weeks after injection shall be used for the indirect ELISA.

Sera from the test and control groups and the reference positive serum 2 (Note 14) shall be diluted 100-fold with a diluent and then serially diluted twofold with the same diluent. A 50 μL of each of these serum dilutions shall be added to each well of the immunosorbent plate 2 (Note 15) and the diluent alone shall be added to separate wells to serve as the serum control. After incubating at 4°C overnight, the plate shall be washed five times with the washing solution. Next, 100 μL of labeled antibody 2 (Note 16) shall be added to each well, followed by incubation at 37°C for 1 hour. The plate shall then be washed five times with the washing solution. Subsequently, 100 μL of substrate solution 2 (Note 17) shall be added to each well, followed by incubation at 37°C for 20 minutes. Then, 50 μL of 0.32w/v% sodium fluoride solution shall be added to each well to stop the reaction, and the absorbance of each well shall be measured at a wavelength of 415 nm.

3.4.8.2.3 Judgment

The cutoff value shall be defined as twice the mean absorbance of each well in the serum control and the maximum dilution factor of serum that exhibits the absorbance equal to or higher than the cutoff value as the antibody titer.

The antibody titer in at least 80% of the test animals shall be at least 6,400-fold or higher. In this case, the antibody titer must be 400-fold or lower in all control animals. Furthermore, the antibody titer in reference positive serum 2 must be between 3,200 and 6,400-fold.

4 Storage and expiry date

The expiry date shall be two years after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

Note 1 Agar plate

Add 4.0 g of purified agar to 1,000 mL of the broth medium in Note 5, dissolve with aid of heat and then sterilize by autoclaving at 115°C for 15 minutes. Cool to approximately 50°C, mix with the additive previously sterilized by filtration as described in Note 6, and then dispense the mixture into a Petri dish with a diameter of 90 mm.

Note 2 Rabbit anti-*Mycoplasma hyopneumoniae* serum

This is serum obtained from immunizing rabbits with the manufacturing strain, aliquoted into small volumes and stored at -20°C. In growth inhibition tests conducted with a bacterial medium at a concentration of approximately 106 cells, the sera should exhibit an inhibition zone of about 5 mm.

Note 3 Mycoplasma growth inhibition test

Drop about 0.05 mL of the test material at one end of an agar plate and tilt the plate to allow the test material to downflow toward the other end. After the surface has dried, place a filter paper disk, which has been previously impregnated with immune serum of rabbit anti-*Mycoplasma hyopneumoniae* and dried, at the center of the flow line. Incubate the plate microaerophilically at 37°C for 14 days. Upon observation after incubation, the antisera diffused from the disk shall inhibit the growth of *Mycoplasma hyopneumoniae* colonies in the surrounding area, forming an inhibition zone. No growth inhibition for Mycoplasma species other than *Mycoplasma hyopneumoniae* must be observed.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 4 | Phosphate-buffered saline |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.0 g | |
|  | Disodium hydrogen phosphate dodecahydrate | 2.9 g | |
|  | Potassium dihydrogen phosphate | 0.2 g | |
|  | Potassium chloride | 0.2 g | |
|  | Water | Residual quantity | |

Adjust the pH to 6.8 - 7.2, sterilize by autoclaving at 121°C for 20 minutes or by filtration.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 5 | BHL medium |  |  |
|  | Hanks’ solution\*1 | 500 mL | |
|  | Lactalbumin | 2 g | |
|  | Brucella broth | 5.8 g | |
|  | 25 w/v% fresh yeast extract | 50 mL | |
|  | Porcine serum | 200 mL | |
|  | Aminobenzyl penicillin | 125 mg | |
|  | Phenol red | 0.1 g | |
|  | Water | 250 mL | |

Adjust the pH with 5 mol/L sodium hydroxide solution to 7.6 - 8.0, sterilize by filtration through a 450 nm membrane filter and then further sterilize by filtration through a 220 nm filter.

|  |  |  |  |
| --- | --- | --- | --- |
| \*1 | In 1,000 mL |  |  |
|  | Sodium chloride | 8 g | |
|  | Potassium chloride | 0.4 g | |
|  | Disodium hydrogen phosphate dodecahydrate | 0.15 g | |
|  | Potassium dihydrogen phosphate | 0.06 g | |
|  | Glucose | 4 g | |
|  | Water | Residual quantity | |

Adjust the pH with 2.5 mol/L sodium hydroxide solution to 7.3 - 7.7, filter through a 450 nm membrane filter and then further sterilize by filtration through a 220 nm filter.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 6 | Broth medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Basal medium |  |  |
|  | Brucella broth | 5.8 g | |
|  | Hanks’ solution powder | 4.9 g | |
|  | Lactalbumin hydrolysate | 2.0 g | |
|  | Water | 750 mL | |
|  | Additive |  |  |
|  | Heat-inactivated porcine serum | 200 mL | |
|  | 5 w/v% yeast extract solution | 50 mL | |
|  | 2.5 w/v% thallous acetate solution | 4 mL | |
|  | Cloxacillin sodium hydrate | 100 mg | |
|  | or |  |  |
|  | Ampicillin sodium | 250 mg | |

After dissolving the basal medium with aid of heat, sterilize by autoclaving at 115°C for 15 minutes. After cooling, mix with the additives that have been previously sterilized by filtration, and adjust the pH of the mixture to 7.5 - 7.7.

Note 7 Polysorbate 20-extracted antigen

Centrifuge the shaking cultured broth of the production strain or an antigenically equivalent strain to collect the bacteria, suspend in diluent, and agitate at 4°C for 24 hours to wash the bacterial cells. After suspending the washed cells in Tris buffer solution (1) to achieve a protein concentration of 1 mg/mL, add an equal volume of Tris buffer solution (2) containing 2 vol% polysorbate 20, and then heat the mixture at 37°C for 30 minutes while agitating. After adding an equal volume of diethyl ether to the supernatant from the centrifugation and agitating, completely remove the ether layer.

(1) Tris buffer solution

Dissolve 3.03 g of Tris aminomethane and 14.61 g of sodium chloride in water to make a total volume of 1,000 mL.

(2) Tris buffer solution containing 2 vol% polysorbate 20

Mix 20 mL of polysorbate 20 with 980 mL of Tris buffer solution.

Note 8 Reference positive serum 1

Serum from mice inoculated with the *Mycoplasma hyopneumoniae* J strain or an immunogenetically equivalent strain, adjusted to achieve a double-antibody sandwich ELISA antibody titer within the range of 2,560 to 5,120-fold and obtained by freezing or freeze-drying.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 9 | Diluent |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.0 g | |
|  | Potassium chloride | 0.2 g | |
|  | Potassium dihydrogen phosphate | 0.2 g | |
|  | Disodium hydrogen phosphate | 1.15 g | |
|  | Water | Residual quantity | |

Sterilize by autoclaving at 121°C for 15 minutes or by filtration.

Note 10 Immunosorbent plate 1

After diluting rabbit immune serum against *Mycoplasma hyopneumoniae* J strain or an immunogenetically equivalent strain (Note 18) 100-fold with carbonate buffer solution (Note 19), add 100 μL of the dilution to each well of a 96-well microplate and incubate at 4°C for 18 hours. Subsequently, wash the plate three times with the washing solution. Add 100 μL of 0.1 w/v% gelatin solution (Note 20) to each well and incubate at 4°C for 18 hours. Furthermore, after washing the plate three times with the washing solution, dilute the polysorbate 20-extracted antigens with a diluent to achieve a protein concentration of 12.5 μg/mL. Add 100 μL of the dilution to each well, incubate at 4°C for 18 hours, and then wash the plate three times with the washing solution. Use the resulting plate as the immunosorbent plate 1.

Note 11 Washing solution

Mix 0.5 mL of polysorbate 20 with 1,000 mL of diluent.

Note 12 Labeled antibody 1

Dilute anti-mouse IgG antibody labeled with alkaline phosphatase to the optimal concentration using a diluent.

Note 13 Substrate solution 1

Dissolve 100 mg of p-nitrophenyl phosphate disodium in 100 mL of the substrate buffer 1 (Note 21).

Note 14 Reference positive serum 2

Serum from mice inoculated with the *Mycoplasma hyopneumoniae* J strain or an immunogenetically equivalent strain, adjusted to achieve an indirect ELISA antibody titer within the range of 3,200 to 6,400-fold and obtained by freeze-drying.

Note 15 Immunosorbent plate 2

Dilute the polysorbate 20-extracted antigens with a carbonate buffer solution to achieve a protein concentration of 25 μg/mL, add 100 μL of the dilution to each well of a 96-well plate and incubate at 37°C for 1 hour. Subsequently, wash the plate three times with the washing solution. Add 100 μL of bovine serum albumin solution (Note 22) to each well, incubate at 37°C for 90 minutes, wash the plate three times with the washing solution and use the plate as Immunosorbent plate 2.

Note 16 Labeled antibody 2

Dilute anti-mouse IgG antibody labeled with peroxidase to the optimal concentration using the bovine serum albumin solution.

Note 17 Substrate solution 2

Dissolve 45 mg of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt in 1 mL of water, then add 0.15 mL of this solution and 0.05 mL of 5 vol% hydrogen peroxide to 10 mL of the substrate buffer solution 2 (Note 23).

Note 18 Rabbit immune serum

Serum from rabbits inoculated with the *Mycoplasma hyopneumoniae* J strain or an immunogenetically equivalent strain, and it exhibits an inhibition zone of at least 3 mm in diameter in growth inhibition tests.

Note 19 Carbonate buffer solution

Solution A: Dissolve 5.3 g of sodium carbonate in water to make 1,000 mL.

Solution B: Dissolve 4.2 g of sodium bicarbonate in water to make 1,000 mL.

Mix equal volumes of Solutions A and B and adjust the pH to 9.6.

Note 20 0.1 w/v% gelatin solution

Dissolve 1.0 g of gelatin in 1,000 mL of diluent.

Note 21 Substrate buffer solution 1

Dissolve 0.049 g of magnesium chloride hexahydrate and 96 mL of diethanolamine in water, adjust the pH to 9.8 with 5 mol/L hydrochloric acid and making the total volume to 1,000 mL

Note 22 Bovine serum albumin solution

This is prepared by dissolving 10 g of bovine serum albumin in 1,000 mL of diluent.

Note 23 Substrate buffer solution 2

Solution A: Dissolve 21 g of citric acid monohydrate in water to make 1,000 mL.

Solution B: Dissolve 29.4 g of trisodium citrate dihydrate in water to make 1,000 mL.

Mix equal volumes of Solutions A and B and adjust the pH to 4.0.

**Porcine Actinobacillus pleuropneumoniae (Serotype 1, 2, 5) Infection, Swine Erysipelas Vaccine (Oil adjuvant), Inactivated, Seed**

1 Definition

This Vaccine (Oil adjuvant), Inactivated, Seed is a vaccine, formulated by inactivating and mixing concentrated supernatant of culture bacteria medium of *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 5 that meet the Seed Lot Specifications, further mixing with inactivated antigens obtained through alkalization and extraction from cultured *Erysipelothrix rhusiopathiae* that meets the specifications, and by adding an oil adjuvant.

2 Production methods

2.1 Bacterial strain used for production

2.1.1 *Actinobacillus pleuropneumoniae* serotype 1

2.1.1.1 Name

*Actinobacillus pleuropneumoniae* serotype 1 Y-1-1 strain or strain approved as equivalent thereof

2.1.1.2 Properties

The bacterial serotype produces cytotoxins Apx I and Apx II. When inoculated into susceptible swine, the strain can induce pleuropneumonia.

2.1.1.3 Master seed bacteria

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a plate and broth media both approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.1.4 Working seed bacteria

2.1.1.4.1 Propagation, passage, and storage

The working seed bacteria shall be propagated and passaged in a plate and broth media both approved as suitable.

The working seed bacteria shall be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.1.5 Production seed bacteria

2.1.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a plate and broth media both approved as suitable.

The production seed bacteria shall be stored freeze-dried at 5°C or lower when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 *Actinobacillus pleuropneumoniae* serotype 2

2.1.2.1 Name

*Actinobacillus pleuropneumoniae* serotype 2 G-4-1 strain or strain approved as equivalent thereof

2.1.2.2 Properties

The bacterial serotype produces cytotoxins Apx II and Apx III. When inoculated into susceptible swine, the strain can induce pleuropneumonia.

2.1.2.3 Master seed bacteria

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a plate and broth media both approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.2.4 Working seed bacteria

2.1.2.4.1 Propagation, passage, and storage

The working seed bacteria shall be propagated and passaged in a plate and broth media both approved as suitable.

The working seed bacteria shall be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.2.5 Production seed bacteria

2.1.2.5.1 Propagation and storage

The production seed bacteria shall be propagated in a plate and broth media both approved as suitable.

The production seed bacteria shall be stored freeze-dried at 5°C or lower when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.3 *Actinobacillus pleuropneumoniae* serotype 5

2.1.3.1 Name

*Actinobacillus pleuropneumoniae* serotype 5 E-3-1 strain or strain approved as equivalent thereof

2.1.3.2 Properties

The bacterial serotype produces cytotoxins Apx I and Apx II. When inoculated into susceptible swine, the strain can induce pleuropneumonia.

2.1.3.3 Master seed bacteria

2.1.3.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a plate and broth media both approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.3.4 Working seed bacteria

2.1.3.4.1 Propagation, passage, and storage

The working seed bacteria shall be propagated and passaged in a plate and broth media both approved as suitable.

The working seed bacteria shall be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.3.5 Production seed bacteria

2.1.3.5.1 Propagation and storage

The production seed bacteria shall be propagated in a plate and broth media both approved as suitable.

The production seed bacteria shall be stored freeze-dried at 5°C or lower when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.4 *Erysipelothrix rhusiopathiae*

2.1.4.1 Name

*Erysipelothrix rhusiopathiae* Kyoto strain (serotype 2) or strain approved as equivalent thereof

2.1.4.2 Properties

When inoculated into susceptible swine, the bacterial strain can induce swine erysipelas.

2.1.4.3 Master seed bacteria

2.1.4.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a plate and broth media both approved as suitable and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored freeze-dried at 5°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.4.4 Working seed bacteria

2.1.4.4.1 Propagation, passage, and storage

The working seed bacteria shall be propagated and passaged in a plate and broth media both approved as suitable.

The working seed bacteria shall be stored freeze-dried at 5°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.4.5 Production seed bacteria

2.1.4.5.1 Propagation and storage

The production seed bacteria shall be propagated in a plate and broth media both approved as suitable.

The production seed bacteria shall be stored freeze-dried at 5°C or lower when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Serotypes of *Actinobacillus pleuropneumoniae*

2.2.1.1 Medium

Plate and broth media both approved as suitable for production shall be used.

2.2.2 *Erysipelothrix rhusiopathiae*

2.2.2.1 Medium

Plate and broth media both approved as suitable for production shall be used.

2.3 Bulk material

2.3.1 Bulk material for each serotype of *Actinobacillus pleuropneumoniae*

2.3.1.1 Cultivation

The production seed bacteria cultured in a plate medium shall be separately inoculated into a separate broth medium for culturing, further inoculated into a broth medium and the resulting product shall serve as cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.1 and 3.2.2.1.

2.3.1.2 Supernatant collection

The cultured bacterial medium shall be centrifuged, and the resulting supernatant shall be collected.

The supernatant shall be tested as specified in 3.3.

2.3.1.3 Antigen solution

The supernatant concentrated in a method approved as suitable shall be used as the antigen solution.

The antigen solution shall be tested as specified in 3.4.1.1 and 3.4.2.

2.3.1.4 Inactivation

The antigen solution shall be inactivated by an addition of formalin and shall serve as the bulk material.

The bulk material shall be tested as specified in 3.5.1 and 3.5.2.1.

2.3.2 *Erysipelothrix rhusiopathiae* bulk material

2.3.2.1 Cultivation

The production seed bacteria cultured in a plate medium shall be inoculated in a broth medium for culturing, then further inoculated to a broth medium. The resulting product shall serve as cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.1 and 3.2.2.2.

2.3.2.2 Antigen extraction

The bacteria obtained through centrifugation from the cultured bacterial medium shall be resuspended in a 0.01 mol/L sodium hydroxide solution, agitated overnight at 2 to 5°C, and then centrifuged to collect the supernatant, which shall serve as the antigen solution.

The antigen solution shall be tested as specified in 3.4.1.2.

2.3.2.3 Inactivation

The antigen solution shall be inactivated by an addition of formalin and shall serve as the bulk material.

The bulk material shall be tested as specified in 3.5.1 and 3.5.2.2.

2.4 Final bulk

The bulk material shall be mixed, its concentration shall be adjusted with a dilute solution approved as suitable, an oil adjuvant approved as suitable shall be added, and this shall serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.6.

3 Test methods

3.1 Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

The tests given in 1.4.2.4.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests shall apply. It should be noted, however, that no bacterial growth other than *Erysipelothrix rhusiopathiae* must be observed in strain for production of *Erysipelothrix rhusiopathiae*.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.1.3 Tests on production seed bacteria

3.1.3.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.2 Tests on cultured bacterial medium

3.2.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test 1 of the General Tests shall apply. It should be noted, however, that no bacterial growth other than *Erysipelothrix rhusiopathiae* must be observed in the cultured broth of *Erysipelothrix rhusiopathiae*.

3.2.2 Viable count test

3.2.2.1 Each serotype of *Actinobacillus pleuropneumoniae*

3.2.2.1.1 Materials

3.2.2.1.1.1 Test materials

The test article shall be serially diluted ten-fold with phosphate-buffered saline, and the dilution at each level shall serve as a test material.

3.2.2.1.1.2 Medium

A test medium 1 (Note 1) or a medium approved as suitable shall be used.

3.2.2.1.2 Test procedures

A 0.5 mL or 1 mL aliquot of the test material shall be inoculated onto at least two plates using the mixed dilution pour plate culture method and incubated at 37°C for 18 hours. After incubation, the number of colonies that have developed shall be counted.

3.2.2.1.3 Judgment

The viable count shall be calculated from the mean colony counts in dilutions at each level, dilution factor, and quantity of inoculum per medium. The viable count in the test article shall be 5.0 × 108 pr higher per mL.

3.2.2.2 *Erysipelothrix rhusiopathiae*

3.2.2.2.1 Materials

3.2.2.2.1.1 Test materials

The test article shall be serially diluted ten-fold with phosphate-buffered saline, and the dilution at each level shall serve as a test material.

3.2.2.2.1.2 Medium

A test medium 2 (Note 2) or a medium approved as suitable shall be used.

3.2.2.2.2 Test procedures

A 0.5 mL or 1 mL aliquot of the test material shall be inoculated onto at least two plates using the mixed dilution pour plate culture method and incubated at 37°C for 48 hours. After incubation, the number of colonies that have developed shall be counted.

3.2.2.2.3 Judgment

The viable count shall be calculated from the mean colony counts in dilutions at each level, dilution factor, and quantity of inoculum per medium. The viable count in the test article shall be 5.0 × 108 or higher per mL.

3.3 Tests on supernatant

3.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.4 Tests on the antigen solution

3.4.1 Enzyme-linked immunosorbent assay (hereafter “ELISA”) test for titer measurement

3.4.1.1 Serotypes of *Actinobacillus pleuropneumoniae*

3.4.1.1.1 Materials

3.4.1.1.1.1 Test materials

The test article shall be sterilized using a 220 nm membrane filter, and this shall serve as the test material.

3.4.1.1.1.2 Reference antigen for ELISA antigen titer measurement

The reference antigen for ELISA antigen titer measurement 1 (Note 3) shall be the antigen matched with the serotype of the test article for ELISA antigen titer measurement.

3.4.1.1.1.3 Serum for ELISA antigen titer measurement

Positive serum for ELISA antigen titer measurement (Note 4) and negative serum for ELISA antigen titer measurement (Note 5) - shall be the serum matched with the serotype of the test article for ELISA antigen titer measurement.

3.4.1.1.2 Test procedures

The test material and the reference antigen for ELISA antigen titer measurement 1 shall be diluted 100-fold with carbonate buffer solution (Note 6), then serially diluted twofold. An aliquot of 100 μL dilution from each level shall be added to U-shaped wells of the ELISA plate and allowed to adsorb overnight at 2ºC to 5°C. Following a wash with the solution for dilution and washing (Note 7), 250 μL of 2 w/v% bovine serum albumin solution (Note 8) shall be added to each well and incubated at 37°C for 1 hour. After another wash, 100 μL of the positive and negative sera for ELISA antigen titer measurement diluted 100-fold with the solution for dilution and washing shall be added to each well and incubated at 37°C for 1 hour. The plate shall be washed, 100 μL of labeled antibody 1 (Note 9) shall be added to each well and incubated at 37°C for 1 hour. After a wash, 100 μL of substrate solution (Note 10) shall be added to each well. The plate shall be shielded from light and allowed to react at 30°C for 30 minutes, and after this, 50 μL of the stopping solution (Note 11) shall be added to stop the reaction. The absorbance shall be measured at two wavelengths, the main wavelength of 490 nm, and secondary wavelength of 630 nm, and the differences shall be treated as the ELISA value.

3.4.1.1.3 Judgment

The highest dilution factor of the antigen that yields an ELISA value of not less than 1.0 shall be defined as the ELISA antigen titer.

For each serotype, the ELISA antigen titer of the test article must be at least 800 times higher than that in the positive serum for ELISA antigen titer measurement and no more than 100 times that of the titer in the negative serum for the measurement.

In this case, the ELISA antigen titer of the reference antigen for ELISA titer measurement 1 shall be in a range of 800 to 1600 times in the positive serum for ELISA antigen titer measurement and no more than 100 times in the negative serum for the measurement.

3.4.1.2 *Erysipelothrix rhusiopathiae*

3.4.1.2.1 Materials

3.4.1.2.1.1 Test materials

The test article shall be sterilized using a 220 nm membrane filter, and this shall serve as the test material.

3.4.1.2.1.2 Reference antigen for ELISA antigen titer measurement

The reference antigen for ELISA antigen titer measurement 2 (Note 12) shall be used.

3.4.1.2.1.3 Antibody for ELISA antigen titer measurement

Monoclonal antibody for ELISA antigen titer measurement (Note 13) shall be used.

3.4.1.2.2 Test procedures

The test material and the reference antigen for ELISA antigen titer measurement 2 shall be diluted ten-fold with carbonate buffer solution, then serially diluted two-fold. An aliquot of 100 μL dilution from each stage shall be added to U-shaped wells of the ELISA plate and allowed to adsorb overnight at 2 to 5°C. Following a wash with the solution for dilution and washing, 250 μL of 2 w/v% bovine serum albumin solution shall be added to each well and incubated at 37°C for 1 hour. After a wash, 100 μL of monoclonal antibody for ELISA antigen measurement shall be added to each well and further incubated at 37°C for 1 hour. The plate shall be washed, 100 μL of labeled antibody 2 (Note 14) shall be added to each well and incubated at 37°C for 1 hour. After a wash, 100 μL of substrate solution shall be added to each well. The plate shall be shielded from light and allowed to react at 30°C for 30 minutes, and after this, 50 μL of the stopping solution shall be added to stop the reaction. The absorbance shall be measured at two wavelengths, the main wavelength of 490 nm and secondary wavelength of 630 nm, and the differences shall be treated as the ELISA value.

3.4.1.2.3 Judgment

The highest dilution factor of the antigen that yields an ELISA value of not less than 0.4 shall be defined as the ELISA antigen titer (U/mL).

The ELISA antigen titer in the test article shall be 80 U or higher per mL.

In this case, the ELISA antigen titer of the reference antigen for ELISA titer measurement 2 shall be in a range of 80 to 160 U per mL.

3.4.2 Lipopolysaccharide (hereinafter “LPS”) content measurement test

3.4.2.1 Materials

3.4.2.1.1 Test materials

The test article shall be adjusted to contain ELISA antigen titer 800 times, and this shall serve as the test material.

3.4.2.2 Test procedures

LPS content shall be determined by measuring keto-deoxyoctonate (hereinafter “KDO”).

To 0.1 mL of the test material, 0.1 mL of 0.25 mol/L sulfuric acid shall be added and boiled for 20 minutes. The mixture shall be cooled to ambient temperature and 0.1 mL of 0.1 mol/L periodic acid solution shall be added to allow reaction at 55°C for 10 minutes. To this, 0.4 mL of 4 w/v% sodium arsenite solution in hydrochloric acid (Note 15) and 1.6 mL of 0.6 w/v% thiobarbituric acid solution shall be added. After boiling for 20 minutes, 2 mL of butanol-concentrated hydrochloric acid solution (Note 16) shall be immediately added and the mixture shall be agitated. The mixture shall be centrifuged at 1,720 *G* for 10 minutes and collect color-developed butanol layer. KDO standard solution shall be processed as directed above. The absorbance in the collected butanol layer shall be measured using a spectrophotometer (at wavelength of 552 nm or 508 nm).

3.4.2.3 Judgment

A calibration curve shall be prepared from the absorbance of the standard KDO solution, and LPS content shall calculated from the absorbance of the test material.

When the test article is adjusted to have ELISA antigen titers 800 times, its LPS content shall be no more than 200 μg per mL.

3.5 Tests on bulk material

3.5.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.5.2 Inactivation test

3.5.2.1 Serotypes of *Actinobacillus pleuropneumoniae*

3.5.2.1.1 Materials

The test article and the test medium 1 shall be used.

3.5.2.1.2 Test procedures

Onto the surface of each of five plate media, 0.1 mL of the test article shall be smeared and incubated at 37°C for 24 hours and then presence of colonies shall be observed.

3.5.2.1.3 Judgment

No growth of *Actinobacillus pleuropneumoniae* shall be observed.

3.5.2.2 *Erysipelothrix rhusiopathiae*

3.5.2.2.1 Materials

The test article and the test medium 2 shall be used.

3.5.2.2.2 Test procedures

Onto the surface of each of five plate media, 0.1 mL of the test article shall be smeared and incubated at 37°C for 48 hours.

3.5.2.2.3 Judgment

No growth of *Erysipelothrix rhusiopathiae* must be observed.

3.6 Tests on final product

3.6.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.6.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6.3 Test for formalin content

The test sample processed by a method approved as suitable shall be used as the test material. When the test given in the Test for Formalin Content of the General Tests applies, the formalin content must be not more than 0.1 vol%.

3.6.4 Safety test

3.6.4.1 Materials

3.6.4.1.1 Injection materials

The test sample shall be used as an injection material.

3.6.4.1.2 Test animals

Swine aged about 30 days shall be used.

3.6.4.2 Test procedures

A group of 3 test animals shall be used as the test group and one test animal as the control group.

A dose of 1 mL of the injection material shall be injected intramuscularly into the cervical region of the test animals and both the test and control groups shall be observed for two weeks.

3.6.4.3 Judgment

No clinical abnormalities shall be observed in the test animals during the observation period. Even if transient fever, lethargy, loss of appetite, small erythema in the injection site are noted post-injection, these must resolve within three days.

3.6.5 Potency test

3.6.5.1 Potency test for Porcine *Actinobacillus pleuropneumoniae* infection

3.6.5.1.1 Materials

3.6.5.1.1.1 Injection materials

The test sample shall be used as an injection material.

3.6.5.1.1.2 Test animals

Guinea pigs weighing about 350 g shall be used.

3.6.5.1.1.3 Antigen for complement fixation test (hereinafter “CF”)

CF antigens (Note 17) prepared separately with *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 5 shall be used.

3.6.5.1.2 Test procedures

A group of 8 test animals shall be used as the test group and two test animals as the control group.

A dose of 0.2 mL of the injection material shall be injected intramuscularly into the thigh of the test animals twice at intervals of three weeks. Sera obtained from each animal in the test and control groups two weeks after the second injection shall be used for CF.

Sera from the test and control groups, the reference positive serum 1 (Note 18) and the reference negative serum 1 (Note 19) shall be diluted four-fold with gelatin veronal buffer (Note 20, hereinafter “GVB”) and then serially diluted two-fold with the GVB. After adding 25 μL of CF antigen to each 25 μL of the dilutions, 50 μL of complement prepared at two units shall be added and the mixture treated at 4°C overnight. After mixing equal volumes of 3 vol% sheep blood cell suspension and three units of hemolysin, 50 μL of sensitized blood cells that were incubated at 37°C for 30 minutes shall be added and incubated at 37°C for 30 minutes.

3.6.5.1.3 Judgment

The maximum dilution factor of serum that results in 50% inhibition of hemolysis shall be defined as the antibody titer.

In the test group, the geometric mean of the antibody titer against each CF antigen must be 32-fold or higher. In this case, the antibody titer against all CF antigens must be fourfold or lower in the control group. In addition, the antibody titer against each CF antigen shall be 32-fold or lower in the reference positive serum 1 and four-fold or lower in the reference negative serum 1.

3.6.5.2 Potency test for swine erysipelas

3.6.5.2.1 Materials

3.6.5.2.1.1 Test animals

The animals used in the test specified in 3.6.5.1 shall be used.

3.6.5.2.1.2 Antigen for ELISA

The antigen for ELISA antibody titer measurement for *Erysipelothrix rhusiopathiae* (Note 21) shall be used.

3.6.5.2.2 Test procedures

Sera obtained from each animal in the test specified in 3.6.5.1.2 shall be used for ELISA.

Sera from the test and control groups, the reference positive serum 2 (Note 22) and the reference negative serum 2 (Note 23) shall be diluted 100-fold with dilution and washing solution and serially diluted two-fold, and 100 μL of the mixtures shall be added to wells of the antigen adsorption plate (Note 24) and incubated at 37°C for 1 hour. After washing the plate with the solution for dilution and washing, 100 μL each of labeled antibody 3 (Note 25) shall be added to each well and incubated at 37°C for 1 hour. After a wash, 100 μL of substrate solution shall be added to each well. The plate shall be shielded from light and allowed to react at 30°C for 30 minutes, and after this, 50 μL of the quenching solution shall be added to stop the reaction. The absorbance shall be measured at two wavelengths, the main wavelength of 490 nm and secondary wavelength of 630 nm, and the differences shall be treated as the ELISA value.

3.6.5.2.3 Judgment

The highest dilution factor of the serum that yields an ELISA value of not less than 0.5 shall be defined as the ELISA antibody titer.

In the test group, the geometric mean of the ELISA antibody titer shall be 300-fold or higher. In this case, the antibody titer must be less than 100-fold in the control group. Furthermore, the antibody titer must be between 400 and 800-fold in the reference positive serum 2 and less than 100-fold in the reference negative serum 2.

4 Storage and expiry date

The expiry date shall be one year and nine months after the manufacturing, unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Test medium 1 |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Casein peptone | 15 g | |
|  | Soy peptone | 5 g | |
|  | Sodium chloride | 5 g | |
|  | Agar | 12 g | |
|  | Water | Residual quantity | |

After dissolving the medium with aid of heat, adjust the pH to 7.1 - 7.5, sterilize by autoclaving at 121°C for 20 minutes.

After cooling to approximately 50°C, add 2 mL of filtered-sterilized 2 w/v% reduced β-nicotinamide adenine dinucleotide (hereinafter “β-NAD”).

|  |  |  |  |
| --- | --- | --- | --- |
| Note 2 | Test medium 2 |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Soybean-casein digest agar broth | 40 g | |
|  | Polysorbate 80 | 1 mL | |
|  | Water | Residual quantity | |

After dissolving the medium, sterilize by autoclaving at 121°C for 20 minutes.

Note 3 Reference antigen for ELISA antigen titer measurement 1

When performing immunoblotting with monoclonal antibodies specific to the capsular antigens of *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 5, using antigens prepared separately from the concentrated culture supernatants of each respective production strain, a specific reaction is observed for each serotype. In addition, when performing immunoblotting using these antigens with monoclonal antibodies specific to Apx I, Apx II and Apx III, the antigens from serotypes 1 and 5 react with the monoclonal antibodies for Apx I and Apx II, while the antigen from serotype 2 reacts with the monoclonal antibodies for Apx II and Apx III. When measuring the ELISA antigen titer using these antigens and positive serum for ELISA antigen titer of serotypes matched with these antigens, as specified in test 3.4.1.1.2, the ELISA antigen titer of these antigens shall range from 800 to 1,600-fold.

Note 4 Positive serum for ELISA antigen titer measurement

Antiserum against serotypes obtained from SPF swine inoculated with production strains of *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 5. This will be adjusted to ensure that CF antibody titer for the serum will be 32-fold when measuring CF antibody titer using CF antigen matched with each positive serum for ELISA antigen titer measurement in the test as specified in 3.6.5.1.2. Dispense into small containers and store freeze-dried.

Note 5 Negative serum for ELISA antigen titer measurement

Serum obtained from swine negative for antibody against *Actinobacillus* *pleuropneumoniae*. When measuring CF antibody titer using CF antigen of *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 5 as specified in 3.6.5.1.2, CF antibody titer against these serotypes will be less than 4-fold. Dispense into small containers and store freeze-dried.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 6 | Carbonate buffer solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium carbonate | 1.59 g | |
|  | Sodium hydrogen carbonate | 2.93 g | |
|  | Water | Residual quantity | |

Adjust the pH to 9.6. Store at 4°C and use within 1 weeks after preparation.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 7 | Solution for dilution and washing |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Disodium hydrogen phosphate dodecahydrate | 1.725 g | |
|  | Potassium dihydrogen phosphate | 0.2 g | |
|  | Sodium chloride | 8.0 g | |
|  | Potassium chloride | 0.2 g | |
|  | Polysorbate 20 | 0.5 mL | |
|  | Water | Residual quantity | |

Adjust the pH to 7.2 - 7.4.

Note 8 2 w/v% bovine serum albumin solution

Dissolve 2.0 g of bovine serum albumin in 100 mL of the solution for dilution and washing immediately before use.

Note 9 Labeled antibody 1

Dilute horseradish peroxidase-labeled anti-swine IgG antibody to the optimal concentration using the solution for dilution and washing.

Note 10 Substrate solution

Dissolve 40 mg of *o*-phenylenediamine dihydrochloride in 100 mL of citrate-phosphate buffer solution (Note 26) and shield from light. Add 40 μL of hydrogen peroxide (30) immediately before use

Note 11 Stopping solution

Measure accurately 56.1 mL of concentrated sulfuric acid, dissolve in 440 mL of water while agitating and cooling and further add water to make 500 mL.

Note 12 Reference antigen for ELISA antigen titer measurement 2

Antigen prepared from the bacterial cell of the production strain of *Erysipelothrix rhusiopathiae*, extracted with sodium hydroxide. When immunoblotting is performed using a monoclonal antibody against the protective antigen of 67 kDa protein, it will show a single reactive band. When measuring the antigen titer in the test specified in 3.4.1.2.2, ELISA antigen titer should be between 80 and 160 U per mL.

Note 13 Monoclonal antibody for ELISA antigen titer measurement

Monoclonal antibody (subclass: IgG1) of 67 kDa protein of protective antigen purified from antigens extracted with sodium hydroxide from *Erysipelothrix rhusiopathiae* Kyoto strain. It possesses passive protective effect in mice. It is produced by intraperitoneally injecting antibody-producing hybridomas into BALB/c mice and purifying the resulting ascites using protein A column chromatography. When analyzed with SDS-polyacrylamide gel electrophoresis, a single band will be observed at about 160 kDa under non-reducing conditions, and a single band each at about 50 kDa and 30 kDa under reducing conditions. Before use, dilute the antigen with the solution for dilution and washing to achieve a protein concentration of 20 μg per mL.

Note 14 Labeled antibody 2

Dilute horseradish peroxidase-labeled anti-mouse IgG antibody to the optimal concentration using the solution for dilution and washing.

Note 15 4 w/v% sodium arsenite solution in hydrochloric acid

A solution of sodium arsenite dissolved at a concentration of 4 w/v% in 0.5 mol/L hydrochloric acid

Note 16 Butanol-concentrated hydrochloric acid solution

A mixture of butanol to concentrated hydrochloric acid at a rate of 5 vol%.

Note 17 CF antigen

Inoculate *Actinobacillus pleuropneumoniae* Shope 4074 strain for serotype 1, S1536 strain for serotype 2, and K-17 strain for serotype 5, or equivalent strains, separately onto plate medium (Note 27) and incubate at 37°C for 20 hours. Suspend these bacterial cells in phosphate-buffered saline (Note 28) and adjust the suspension to the concentration of McFarland turbidity standard No. 4. These preparations or their equivalents are CF antigen. Store at 4°C and dilute 16-fold with GVB buffer before use.

Note 18 Reference positive serum 1

Antiserum against serotypes obtained from guinea pigs inoculated with production strains for *Actinobacillus pleuropneumoniae* serotypes 1, 2, and 5. This will be adjusted to ensure that CF antibody titer for the serum will be 32-fold when measuring antibody titer using CF antigen matched with each serum in the test as specified in 3.6.5.1.2. Dispense into small containers and store frozen.

Note 19 Reference negative serum 1

Serum obtained from guinea pigs with negative antibody to *Actinobacillus pleuropneumoniae*. When measuring antibody titer using CF antigen for either serotypes 1, 2, or 5 in the test specified in 3.6.5.1.2, CF antibody titer will be no more than 4-fold. Dispense into small containers and store frozen.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Note 20 | Gelatin veronal buffer (GVB) | |  |  |
|  | Solution A | Veronal buffer saline |  |  |
|  |  | 1,000 mL consists of the following: |  |  |
|  |  | Sodium chloride | 42.50 g | |
|  |  | Barbital | 2.88 g | |
|  |  | Barbital sodium | 1.88 g | |
|  |  | Water | Residual quantity | |
|  |  | After dissolve with aid of heat, cool the solution and make 1000 mL | | |
|  | Solution B | 2 w/v% gelatin solution |  |  |
|  |  | 1,000 mL consists of the following: |  |  |
|  |  | Purified gelatin | 20 g | |
|  |  | Water | Residual quantity | |
|  |  | Dissolve with aid of heat. | | |

Before use, mix 400 mL of Solution A, 100 mL of Solution B, 10 mL of 0.03 mol/L calcium chloride solution and 10 mL of 0.1 mol/L magnesium chloride solution and add water to make 2,000 mL.

Note 21 Antigen for ELISA antibody titer measurement for *Erysipelothrix rhusiopathiae*

This is prepared from the bacterial cell of the production strain of *Erysipelothrix rhusiopathiae*, extracted with sodium hydroxide. When immunoblotting is performed using a monoclonal antibody against the protective antigen of 67 kDa protein, it will show a single reactive band. When measuring the antigen titer in the test specified in 3.4.1.2.2, ELISA antigen titer should be between 160 and 320 U per mL.

Note 22 Reference positive serum 2

Serum obtained by immunizing guinea pigs with an antigen prepared from the bacterial cells of the production strain of *Erysipelothrix rhusiopathiae*, extracted with sodium hydroxide. When measuring the antibody titer in the test specified in 3.6.5.2.2, ELISA antibody titer should be between 400 and 800-fold. Dispense into small containers and store frozen.

Note 23 Reference negative serum 2

Serum of guinea pigs with negative antibody to *Erysipelothrix rhusiopathiae*. When measuring the antibody titer in the test specified in 3.6.5.2.2, ELISA antibody titer will be less than 100-fold. Dispense into small containers and store frozen.

Note 24 Antigen adsorption plate

Dilute antigen for ELISA antigen titer measurement 100-fold with carbonate buffer solution, add 100 μL of the dilution to each U-shaped well of the ELISA plate and allowed to adsorb overnight at 2 to 5°C. Following a wash with solution for dilution and washing, add 250 μL each of 2 w/v% bovine serum albumin solution to each well and incubate at 37°C for 1 hour, and then wash with solution for dilution and washing.

Note 25 Labeled antibody 3

Dilute horseradish peroxidase-labeled anti-guinea pig IgG antibody to the optimal concentration using the solution for dilution and washing.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 26 | Citrate-phosphate buffer solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Citric acid anhydrous | 4.67 g |  |
|  | Disodium hydrogen phosphate dodecahydrate | 19.95 g |  |
|  | Water | Residual quantity | |

Adjust the pH to 5.0.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 27 | Plate medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Chicken broth | 100 mL | |
|  | Casamino acid | 5 g | |
|  | Soy peptone | 5 g | |
|  | Yeast extract | 5 g | |
|  | Sodium chloride | 5 g | |
|  | Agar | 12 g | |
|  | Water | Residual quantity | |

After dissolving the medium sterilize by autoclaving at 121°C for 20 minutes. After cooling to approximately 50°C, add 2 mL of filtered-sterilized 2 w/v% reduced β-NAD.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 28 | Phosphate-buffered saline |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.0 g | |
|  | Potassium chloride | 0.2 g | |
|  | Disodium hydrogen phosphate dodecahydrate | 2.9 g | |
|  | Potassium dihydrogen phosphate | 0.2 g | |
|  | Water | Residual quantity | |

Adjust the pH to 6.9 - 7.1.

**Swine Bordetellosis Vaccine inactivated, Pasteurella multocida toxoid, Swine Erysipelas Vaccine inactivated (adjuvant), Seed**

1 Definition

This Vaccine inactivated (adjuvant), Seed is a vaccine comprising the inactivated culture bacteria medium of *Bordetella bronchiseptica* with added aluminum gel adjuvant, the partially purified and inactivated dermonecrotic toxin from *Pasteurella multocida* with aluminum gel adjuvant and inactivated culture bacteria medium of *Erysipelothrix rhusiopathiae* with aluminum gel adjuvant, all meeting the Seed Lot Specifications.

2 Production methods

2.1 Bacterial strain used for production

2.1.1 *Bordetella bronchiseptica*

2.1.1.1 Name

Phase I *Bordetella bronchiseptica* N-40 strain or a strain approved as equivalent thereof

2.1.1.2 Properties

This bacterial strain forms raised, small, circular colonies on Bordet-Gengou medium (Note 1) and exhibits beta-hemolysis. In addition, it possesses K antigen and is specifically agglutinated by immune serum from Phase I *Bordetella bronchiseptica*.

When inoculated intranasally into pigs no older than seven days, it causes atrophic rhinitis of swine. When live *Bordetella bronchiseptica* or ultrasonicated organisms are injected intradermally into guinea pigs, they cause hemorrhage and necrosis at the injection site.

2.1.1.3 Master seed bacteria

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a Bordet-Gengou medium or a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.1.4 Working seed bacteria

2.1.1.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a Bordet-Gengou medium or a medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.1.5 Production seed bacteria

2.1.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a Bordet-Gengou medium or a medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 *Pasteurella multocida*

2.1.2.1 Name

*Pasteurella multocida* G-7 strain (capsular antigen D) or a strain approved as equivalent thereof

2.1.2.2 Properties

This bacterial strain forms mucoid, circular colonies on heart infusion agar medium with chicken serum.

When live bacteria is inoculated intranasally into the acetic acid-pretreated nasal membrane of approximately 3-week-old pigs, it causes atrophic rhinitis of swine. When dermonecrotic toxin, prepared from cultured bacteria, is injected intradermally into guinea pigs, it causes necrosis at the injection site. When injected intramuscularly into pigs, it causes atrophic rhinitis of swine.

2.1.2.3 Master seed bacteria

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a PPLO agar medium with chicken serum (Note 2) or a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.2.4 Working seed bacteria

2.1.2.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a PPLO agar medium supplemented with chicken serum or a medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.2.5 Production seed bacteria

2.1.2.5.1 Propagation and storage

The production seed bacteria shall be propagated in a PPLO agar medium supplemented with chicken serum or a medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.3 *Erysipelothrix rhusiopathiae*

2.1.3.1 Name

*Erysipelothrix rhusiopathiae* Tama-96 strain (serotype 2) or a strain approved as equivalent thereof

2.1.3.2 Properties

When inoculated into susceptible swine, the bacterial strain can induce swine erysipelas.

2.1.3.3 Master seed bacteria

2.1.3.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a plate medium for *Erysipelothrix rhusiopathiae* (Note 3) or a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.3.4 Working seed bacteria

2.1.3.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a plate medium for *Erysipelothrix rhusiopathiae* or a medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.3.5 Production seed bacteria

2.1.3.5.1 Propagation and storage

The production seed bacteria shall be propagated in a plate medium for *Erysipelothrix rhusiopathiae* or a medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 *Bordetella bronchiseptica*

2.2.1.1 Medium

Bordet-Gengou medium and production medium 1 (Note 4), or a medium approved as suitable for production shall be used.

2.2.2 *Pasteurella multocida*

2.2.2.1 Medium

PPLO agar medium with chicken serum and production medium 2 (Note 5), or a medium approved as suitable for production shall be used.

2.2.3 *Erysipelothrix rhusiopathiae*

2.2.3.1 Medium

A medium for *Erysipelothrix rhusiopathiae* (Note 6) and production medium 3 (Note 7), or a medium approved as suitable shall be used.

2.3 Bulk material

2.3.1 *Bordetella bronchiseptica* bulk material

2.3.1.1 Cultivation

Working seed bacteria or production seed bacteria cultured on a Bordet-Gengou medium or medium approved as suitable shall be inoculated onto production medium 1 or medium approved as suitable and this shall serve as the cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.1.1 and 3.2.2.

2.3.1.2 Inactivation

After the cultured bacterial medium is inactivated by an addition of formalin or an inactivator approved as appropriate, the bacterial cell pellet obtained from centrifugation shall be suspended in phosphate-buffered saline (Note 8:hereinafter “PBS” within this section) or one supplemented with a preservative approved as suitable. This shall serve as inactivated solution of *Bordetella bronchiseptica*.

The inactivated solution shall be tested as specified in 3.3.

2.3.1.3 Concentration adjustment

The concentration of the inactivated solution of *Bordetella bronchiseptica* shall be adjusted with PBS or one supplemented with a preservative approved as suitable and this shall serve as the bulk material.

The bulk material shall be tested as specified in 3.6.1.

2.3.2 *Pasteurella multocida* balk material

2.3.2.1 Cultivation

Working seed bacteria or production seed bacteria cultured on a PPLO agar medium with chicken serum or medium approved as suitable shall be inoculated onto production medium 2 or medium approved as suitable and this shall serve as the cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.1.2 and 3.2.3.1.

2.3.2.2 Bacterial harvesting and homogenization

The cultured bacterial medium shall be centrifuged, the resulting bacterial cell pellet shall be suspended in an adequate volume of phosphate-buffered solution (Note 9), and the bacterial cells shall be homogenized using physical treatment. This shall serve as homogenized solution of *Pasteurella multocida*.

The homogenized solution shall be tested as specified in 3.4.

2.3.2.3 Partial purification and concentration

The active toxin component shall be fractionated from the homogenized solution of *Pasteurella multocida* using a column chromatography and concentrated. This shall serve as the *Pasteurella multocida* dermonecrotic toxin solution.

The dermonecrotic toxin solution shall be tested as specified in 3.5.

2.3.2.4 Inactivation

*Pasteurella multocida* dermonecrotic toxin solution shall be inactivated by an addition of formalin or an inactivator approved as appropriate and shall serve as the bulk material.

The bulk material shall be tested as specified in 3.6.1.

2.3.3 *Erysipelothrix rhusiopathiae* bulk material

2.3.3.1 Cultivation

The working seed bacteria or the production seed bacteria shall be inoculated into a medium for *Erysipelothrix rhusiopathiae* or a medium approved as suitable to enrich the culture, and the culture shall be further inoculated into production medium 3 or a medium approved as suitable. Then, the resulting broth shall serve as the cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.1.3 and 3.2.3.2.

2.3.3.2 Bulk material preparation

After the cultured bacterial medium is inactivated by an addition of formalin, the bacterial cells obtained from centrifugation shall be suspended in PBS or one supplemented with a preservative approved as suitable. This shall serve as the bulk material.

The bulk material shall be tested as specified in 3.6.1 and 3.6.2.

2.4 Final bulk

2.4.1 *Bordetella bronchiseptica* bulk

The concentration of the bulk material shall be adjusted with an adequate volume of PBS and aluminum gel adjuvant. This shall serve as *Bordetella bronchiseptica* bulk. In this procedure, preservatives approved as suitable may be added.

2.4.2 *Pasteurella multocida* bulk

The concentration of the bulk material shall be adjusted with an adequate volume of PBS and aluminum gel adjuvant. This shall serve as *Pasteurella multocida* bulk. In this procedure, preservatives approved as suitable may be added.

2.4.3 *Erysipelothrix rhusiopathiae* bulk

The concentration of the bulk material shall be adjusted with an adequate volume of PBS and aluminum gel adjuvant. This shall serve as *Erysipelothrix rhusiopathiae* bulk. In this procedure, preservatives approved as suitable may be added.

2.4.4 Final bulk

The mixture of *Bordetella bronchiseptica* bulk, *Pasteurella multocida* bulk and *Erysipelothrix rhusiopathiae* bulk shall serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product. The final product shall be tested as specified in 3.7.

3 Test methods

3.1 Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

The tests given in 1.4.2.4.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

3.1.1.2.1 Test for freedom from contaminant microorganisms for *Bordetella bronchiseptica*

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than *Bordetella bronchiseptica*.

3.1.1.2.2 Test for freedom from contaminant microorganisms for *Pasteurella multocida*

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than *Pasteurella multocida*.

3.1.1.2.3 Test for freedom from contaminant microorganisms for *Erysipelothrix rhusiopathiae*

3.1.1.2.3.1 Culturing method using broth medium

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than *Erysipelothrix rhusiopathiae*.

3.1.1.2.3.2 Culturing method using nutrient agar slant medium

3.1.1.2.3.2.1 Medium

A nutrient agar slant medium shall be used.

3.1.1.2.3.2.2 Test procedures

A 0.5 mL portion each of the test article shall be inoculated into four tubes of a medium, cultured at 37°C for 7 days and observed.

3.1.1.2.3.2.3 Judgment

No bacterial growth other than *Erysipelothrix rhusiopathiae* must be observed.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from contaminant microorganisms

3.1.2.1.1 Test for freedom from contaminant microorganisms for *Bordetella bronchiseptica*

The test given in 3.1.1.2.1 shall apply.

3.1.2.1.2 Test for freedom from contaminant microorganisms for *Pasteurella multocida*

The test given in 3.1.1.2.2 shall apply.

3.1.2.1.3 Test for freedom from contaminant microorganisms for *Erysipelothrix rhusiopathiae*

The test given in 3.1.1.2.3 shall apply.

3.1.3 Tests on production seed bacteria

3.1.3.1 Test for freedom from contaminant microorganisms

3.1.3.1.1 Test for freedom from contaminant microorganisms for *Bordetella bronchiseptica*

The test given in 3.1.1.2.1 shall apply.

3.1.3.1.2 Test for freedom from contaminant microorganisms for *Pasteurella multocida*

The test given in 3.1.1.2.2 shall apply.

3.1.3.1.3 Test for freedom from contaminant microorganisms for *Erysipelothrix rhusiopathiae*

The test given in 3.1.1.2.3 shall apply.

3.2 Tests on cultured bacterial medium

3.2.1 Test for freedom from contaminant microorganisms

3.2.1.1 Test for freedom from contaminant microorganisms for *Bordetella bronchiseptica*

The test given in the Sterility Test 1 of the General Tests must not show any bacterial growth other than *Bordetella bronchiseptica*.

3.2.1.2 Test for freedom from contaminant microorganisms for *Pasteurella multocida*

The test given in the Sterility Test 1 of the General Tests must not show any bacterial growth other than *Pasteurella multocida*.

3.2.1.3 Test for freedom from contaminant microorganisms for *Erysipelothrix rhusiopathiae*

The test given in 3.1.1.2.3 shall apply.

3.2.2 Test for *Bordetella bronchiseptica* serotyping

3.2.2.1 Materials

3.2.2.1.1 Test materials

The test article shall be used as the test material.

3.2.2.1.2 Factor serum (Note 10)

K factor serum and O factor serum against *Bordetella bronchiseptica* shall be used.

3.2.2.2 Test procedures

Approximately 0.03 mL of the test material and approximately 0.03 mL of either K factor serum or O factor serum shall be mixed on a slide glass to allow the mixture for rapid agglutination reaction.

3.2.2.3 Judgment

The test material shall exhibit rapid agglutinability with K factor serum while poor agglutinability with the O factor serum.

3.2.3 Viable count test

3.2.3.1 Viable count test for *Pasteurella multocida*

3.2.3.1.1 Materials

3.2.3.1.1.1 Test materials

The test article shall be serially diluted ten-fold with soybean-casein digest broth (Note 11) and the dilution at each level shall serve as a test material.

3.2.3.1.1.2 Medium

A CHI agar medium (Note 12) or a medium approved as suitable shall be used.

3.2.3.1.2 Test procedures

A 0.1 mL portion of the test material shall be inoculated onto at least two medium plates each, spread over the surface of the plates, incubated at 37°C overnight and then the resulting colonies shall be counted.

3.2.3.1.3 Judgment

The viable count shall be at least 5 × 109 or higher per mL when calculated from the mean colony counts in dilutions at each level, dilution factor, and quantity of inoculum per medium.

3.2.3.2 Viable count test for *Erysipelothrix rhusiopathiae*

3.2.3.2.1 Materials

3.2.3.2.1.1 Test materials

The test article shall be serially diluted ten-fold with nutrient broth and each level dilution shall serve as a test material.

3.2.3.2.1.2 Medium

A nutrient agar medium shall be used.

3.2.3.2.2 Test procedures

A 1 mL portion of the test material shall be dispensed onto each of two Petri dishes, cultured using the pour plate culture method at 37°C for 48 hours, and the resulting number of *Erysipelothrix rhusiopathiae* colonies shall be counted.

3.2.3.2.3 Judgment

The viable count shall be calculated from the mean of colony counts in dilutions at each level, dilution factor, and quantity of inoculum per medium.

The viable count in the test article shall be 1.5 × 109 or higher per mL.

3.3 Tests on inactivated solution of *Bordetella bronchiseptica*

3.3.1 Inactivation test

The test given in the Sterility Test 1 of the General Tests shall apply.

3.3.2 Total bacterial count test

3.3.2.1 Test material

The test article shall be diluted appropriately with PBS to serve as the test material.

3.3.2.2 Test procedures

The absorbance of the test material shall be measured using a spectrophotometer.

3.3.2.3 Judgment

The total count in the test article calculated using the standard curve, the absorbance and the factor for diluting the test article shall be at least 2 × 1011 or higher per mL.

3.4 Tests on homogenized solution of *Pasteurella multocida*

3.4.1 Test for toxin measurement

3.4.1.1 Materials

3.4.1.1.1 Test materials

The test article filtered shall be serially diluted two-fold with PBS, and the dilution at each level shall serve as test materials.

3.4.1.1.2 Test animals

Guinea pigs weighing about 350 g shall be used.

3.4.1.2 Test procedures

A 0.2 mL of the test material shall be injected intradermally into the dorsal skin of each of the test animals at appropriate distance. Injections shall be limited to eight dilutions of the test material per animal.

3.4.1.3 Judgment

On the 2nd day of the injection, necrotic spots shall be measured. The dilution that induces necrotic spots with a diameter of at least 5 mm shall be defined as one dermonecrotic toxin unit, and the dilution factor shall be the unit per 0.2 mL of the test material.

Dermonecrotic toxin unit of the test article shall be 640 unit or higher per mL.

3.5 Tests on dermonecrotic toxin solution of *Pasteurella multocida*

3.5.1 Identification test

3.5.1.1 Materials

3.5.1.1.1 Test materials

The test material shall be prepared by adding 50 μL of sample buffer (Note 13) to an equal volume of the test article and boiling the mixture for 3 minutes.

3.5.1.2 Test procedures

The test is conducted in accordance with Sodium dodecyl sulfate (hereinafter referred to as “SDS” within this section)-polyacrylamide gel electrophoresis. After loading 10 μL of the test material onto a 10 w/v% acrylamide gel (Note 14) and a commercial molecular marker to the adjacent well for electrophoresis, Coomassie blue staining shall be performed to observe the electrophoresis image.

3.5.1.3 Judgment

The test article shall exhibit the primary band at a molecular weight of approximately 140 kDa.

3.5.2 Dermonecrotic toxin assay test

3.5.2.1 Materials

3.5.2.1.1 Test article and test material

For toxin measurement test, the test article shall be serially diluted two-fold with PBS, and the dilution at each level shall serve as test materials. For protein measurement test, the test article shall be used.

3.5.2.1.2 Test animals

For the toxin measurement test, guinea pigs weighing about 350 g shall be used.

3.5.2.2 Test procedures

3.5.2.2.1 Toxin measurement test

The test given in 3.4.1.2 shall apply and dermonecrotic toxin unit per mL of the test article shall be measured.

3.5.2.2.2 Protein measurement test

The protein content in 1 mL of the test article shall be measured using the Lowry method.

3.5.2.3 Judgment

The dermonecrotic toxin unit per μg of protein shall be calculated based on the dermonecrotic toxin units and protein content per mL of the test article.

The dermonecrotic toxin unit of the test article shall be 30 unit per μg of protein content or higher.

3.6 Tests on bulk material

3.6.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.6.2 Inactivation test

The test given in the Sterility Test 1 of the General Tests shall apply. Provided, the judgment shall be made 3 days after the inoculation.

3.7 Tests on final product

3.7.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.7.2 Test for pH

When the test given in the Test for pH of the General Tests shall apply, the pH shall have an intrinsic value.

3.7.3 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.7.4 Detoxification test

3.7.4.1 Materials

3.7.4.1.1 Injection materials

The test sample shall be used as an injection material.

3.7.4.1.2 Test animals

Guinea pigs weighing about 350 g shall be used.

3.7.4.2 Test procedures

A dose of 0.1 mL of the injection material shall be injected intradermally into the dorsal skin of the two test animals and observed for 10 days.

3.7.4.3 Judgment

Any injection reactions shall be of negligible severity and all the test animals shall survive.

3.7.5 Test for thimerosal content

The test given in the Test for Thimerosal Content of the General Tests shall apply.

3.7.6 Test for formalin content

When the test given in the Test for Formalin Content of the General Tests applies, the formalin content shall be not more than 0.25 vol%.

3.7.7 Test for aluminum content

When the test given in the Test for Aluminum Content of the General Tests applies, the aluminum content shall be not more than 2.4 mg per mL.

3.7.8 Safety test

3.7.8.1 Materials

3.7.8.1.1 Injection materials

The test sample shall be used as an injection material.

3.7.8.1.2 Test animals

Swine aged about 5 weeks shall be used.

3.7.8.2 Test procedures

A group of two test animals shall be used as the test group and one test animal as the control group.

A dose of 1 mL of the injection material shall be administered intramuscularly to the animals in the test group twice, with a three-week interval between injections. Both the test and control groups shall be observed for five weeks following the initial injection.

3.7.8.3 Judgment

No clinical abnormalities shall be observed in the test or control group during the observation period. In addition, any injection reactions in the test group shall be of negligible severity.

3.7.9 Potency test

3.7.9.1 Potency test for swine bordetellosis

3.7.9.1.1 Materials

3.7.9.1.1.1 Test animals

The animals used in the test specified in 3.7.8 shall be used.

3.7.9.1.1.2 Antigen for agglutination reaction

Antigen for agglutination reaction of *Bordetella bronchiseptica* (Note 15) shall be used.

3.7.9.1.2 Test procedures

Agglutination reaction shall be performed on sera obtained from each animal in the test and control groups on the last day of the test specified in 3.7.8.

The sera shall be diluted five-fold with PBS for the agglutination reaction (Note 16). After further serial two-fold dilution, the antigen for the agglutination reaction shall be used to perform the *in vitro* agglutination reaction.

3.7.9.1.3 Judgment

The maximum dilution factor of the serum that results in agglutination shall be defined as the agglutination titer.

The agglutination titer in all of the test group shall be at least 40-fold. In this case, the titer shall be ten-fold or lower in the control group.

3.7.9.2 Potency test for swine pasteurellosis

3.7.9.2.1 Materials

3.7.9.2.1.1 Test animals

The animals used in the test specified in 3.7.8 shall be used.

3.7.9.2.1.2 Antigen for enzyme-linked immunosorbent assay (hereinafter “ELISA” within this section)

Recombinant nontoxic dermonecrotic toxin protein (hereinafter “mrPMT” within this section: Note 17) shall be used.

3.7.9.2.2 Test procedures

ELISA shall be performed on sera obtained from each animal in the test and control groups on the last day of the test specified in 3.7.8.

To the mrPMT adsorption plate (Note 18), specifically to each of four wells (two in even-numbered lanes and two in odd-numbered lanes), 50 μL of serum from the test and control groups, diluted 100-fold with serum diluent (Note 19), positive indicator serum (Note 20) and negative indicator serum (Note 21) shall be added. After incubating at 37°C for 30 minutes, the plate shall be washed three times with the washing solution (Note 22).

Next, 50 μL of enzyme-labeled antibody solution (Note 23) shall be added to each well, followed by incubation at 37°C for 15 minutes. The plate shall then be washed three times with the washing solution. Then, 50 μL of chromogenic substrate solution (Note 24) shall be added to each well. After sensitizing the indicator serum at 30°C shielded from light to achieve the designated absorbance, 50 μL of 2 mol/L sulfuric acid solution shall be added to each well to stop the reaction, and the absorbance of each well shall be measured at the primary wavelength of 490 nm to 492 nm and the secondary wavelength of 630 nm.

3.7.9.2.3 Judgment

For the sera and positive and negative indicator sera from the test and control groups, the absorbance for each serum shall be calculated by subtracting the absorbance values in wells of the even-numbered lanes from those in the odd-numbered lanes and averaging these differences.

When the absorbance of the positive indicator serum is between 0.8 and 1.3, and that of the negative indicator serum is below 0.1, the test shall be considered valid. When calculating the E value for the sera from the test and control groups using the following equation, a value of no less than 0.1 shall be determined as positive.

E value = (S-N)/(P-N)

S: Absorbance of tested serum

N: Absorbance of negative indicator serum

P: Absorbance of positive indicator serum

All E values shall be positive in the test group. In this case, all the value shall be below 0.1 in the control group.

3.7.9.3 Potency test for swine erysipelas

3.7.9.3.1 Materials

3.7.9.3.1.1 Injection materials

The test sample shall be used as an injection material.

3.7.9.3.1.2 Test animals

Mice aged 5 weeks shall be used.

3.7.9.3.1.3 Challenge medium

Freeze-dried *Erysipelothrix rhusiopathiae* Fujisawa strain, or any strain with equivalent toxicity, shall be inoculated into a medium for *Erysipelothrix rhusiopathiae* and cultured at 37°C for 14 to 20 hours. The resulting culture shall be diluted with nutrient broth to achieve a bacterial amount of 103 per mL, and this mixture shall serve as the challenge medium.

3.7.9.3.2 Test procedures

A group of ten test animals shall be used as the test group and ten test animals as the control group.

A dose of 0.5 mL of the injection material shall be injected subcutaneously into the inner thigh of the animals in the test group twice at intervals of 14 days. On 14 days after the second injection, a dose of 0.1 mL each of the challenge medium shall be injected subcutaneously into the inner thigh for the challenge, and the animals shall be observed for seven days.

3.7.9.3.3 Judgment

The survival rate of the test group must be 70% or higher. In this case, the mortality rate of the control group must be 90% or higher.

4 Storage and expiry date

The expiry date shall be three years after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Bordet-Gengou medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Potato extract | 4.5 g | |
|  | Sodium chloride | 5.5 g | |
|  | Agar | 20 g | |
|  | Glycerin | 10 mL | |
|  | Water | Residual quantity | |

Dissolve 10 mL of glycerin in 990 mL of water. Dissolve other ingredients with aid of heat. Adjust the pH to 6.6 - 7.0, sterilize by autoclaving at 121°C for 15 minutes. After cooling to about 50°C, add either horse or sheep blood to achieve a concentration of 5 to 20 vol% as needed.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 2 | PPLO agar medium with chicken serum |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | PPLO medium | 21 g | |
|  | Agar | 10 g | |
|  | Chicken serum | 50 mL | |
|  | Water | Residual quantity | |

After dissolving the ingredients, except chicken serum, sterilize by autoclaving at 121°C for 15 minutes. After cooling to about 50°C, add 50 mL of chicken serum sterilized by filtration.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 3 | Plate medium for *Erysipelothrix rhusiopathiae* |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 30 g | |
|  | Proteose peptone No. 3 | 10 g | |
|  | Polysorbate 80 | 1 mL | |
|  | Agar | 10 g | |
|  | Water | Residual quantity | |

Adjust the pH to 7.4 - 7.8, sterilize by autoclaving at 121°C for 15 minutes.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 4 | Production medium 1 |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Soybean-casein digest broth | 30 g | |
|  | Meat extract powder | 2 g | |
|  | Water | Residual quantity | |

Adjust the pH to 7.0 - 7.4, remove any sediment by filtering, sterilize by autoclaving at 121°C for 15 minutes.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 5 | Production medium 2 |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Soybean-casein digest broth | 30 g | |
|  | Peptone | 5 g | |
|  | 10 w/v% yeast extract solution | 35 mL | |
|  | Water | Residual quantity | |

After dissolving the ingredients, except the yeast extract, with aid of heat, adjust the pH to 7.4 - 7.6, remove any sediment by filtering, sterilize by autoclaving at 121°C for 15 minutes. After cooling, add yeast extract sterilized by filtration.

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| --- | --- | --- | --- |
| Note 6 | Medium for *Erysipelothrix rhusiopathiae* |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 30 g | |
|  | Proteose peptone No. 3 | 10 g | |
|  | Polysorbate 80 | 1 mL | |
|  | Water | Residual quantity | |

After adjusting the pH to 7.4 - 7.8, sterilize by autoclaving at 121°C for 15 minutes.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 7 | Production medium 3 |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 30 g | |
|  | Polysorbate 80 | 1 mL | |
|  | Water | Residual quantity | |

Adjust the pH to 8.0 and autoclave at 121°C for 15 minutes. After cooling, add 10 to 50 mL of bovine serum sterilized by filtration.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 8 | PBS |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.5 g | |
|  | Disodium hydrogen phosphate dodecahydrate | 2.435 g | |
|  | Potassium dihydrogen phosphate | 0.435 g | |
|  | Water | Residual quantity | |

After adjusting the pH to 6.9 - 7.1, sterilize by autoclaving at 121°C for 20 minutes.

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| --- | --- | --- | --- |
| Note 9 | Phosphate-buffered solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Disodium hydrogen phosphate dodecahydrate | 2.256 g | |
|  | Sodium dihydrogen phosphate dihydrate | 2.137 g | |
|  | Water | Residual quantity | |

After adjusting the pH to 6.4 - 6.6, sterilize by autoclaving at 121°C for 20 minutes.

Note 10 Factor serum

K factor serum: Rabbit serum immunized against Phase I *Bordetella bronchiseptica*, with an agglutination titer adjusted to at least 80 times higher than the Phase I strain and no more than 10 times that of the Phase III strain.

O factor serum: Rabbit serum immunized against Phase III *Bordetella bronchiseptica*, with an agglutination titer adjusted to at least 80 times higher than the Phase III strain and no more than 10 times that of the Phase I strain.

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| --- | --- | --- | --- |
| Note 11 | Soybean-casein digest broth |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Soybean-casein digest broth | 30 g | |
|  | Water | Residual quantity | |

After dissolving with aid of heat, adjust the pH to 7.0 - 7.4, remove any sediment by filtering, sterilize by autoclaving at 121°C for 15 minutes.

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| --- | --- | --- | --- |
| Note 12 | CHI agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Heart infusion broth | 25 g | |
|  | Agar | 10 g | |
|  | Water | Residual quantity | |

After dissolving the medium, sterilize by autoclaving at 121°C for 15 minutes. After cooling to about 50°C, add chicken serum to achieve a concentration of 5 vol%.

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| --- | --- | --- | --- |
| Note 13 | Sample buffer |  |  |
|  | 50 mL consists of the following: |  |  |
|  | 0.25 mol/L Tris HCl buffer, pH 6.8 | 25 mL | |
|  | 20w/v%SDS | 13 mL | |
|  | Glycerin | 10 mL | |
|  | Dithiothreitol | 0.77 g | |
|  | Bromophenol blue | 0.05 g | |
|  | Water | Residual quantity | |

|  |  |  |  |
| --- | --- | --- | --- |
| Note 14 | 10 w/v% acrylamide gel |  |  |
|  | In 5 mL of concentrated gel |  |  |
|  | 0.25 mol/L Tris HCl buffer, pH 6.8 | 1.3 mL | |
|  | 30 w/v% acrylamide gel/0.8 w/v% bis | 0.9 mL | |
|  | 20w/v%SDS | 0.05 mL | |
|  | Water | Residual quantity | |
|  | Immediately before use, add the following reagents to allow polymerization. | | |
|  | 10 w/v% Ammonium persulfate | 0.025 mL | |
|  | N,N,N’,N’-Tetramethylethylenediamine (TEMED) | 0.01 mL | |
|  |  |  |  |
|  |  |  |  |
|  | In 10 mL of separating gel |  |  |
|  | 1.5 mol/L Tris HCl buffer, pH 8.8 | 2.5 mL | |
|  | 30 w/v% acrylamide gel/0.8 w/v% bis | 3.3 mL | |
|  | 20w/v%SDS | 0.1 mL | |
|  | Water | Residual quantity | |
|  | Immediately before use, add the following reagents to allow polymerization. | | |
|  | 10 w/v% Ammonium persulfate | 0.1 mL | |
|  | TEMED | 0.01 mL | |

Note 15 Antigen for agglutination reaction of *Bordetella bronchiseptica*

Suspension of formalin-inactivated Phase-I *Bordetella bronchiseptica* at a concentration of 1×1010 per mL in a diluent approved as suitable. Its antibody titer has been confirmed to exhibit the designated agglutination titer against a known positive serum while showing no agglutination titer to a negative serum.

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| --- | --- | --- | --- |
| Note 16 | PBS for agglutination reaction |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 6.8 g | |
|  | Disodium hydrogen phosphate dodecahydrate | 2.4 g | |
|  | Potassium dihydrogen phosphate | 0.7 g | |
|  | Water | Residual quantity | |

After adjusting the pH to 6.8 - 7.2, sterilize by autoclaving at 121°C for 20 minutes.

Note 17 mrPMT

Recombinant nontoxic dermonecrotic toxin protein (mrPMT) is expressed in *Escherichia coli* transformed with *Pasteurella multocida*-derived nontoxic dermonecrotic toxin gene. Culture the *Escherichia coli*, harvest the cultured bacteria, ultrasonicate to homogenize the bacteria, collect the mrPMT by ammonium sulfate precipitation, and purify the collected protein.

When performing SDS-polyacrylamide gel electrophoresis on the purified mrPMT, a specific band at approximately 140 kDa should be observed. Inactivate the purified mrPMT with formalin, and dialyze it against a phosphate-buffered solution. The resulting product shall be used as the ELISA antigen.

Note 18 mrPMT adsorption plate

Dilute the ELISA antigen with 0.05 mol/L carbonate-bicarbonate buffer solution (Note 25) to ensure the absorbance of the positive indicator serum between 0.8 and 1.3. Dispense 50 μL each of the dilution into the odd-numbered lanes and 0.05 mol/L carbonate-bicarbonate buffer solution into the even-numbered lanes of the plates. Immobilize the antigens overnight at 2ºC to 10°C. Wash the antigen-immobilized plate with washing solution and add 50 μL of blocking solution (Note 26) to each well after blocking.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 19 | Serum diluent |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Gelatin | 10 g | |
|  | Ten-fold PBS concentrate | 100 mL | |
|  | Polysorbate 80 | 1 mL | |
|  | Water | Residual quantity | |

After dissolving gelatin in water with aid of heat, cool and add ten-fold PBS concentrate and polysorbate 80.

Note 20 Positive indicator serum

Serum from swine immunized with purified mrPMT and adjusted to show an absorbance of approximately 1.0 in ELISA.

The swine for immunization shall be those of a specification approved as suitable.

Note 21 Negative indicator serum

Serum of healthy swine that shows absorbance below 0.1 in ELISA.

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| --- | --- | --- | --- |
| Note 22 | Washing solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.5 g | |
|  | Polysorbate 80 | 0.5 mL | |
|  | Water | Residual quantity | |

|  |  |  |  |
| --- | --- | --- | --- |
| Note 23 | Enzyme-labeled antibody solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Peroxidase-labeled protein A | 1 mL | |
|  | Ten-fold PBS concentrate | 100 mL | |
|  | Polysorbate 80 | 1 mL | |
|  | Water | Residual quantity | |

Prepare immediately before use.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 24 | Chromogenic substrate solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | *o*-phenylenediamine dihydrochloride | 0.4 g | |
|  | 30% hydrogen peroxide solution | 0.2 mL | |
|  | Substrate buffer solution (Note 27) | Residual quantity | |

Prepare immediately before use.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 25 | 0.05 mol/L Carbonate-bicarbonate buffer solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium hydrogen carbonate | 3 g | |
|  | Sodium carbonate | 1.5 g | |
|  | Water | Residual quantity | |

Adjust the pH to 9.6.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 26 | Blocking solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Gelatin | 10 g | |
|  | Water | Residual quantity | |

Dissolve gelatin with aid of heat and use after cooling.

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| --- | --- | --- | --- |
| Note 27 | Substrate buffer solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Citric acid | 2.55 g | |
|  | Disodium hydrogen phosphate, anhydrous | 3.65 g | |
|  | Water | Residual quantity | |

Adjust the pH to 5.0.

**Avian Infectious Bursal Disease Vaccine, Live (for chicks), Seed**

1 Definition

Avian Infectious Bursal Disease Vaccine, Live (for chicks), Seed is a freeze-dried vaccine of virus suspension obtained by propagating attenuated Infectious bursal disease virus that meets the Seed Lot Specifications in embryonated chicken eggs or primary cells that meet the said specifications, and the vaccine is applied to chicks including neonatal chicks.

2 Production methods

2.1 Virus strain used for production

2.1.1 Name

Attenuated Infectious bursal disease virus D78 strain or strain approved as equivalent thereof

2.1.2 Properties

Subcutaneous or oral inoculation of this virus strain in 1-day-old chicks shall not show clinical signs or immunosuppressive effects.

When inoculated into primary cultured cells of chick embryos, the strain proliferates with CPE.

2.1.3 Master seed virus

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in embryonated chicken eggs that meet 1.1 in the SPF Animal Specifications, chick primary embryo cells that meet 2.6 in the said specifications or cultured cells approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than production of vaccines. The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.4 Working seed virus

2.1.4.1 Propagation, passage and storage

The working seed virus shall be propagated in embryonated chicken eggs that meet 1.1 in the SPF Animal Specifications, chick primary cultured cells of chicken embryos that meet 2.6 in the said specifications or cultured cells approved as suitable.

The working seed virus shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.5 Production seed virus

2.1.5.1 Propagation and storage

The production seed virus shall be propagated in embryonated chicken eggs that meet 1.1 in the SPF Animal Specifications, primary cultured cells of chick embryos that meet 2.6 in the said specifications or cells approved as suitable.

The production seed virus shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 When using embryonated chicken eggs

2.2.1.1 Embryonated chicken eggs

Embryonated eggs aged 10 to 12 days that meet 1.1 in the SPF Animal Specifications shall be used.

Embryonated chicken eggs to be used for propagating, passaging, and storing master seed virus and working seed virus or those for propagating and storing production seed viruses shall be tested as specified in 3.2.

2.2.2 When using primary cultured cells

2.2.2.1 Primary cultured cells

The chick primary cultured cells of chicken embryos that meet 2.6 in the SPF Animal Specifications or primary cultured cells approved as suitable for production shall be used.

2.2.2.2 Culture medium

The culture medium approved as suitable for production shall be used.

2.2.2.3 Master primary cell seeds (production primary cell seeds).

2.2.2.3.1 Propagation, passage, and storage

The master primary cell seeds (production primary cell seeds) shall be propagated using culture medium in 2.2.2.2 and will not be passaged or stored.

Master primary cell seeds (production primary cell seeds) shall be tested as specified in 3.3.

2.3 Bulk material

2.3.1 Culture of Embryonated chicken eggs

Embryonated chicken eggs processed in one session shall be regarded as individual embryonated chicken eggs.

The individual embryonated chicken eggs shall be tested as specified in 3.4.

2.3.2 Culture of master primary cell seeds (production primary cell seeds)

The cultured cells obtained in one session shall be regarded as an individual cultured cells. No abnormalities shall be detected prior to the inoculation of the virus.

The individual cultured cells shall be tested as specified in 3.5.

2.3.3 Virus cultivation

2.3.3.1 When using embryonated chicken eggs

The production seed virus shall be inoculated into the yolk sac or the allantoic cavity of embryonated chicken eggs given in 2.3.1. After incubation, the infected chick embryos shall be harvested and emulsified. The filtrate or centrifugal supernatant shall be used as the bulk material. In this case, a stabilizer approved as suitable, or a minimal amount of antibiotics approved as suitable may be added.

The bulk material shall be tested as specified in 3.6.

2.3.3.2 When using cultured cells

The production seed virus shall be cultivated in the cells given in 2.3.2. The culture medium collected from individual cultured cells at the timing approved as suitable, its filtrate or centrifugal supernatant shall serve as the bulk material. In this case, a stabilizer approved as suitable, or a minimal amount of antibiotics approved as suitable may be added.

The bulk material shall be tested as specified in 3.6.

2.4 Final bulk

The bulk material shall be mixed and adjusted by adding a diluent and a stabilizer approved as appropriate, and the mixture shall serve as the final bulk. In this case, a minimal amount of antibiotics approved as suitable may be added.

For final product intended for tablet, the bulk material shall be mixed and added a diluent and a stabilizer approved as appropriate, freeze-drying, and adjusted by adding excipients and a lubricant approved as suitable, and then the resulting product shall serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product. For final product intended for tablet, the final bulk shall be formed into tablets to serve as the final product.

The final product shall be tested as specified in 3.7.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The test given in 1.4.2.1.1.1.2 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

For Avian encephalomyelitis virus, the tests given in 1.1 and 3.1.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply. However, the tests may not apply to the products that are subjected to the tests specified in 3.1.1.4.2.2 for testing Avian encephalomyelitis virus.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For Avian leukosis virus, Reticuloendotheliosis virus, and Avian encephalomyelitis virus, the tests given in 3.2.1, 3.2.2 and 3.2.10 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply. However, the test given in 3.2.10 may not apply to products that are subjected to the tests specified in 3.1.1.4.2.1 for testing Avian encephalomyelitis virus.

3.1.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.7 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Test on embryonated chicken egg

3.2.1 Observation of embryonated eggs

The test given in 3.2.1 in the Seed Lot Specifications shall apply.

3.3 Tests on primary cultured cells

3.3.1 Tests on master primary cell seeds (production primary cell seeds)

3.3.1.1 Test for confirmation of cell properties

The test given in 2.2.4.2.1.1 in the Seed Lot Specifications shall apply.

3.3.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.3.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.4 Tests on individual embryonated chicken eggs

A minimum of 1% of individual embryonated chicken eggs or at least 30 eggs shall serve as control embryonated eggs and shall be tested as specified below.

3.4.1 Culture observation

The control embryonated eggs shall be incubated without virus inoculation under the same condition. Upon observation, no abnormalities shall be observed in the chick embryos.

3.4.2 Chicken hemagglutination test

Allantoic fluid shall be collected on the final day of the tests given in 3.4.1, an equal volume of 0.5 vol% chicken red blood cell suspension shall be added and allowed to stand for 60 minutes　and be observed. No hemagglutination shall be observed.

3.5 Tests on cultured cells

A minimum of 1% of individual cultured cells for standing culture or at least 1 vol% or 500 mL of the cell for fermenter culture shall serve as control cultured cell and shall be tested as specified below.

3.5.1 Culture observation

The control cultured cell shall be incubated without virus inoculation under the same condition and be observed. No CPE shall be observed.

3.5.2 Chicken hemagglutination test

Culture medium shall be collected on the final day of the tests given in 3.5.1, an equal volume of 0.5 vol% chicken red blood cell suspension shall be added and allowed to stand for 60 minutes and be observed. No hemagglutination shall be observed.

3.6 Tests on bulk material

3.6.1 Microbial limit test

The test given in the Microbial Limit Test or the Sterility Test of the General Tests shall apply.

3.6.2 Test for virus content

3.6.2.1 Materials

3.6.2.1.1 Test materials

The test sample shall be serially diluted ten-fold with phosphate-buffered saline and each level dilution shall serve as a test material.

3.6.2.1.2 Cultured cells

Primary cultured cells of chicken embryos specified in 2.1.1 in the Materials for Live Vaccine Production and monolayer cultures shall be used.

3.6.2.2 Test procedures

A 0.2 mL portion of the test material shall be inoculated into at least four plates of cultured cells and allowed to stand for adsorption at 37°C for 60 minutes. Cell maintenance medium(Note 1) shall be added, incubated at 37°C for six days and observed.

3.6.2.3 Judgment

The cultured cells where CPE is observed shall be regarded as infected to calculate TCID50. The virus content of the test article shall be 106.8TCID50 or higher per mL unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.7 Tests on final product

3.7.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.7.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.7.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.7.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.7.5 Test for Freedom from Salmonella contamination

The test given in the Test for Freedom from Salmonella Contamination of the General Tests shall apply.

3.7.6 Microbial Limit Test

The test given in the Microbial Limit Test given of the General Tests shall apply.

3.7.7 Test for virus content

When the test is performed as specified in 3.6.2, the virus content of the test sample shall be 104.0TCID50 or higher per animal unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.7.8 Marker test

3.7.8.1 Materials

3.7.8.1.1 Test materials

The test sample shall be adjusted with phosphate-buffered saline to ensure that viruses sufficient for one dose are contained in 0.1 mL, and this shall serve as the test material. The virulent infectious bursal disease virus shall be adjusted to contain 104.0EID50 per 0.1 mL and this shall serve as the control.

3.7.8.1.2 Cultured cells

Primary cultured cells of chicken embryos specified in 2.1.1 in the Materials for Live Vaccine Production and monolayer cultures shall be used.

3.7.8.2 Test procedure

A 0.1 mL portion of the test material and the control shall be separately inoculated into each of at least four cultured cells, incubated at 37°C and observed for 5 to 7 days.

3.7.8.3 Judgment

CPE shall be observed in cultured cells. In this case, no CPE shall be observed in the cultured cells inoculated with the control.

3.7.9 Safety test

3.7.9.1 Materials

3.7.9.1.1 Inoculation material

The test sample shall be adjusted with phosphate-buffered saline to ensure that viruses sufficient for five doses are contained in 0.2 mL, and this shall serve as the inoculation material.

3.7.9.1.2 Test animals

Chicks aged 1 to 4 days derived from 1.1 in the Materials for Live Vaccine Production shall be used.

3.7.9.2 Test procedures

A group of ten test animals shall be used as the test group and five test animals as the control group.

A 0.2 mL portion of the inoculation material shall be orally inoculated into the test group, both the test and control groups shall be observed for five weeks. On the last day of the test, the bursa of Fabricius shall be autopsied.

3.7.9.3 Judgment

No clinical abnormalities shall be observed in the test and control group during the observation period. In addition, no significant atrophy of the bursa of Fabricius shall be observed upon autopsy.

3.7.10 Potency test

3.7.10.1 Materials

3.7.10.1.1 Inoculation material

The test sample shall be adjusted with phosphate-buffered saline to ensure that viruses sufficient for one dose are contained in 0.2 mL, and this shall serve as the inoculation material.

3.7.10.1.2 Test animals

Chicks aged 1 to 4 days derived from 1.1 in the Materials for Live Vaccine Production shall be used.

3.7.10.1.3 Virus for neutralization test

Infectious bursal disease virus D78 strain or a strain approved as suitable, which was inoculated into primary cultured cells of chicken embryos specified in 2.1.1 of the Materials for Live Vaccine Production and cultured shall be used.

3.7.10.1.4 Cultured cells

Primary cultured cells of chicken embryos specified in 2.1.1 of the Materials for Live Vaccine Production shall be cultured in Petri dishes, and the resulting monolayer cultures shall be used.

3.7.10.2 Test procedures

A 0.2 mL portion of the inoculation material shall be orally inoculated into each of the ten test animals. The serum obtained from each animal three weeks after inoculation shall be used for the neutralization test. In addition, three uninoculated animals shall serve as the control and shall be housed separately from the inoculated group. The serum obtained from each control animal three weeks after inoculation shall be also used for the neutralization test.

The serum shall be heat-inactivated and serially diluted two-fold with phosphate-buffered saline. Each diluted serum shall be mixed in equal volumes with virus suspension for neutralization test containing 100 to 200 PFU per 0.1 mL and the mixtures shall be reacted at 4°C for 18 to 24 hours. A 0.1 mL portion of each mixture shall be inoculated onto two separate cultured cell plates. After the plates shall be allowed to stand at 37°C for 60 minutes, the first overlaying agar medium (Note 2) shall be applied and statically incubated at 37°C for two to three days. Subsequently, the second overlaying agar medium (Note 3) shall be applied, statically incubated at 37°C for 24 hours and observed.

3.7.10.3 Judgment

The maximum dilution factor of the serum that results in a reduction of the plaque count to 50% shall be defined as the neutralizing antibody titer.

The antibody titer in at least 80% of the test animals shall be 200-fold or higher. In this case, the titer in the control group shall be ten-fold or lower.

3.7.11 Test for Freedom from Immunosuppression

3.7.11.1 Materials

3.7.11.1.1 Inoculation material

The test sample shall be adjusted with phosphate-buffered saline to ensure that viruses sufficient for five doses are contained in 0.2 mL, and this shall serve as the inoculation material.

3.7.11.1.2 Test animals

Chicks aged 1 to 4 days derived from 1.1 in the Materials for Live Vaccine Production shall be used.

3.7.11.2 Test procedures

A group of ten test animals shall be used as the test group and ten test animals as the control group, and one of the tests given below shall then be conducted.

3.7.11.2.1 Hemagglutination inhibition test

A 0.2 mL portion of inoculation material shall be subcutaneously inoculated into the test group. On the 7th day after inoculation, “Newcastle Disease Live Vaccine” of the B1 strain for one animal shall be intranasally inoculated to the test group and the control group. Blood will be collected on the 21st day for a hemagglutination inhibition test against Newcastle disease virus.

3.7.11.2.2 Challenge test

A 0.2 mL portion of inoculation material shall be orally inoculated into the test group. On the 14th day after inoculation, “Newcastle Disease Live Vaccine” of the B1 strain for one animal shall be intranasally inoculated to the test group and the control group. On the 21st day of intranasal inoculation, 1 mL of virulent Newcastle disease virus Sato strain, adjusted to 104.0 lethal dose per mL, shall be injected intramuscularly in all animals and observed for 14 days.

3.7.11.3 Judgment

In the hemagglutination inhibition test given in 3.5.11.2.1, hemagglutination inhibition antibodies shall increase in both the test and control groups, and no significant difference (P < 0.05) shall be found in their hemagglutination inhibition antibody titer.

In the challenge test given in 3.5.11.2.2, the tolerability at the end of the test must be 80% or higher in both the test and control groups without abnormalities.

3.7.12 Disintegration test

For the final product in tablet, the test given below shall be conducted.

3.7.12.1 Test procedures

One test sample tablet shall be placed in a beaker containing 200 mL of water at 15 to 25°C, and the time until the bubbling stops shall be measured.

This procedure shall be repeated on six tablets.

3.7.12.2 Judgment

Once the bubbling stops, the state where the tablet is dissolved or dispersed in water without any mass of particles shall be considered as disintegration.

All tablets shall disintegrate in 10 minutes.

4 Storage and expiry date

The expiry date shall be two years and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Note 1 | Cell maintenance medium |  |  |  |
|  | 1,000 mL consists of the following: |  |  |  |
|  | Tryptose phosphate broth | 2.95 g | | |
|  | Bovine serum | Appropriate amount | | |
|  | Eagle’s MEM | Residual quantity | | |

Adjust the pH to 7.0 - 7.4.

Minimal amounts of antibiotics may be added.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 2 | First overlaying agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Agar | 8-10 g | |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Bovine serum | 0-20 mL | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 7.0 - 7.4.

Minimal amounts of antibiotics may be added.

Note 3 Second overlaying agar medium

To the first overlaying agar medium, add 0.5 w/v% neutral red to achieve the concentration of 2 vol%.

**Newcastle Disease, Avian Infectious Bronchitis Vaccine, Live, Seed**

1 Definition

This Vaccine, Live, Seed is a freeze-dried vaccine of mixed virus suspensions obtained by propagating attenuated Newcastle disease virus and attenuated Avian infectious bronchitis virus that meet the Seed Lot Specifications in embryonated eggs that meet the said specifications.

2 Production methods

2.1 Virus strain used for production

2.1.1 Newcastle disease virus

2.1.1.1 Name

Attenuated Newcastle disease virus B1 strain or strain approved as equivalent thereof

2.1.1.2 Properties

This virus strain does not exhibit pathogenicity at a dose of 106.0EID50 in chicken aged 8 weeks, even when inoculated ophthalmically or smeared into the cloaca.

It propagates when injected at a dose of 1 EID50 in an embryonated chicken egg aged 10 days and will cause death in chicken embryo in about five days.

2.1.1.3 Master seed virus

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in embryonated chicken eggs that meet 1.1 in the SPF Animal Specifications, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines. The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.1.4 Working seed virus

2.1.1.4.1 Propagation, passage, and storage

The working seed virus shall be propagated and passaged in embryonated chicken eggs that meet 1.1 in the SPF Animal Specifications.

The working seed virus shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.1.5 Production seed virus

2.1.1.5.1 Propagation and storage

The production seed virus shall be propagated in embryonated chicken eggs that meet 1.1 in the SPF Animal Specifications.

The production seed virus shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 Avian infectious bronchitis virus

2.1.2.1 Name

Attenuated Avian infectious bronchitis virus H120 strain or a strain approved as suitable for manufacturing

2.1.2.2 Properties

This virus strain may cause transient mild respiratory symptoms when inoculated intranasally or ophthalmically at 103.0 EID50 in chicks aged 4 days. When injected into the allantoic cavity of an embryonated chicken egg aged 8 to 10 days, it propagates and causes the chicken embryo to die within two to seven days, or it induces insufficient embryonic growth or curling.

2.1.2.3 Master seed virus

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed virus shall be propagated in embryonated chicken eggs that meet 1.1 of the SPF Animal Specifications, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed virus shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower.

The master seed virus shall be tested as specified in 3.1.1.

The master seed virus shall not be passaged for purposes other than the production of vaccines. The master seed virus shall not be passaged more than five times to obtain the final product.

2.1.2.4 Working seed virus

2.1.2.4.1 Propagation, passage and storage

The working seed virus shall be propagated and passaged in embryonated chicken eggs that meet 1.1 of the SPF Animal Specifications.

The working seed virus shall be stored frozen at -70°C or lower or be stored freeze-dried at 5°C or lower.

The working seed virus shall be tested as specified in 3.1.2.

2.1.2.5 Production seed virus

2.1.2.5.1 Propagation and storage

The production seed virus shall be propagated in embryonated chicken eggs that meet 1.1 in the SPF Animal Specifications.

The production seed virus shall be stored frozen at -70°C or lower or stored freeze-dried at 5°C or lower, when it needs to be stored.

The production seed virus shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Newcastle disease virus

2.2.1.1 Embryonated chicken egg

Embryonated eggs aged 9 to 11 days that meet 1.1 in the SPF Animal Specifications shall be used.

Embryonated chicken eggs to be used for propagating, passaging and storing master seed viruses and working seed viruses as well as those for propagating and storing production seed viruses shall be tested as specified in 3.2.

2.2.2 Avian infectious bronchitis virus

2.2.2.1 Embryonated chicken egg

Embryonated eggs aged 10 to 12 days that meet 1.1 in the SPF Animal Specifications shall be used.

Embryonated chicken eggs to be used for propagating, passaging, and storing master seed viruses and working seed viruses as well as those for propagating and storing production seed viruses shall be subjected to the tests in 3.2.

2.3 Bulk material

2.3.1 Newcastle disease virus bulk material

2.3.1.1 Culture of embryonated chicken egg

Embryonated chicken eggs processed in one session shall be regarded as individual embryonated chicken eggs.

The individual embryonated chicken eggs shall be subjected to the test given in 3.3.

2.3.1.2 Virus cultivation

The production seed virus shall be cultivated in the embryonated eggs specified in 2.3.1.1. The allantoic fluid shall be collected, and its filtrate or centrifugal supernatant or the concentrate of this shall serve as the bulk material.

A minimal amount of antibiotics approved as suitable for the bulk material may be added.

The bulk material shall be tested as specified in 3.4.1 and 3.4.2.1.

2.3.2 Avian infectious bronchitis virus bulk material

2.3.2.1 Culture of embryonated chicken egg

Embryonated chicken eggs processed in one session shall be regarded as individual embryonated chicken eggs.

The individual embryonated chicken eggs shall be subjected to the test given in 3.3.

2.3.2.2 Virus cultivation

The production seed virus shall be cultivated in the embryonated eggs specified in 2.3.2.1. The allantoic fluid shall be collected, and its filtrate or centrifugal supernatant or concentrate of supernatant shall serve as the bulk material.

A minimal amount of antibiotics approved as suitable for the bulk material may be added.

The bulk material shall be tested as specified in 3.4.1 and 3.4.2.2.

2.4 Final bulk

The bulk materials of Newcastle disease virus and Avian infectious bronchitis virus shall be mixed and adjusted by adding a diluent or a stabilizer approved as suitable, and the mixture shall serve as the final bulk. In this case, a minimal amount of antibiotics approved as suitable may be added.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product.

The final product shall be tested as specified in 3.5.

3 Test methods

3.1 Tests on virus strain used for production

3.1.1 Tests on master seed virus

3.1.1.1 Identification test

The tests given in 1.4.2.1.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.1.3 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.1.4 Test for freedom from extraneous viruses

3.1.1.4.1 Common test for freedom from extraneous viruses

The tests given in 1.1, 2.1 and 2.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply.

3.1.1.4.2 Test for freedom from specific viruses

3.1.1.4.2.1 General test for freedom from specific viruses

For Avian encephalomyelitis virus, the tests given in 1.1 and 3.1.2 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply. However, this test may not apply to products that are subjected to the tests specified in 3.1.1.4.2.2 for testing Avian encephalomyelitis virus.

3.1.1.4.2.2 Individual test for freedom from specific viruses

For Avian leukosis virus, Reticuloendotheliosis virus and Avian encephalomyelitis virus, the tests given in 1.1, 3.2.1, 3.2.2 and 3.2.10 in the Test for Freedom from Extraneous Viruses of the General Tests shall apply. However, the test given in 3.2.10 may not apply to products that are subjected to tests specified in 3.1.1.4.2.1 for testing Avian encephalomyelitis virus.

3.1.1.5 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.6 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.7 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.1.8 Marker test

The test shall be conducted for Newcastle disease virus. This test may be omitted if it is conducted for final products.

3.1.1.8.1 Materials

3.1.1.8.1.1 Test materials

The virus content of the test article shall be adjusted with a diluent approved as suitable so that each 0.5 mL contains viruses for one dose and for 1/10 of a dose. These shall serve as the test materials.

3.1.1.8.1.2 Cultured cells

Cells obtained from embryo of chicken embryonated eggs aged 9 to 10 days that meet 1.1 in the Materials for Live Vaccine Production shall be suspended in cell growth medium (Note 1), dispensed in Petri dishes of about 20 cm2 or larger and the resulting monolayer cells shall be used.

3.1.1.8.2 Test procedures

A 0.5 mL portion of the test material shall be inoculated onto at least two separate culture cell plates. After the plates shall be allowed to stand at 37°C for 60 minutes, the overlaying agar medium (Note 2) shall be applied, incubated at 37°C for four days and their plaque-forming ability shall be tested.

3.1.1.8.3 Judgment

When the cells are observed on four days of the incubation, no plaque formation shall be found.

3.1.2 Tests on working seed virus

3.1.2.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.2.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.1.3 Tests on production seed virus

3.1.3.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.1.3.2 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.2 Test on embryonated chicken egg

3.2.1 Observation of embryonated eggs

The test given in 3.2.1 in the Seed Lot Specifications shall apply.

3.3 Tests on individual embryonated chicken eggs

A minimum of 1% of individual embryonated chicken eggs or at least 30 eggs shall serve as control embryonated eggs and shall be subjected to the tests given below.

3.3.1 Culture observation

The control embryonated eggs shall be incubated without virus inoculation under the same condition. Upon observation, no abnormalities shall be observed in the chicken embryos.

3.3.2 Chicken hemadsorption test

Allantoic fluid shall be collected on the final day of the tests given in 3.3.1, an equal volume of 0.5 vol% chicken red blood cell suspension shall be added and allowed to stand for 60 minutes and be observed. No hemagglutination shall be observed.

3.4 Tests on bulk material

3.4.1 Microbial limit test

The test given in the Microbial Limit Test of the General Tests shall apply.

3.4.2 Test for virus content

3.4.2.1 Newcastle disease virus

3.4.2.1.1 Materials

3.4.2.1.1.1 Test materials

The test article shall be serially diluted ten-fold with phosphate-buffered saline and each level dilution shall serve as a test material.

3.4.2.1.1.2 Embryonated chicken eggs

Eggs aged 9 to 11 days that meet 1.1 in the Materials for Live Vaccine Production shall be used.

3.4.2.1.2 Test procedures

A 0.1 mL portions of the test material shall be injected into the allantoic cavity of at least five embryonated chicken eggs, cultured at 37°C for five days and observed. Allantoic fluid shall be collected on the final day of the test and subjected to hemagglutination test using 0.5 vol% chicken red blood cell suspension.

3.4.2.1.3 Judgment

The allantoic fluid in which hemagglutination is observed shall be regarded as infected to calculate EID50. However, eggs died within 24 hours in the test shall be excluded.

The virus content of the test article shall be 108.8EID50 or higher per mL unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.4.2.2 Avian infectious bronchitis virus

3.4.2.2.1 Materials

3.4.2.2.1.1 Test materials

The test article shall be serially diluted ten-fold with phosphate-buffered saline and each level dilution shall serve as a test material.

3.4.2.2.1.2 Embryonated chicken eggs

Eggs aged 9 to 10 days that meet 1.1 in the Materials for Live Vaccine Production shall be used.

3.4.2.2.2 Test procedures

A 0.1 mL portions of the test material shall be injected into the allantoic cavity of at least five embryonated chicken eggs, cultured at 37°C for seven days and observed. On the final day of the test, these eggs shall be observed for any changes in the chicken embryos.

3.4.2.2.3 Judgment

The chicken embryos in which death or degeneration (insufficient growth, curling) is observed shall be regarded as being infected to calculate EID50. However, eggs died within 24 hours in the test shall be excluded.

The virus content of the test article shall be 106.8EID50 or higher per mL.

3.5 Tests on final product

3.5.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a liquid with a specific color or homogeneous suspension, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.5.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.5.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.5.4 Test for freedom from Mycoplasma contamination

The test given in the Test for Freedom from Mycoplasma Contamination of the General Tests shall apply.

3.5.5 Test for freedom from Salmonella contamination

The test given in the Test for Freedom from Salmonella Contamination of the General Tests shall apply.

3.5.6 Microbial limit test

The test given in the Microbial Limit Test of the General Tests shall apply.

3.5.7 Test for virus content

3.5.7.1 Newcastle disease virus

Avian infectious bronchitis virus in the test sample shall be neutralized with heat-inactivated anti-Avian infectious bronchitis virus serum (Note 3) to serve as the test material. When the test given in 3.4.2.1 is conducted, the virus contents in the test sample shall be 105.5 EID50 or higher per animal unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.5.7.2 Avian infectious bronchitis virus

Newcastle disease virus in the test sample shall be neutralized with inactivated anti-Newcastle disease virus serum (Note 4) and serve as the test material. When the test given in 3.4.2.2 is conducted, the virus contents in the test sample shall be 103.0 EID50 or higher per animal.

3.5.8 Marker test

The test given in 3.1.1.8 shall apply. This test may be omitted if it is conducted for Newcastle disease master seed virus.

3.5.8.1 Materials

3.5.8.1.1 Test materials

Avian infectious bronchitis virus in the test sample shall be neutralized with heat-inactivated anti-Avian infectious bronchitis virus serum. The concentration shall be adjusted using phosphate-buffered saline to ensure that the mixture contains Newcastle disease virus at a concentration for one dose and 1/10 of a dose per 0.5 mL. This preparation shall serve as the test material.

3.5.8.1.2 Cultured cells

Cells obtained from embryo of chicken embryonated eggs aged 9 to 10 days that meet 1.1 in the Materials for Live Vaccine Production shall be suspended in cell growth medium, dispensed in Petri dishes of about 20 cm2 or larger and the resulting monolayer cells shall be used.

3.5.8.2 Test procedures

A 0.5 mL portion of the test material shall be inoculated onto at least two separate culture cell plates. After the plates shall be allowed to stand at 37°C for 60 minutes, the overlaying agar medium shall be applied, incubated at 37°C for four days and their plaque-forming ability shall be tested.

3.5.8.3 Judgment

When the cells are observed on four days of the incubation, no plaque formation shall be found.

3.5.9 Safety test

3.5.9.1 Materials

3.5.9.1.1 Inoculation material

The test sample shall be adjusted with phosphate-buffered saline to ensure that viruses sufficient for ten doses are contained in 0.03 mL, and this shall serve as the inoculation material.

3.5.9.1.2 Test animals

Chickens aged 4 days derived from embryonated eggs that meet 1.1 in the Materials for Live Vaccine Production shall be used.

3.5.9.2 Test procedures

A group of ten test animals shall be used as the test group and five test animals as the control group.

A 0.03 mL portion of the inoculation material shall be ophthalmically inoculated into the test animals, and both the test and control groups shall be observed for three weeks.

Body weight shall be measured at the start and at the end of the test.

3.5.9.3 Judgment

During the observation period, in the control group, no clinical abnormalities should be observed, and in the test group, even if transient respiratory symptoms and conjunctival hyperemia are observed, no other abnormalities should be observed.

3.5.10 Potency test

3.5.10.1 Potency test for Newcastle disease

3.5.10.1.1 Materials

3.5.10.1.1.1 Inoculation material

The test sample shall be adjusted with phosphate-buffered saline or a diluent approved as suitable to ensure that viruses sufficient for one dose are contained in 0.03 mL, and this shall serve as the inoculation material.

3.5.10.1.1.2 Test animals

Chickens aged 4 to 5 weeks derived from embryonated eggs that meet 1.1 in the Materials for Live Vaccine Production shall be used.

3.5.10.1.1.3 Challenge virus

This is allantoic fluid infected with the virulent Newcastle disease virus Sato strain. When the viral titer shall be measured by injection intramuscularly into chickens aged approximately 40 days, it shall be at least 106.0 lethal doses per mL.

At the time of use, its concentration shall be adjusted with phosphate-buffered saline to ensure that the viral titer shall be 104.0 lethal dose per mL.

3.5.10.1.2 Test procedures

A group of ten test animals shall be used as the test group and five test animals as the control group.

A 0.03 mL portion of the inoculation material shall be intranasally inoculated to the test group. After two weeks, all animals in the test and control groups shall be injected intramuscularly with 1 mL of the challenge virus and observed for two weeks.

3.5.10.1.3 Judgment

At the end of the test, the survival rate of the test group must be 80% or higher without abnormalities. In this case, the incidence and mortality rate of the control group must be 100%.

3.5.10.2 Potency test for Avian infectious bronchitis

3.5.10.2.1 Materials

3.5.10.2.1.1 Inoculation material

The test sample shall be adjusted with phosphate-buffered saline or a diluent approved as suitable to ensure that viruses sufficient for one dose are contained in 0.03 mL, and this shall serve as the inoculation material.

3.5.10.2.1.2 Test animals

Chicks aged 4 days derived from embryonated eggs that meet 1.1 in the Materials for Live Vaccine Production shall be used.

3.5.10.2.1.3 Virus for neutralization test

The strain used for production shall be used. However, when allantoic fluid infected with the virus is injected into the allantoic cavity of embryonated chicken eggs aged 9 to 10 days that meet 1.1 in the Materials for Live Vaccine Production, the viral titer measured shall be at least 105.0 EID50 per mL.

3.5.10.2.1.4 Embryonated chicken eggs

Eggs aged 9 to 10 days that meet 1.1 in the Materials for Live Vaccine Production and shall be used.

3.5.10.2.2 Test procedures

A group of ten test animals shall be used as the test group and three test animals as the control group.

A 0.03 mL portion of the inoculation material shall be ophthalmically inoculated into the test group, and both the test and control groups shall be observed for three to four weeks. No abnormalities shall be observed in the test or control group during the observation period.

Neutralization test shall be performed using virus dilution method on sera obtained at the end of the test from each animal in the test and control groups. The sera from both groups shall be pooled at equal volumes and heat-inactivated.

The virus for the neutralization test shall be serially diluted ten-fold, and each level dilutions shall be divided into three groups. Pooled serum from the test group shall be added to Group 1, pooled serum from the control group to Group 2, and phosphate-buffered saline as a virus control to Group 3, each in equal volumes to the virus dilutions and mixed. After treating these mixtures at 4°C for 18 to 24 hours or at 37°C for 60 minutes, 0.1 mL of each level dilution shall be injected into the allantoic cavity of at least five embryonated chicken eggs, and these eggs shall be incubated at 37°C for seven days and observed. On the final day of the test, these eggs shall be observed for any changes in the chicken embryos.

3.5.10.2.3 Judgment

The chicken embryos in which death or degeneration (insufficient growth, curling) is observed shall be regarded as being infected to calculate neutralization index. However, eggs died within 24 hours in the test shall be excluded.

The neutralization index in the test group shall be at least 2.0 higher than in the control group. In this case, the index in the control group must be not more than 1.0 against the virus control.

4 Storage and expiry date

The expiry date shall be one year after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Cell growth medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Bovine serum | 50 mL | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 6.8 - 7.2.

Minimal amounts of antibiotics may be added.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 2 | Overlaying agar medium |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Tryptose phosphate broth | 2.95 g | |
|  | Bovine serum | 10 mL | |
|  | Neutral red | 50 mg | |
|  | Agar | 9 g | |
|  | Eagle’s MEM | Residual quantity | |

Adjust the pH to 6.8 -7.2.

Minimal amounts of antibiotics may be added.

Note 3 Anti-Avian infectious bronchitis virus serum

Serum obtained by immunizing the chicken derived from embryonated chicken eggs that meet 1.1 in the Materials for Live Vaccine Production with the strain for production or immunizing the mammalian with one, possessing a titer sufficient to fully neutralize the viruses contained in the test samples.

Note 4 Anti-Newcastle disease virus serum

Serum obtained by immunizing the chicken derived from embryonated chicken eggs that meet 1.1 in the Materials for Live Vaccine Production with the strain for production or immunizing the mammalian with one, possessing a titer sufficient to fully neutralize the viruses contained in the test samples.

**Poultry Salmonellosis (S. infantis, S. enteritidis, S. typhimurium) Vaccine (Oil adjuvant), Inactivated, Seed**

1 Definition

This Vaccine (Oil adjuvant), Inactivated, Seed is a vaccine comprising inactivated and concentrated culture bacteria media of *Salmonella infantis* (hereinafter “SI” within this section), *Salmonella enteritidis* (‘SE’) and *Salmonella typhimurium* (‘ST’), meeting the Seed Lot Specifications, and by adding an oil gel adjuvant.

2 Production methods

2.1 Bacterial strain used for production

2.1.1 SI

2.1.1.1 Name

SI I-178 strain or strain approved as equivalent thereof

2.1.1.2 Properties

The biological properties shall conform to the SI standard strain.

2.1.1.3 Master seed bacteria

2.1.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.1.4 Working seed bacteria

2.1.1.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.1.5 Production seed bacteria

2.1.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.2 SE

2.1.2.1 Name

SE E-926 strain or strain approved as equivalent thereof

2.1.2.2 Properties

The biological properties shall conform to the SE standard strain.

2.1.2.3 Master seed bacteria

2.1.2.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.2.4 Working seed bacteria

2.1.2.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.2.5 Production seed bacteria

2.1.2.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.1.3 ST

2.1.3.1 Name

ST T-023 strain or strain approved as equivalent thereof

2.1.3.2 Properties

The biological properties shall conform to the ST standard strain.

2.1.3.3 Master seed bacteria

2.1.3.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated on a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be given a specific manufacturing number or manufacturing code and stored frozen at -70°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.3.4 Working seed bacteria

2.1.3.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a medium approved as suitable.

The working seed bacteria shall be stored frozen at -70°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.3.5 Production seed bacteria

2.1.3.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable.

The production seed bacteria shall be stored frozen at -70°C or lower when it needs to be stored.

The production seed bacteria shall be tested as specified in 3.1.3 if it needs to be stored.

2.2 Materials for production

2.2.1 Medium

A medium approved as suitable for production shall be used.

2.3 Bulk material

2.3.1 SI bulk material

2.3.1.1 Cultivation

Production seed bacteria shall be inoculated in a medium and cultured. The resulting product shall serve as cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.1.2 Inactivation

Formalin shall be added to the cultured bacterial medium to inactivate the bacteria, then washed with phosphate-buffered saline (Note 1; “PBS” in this section) and concentrated to serve as an inactivated bacterial solution.

The inactivated bacterial solution shall be tested as specified in 3.3.

2.3.1.3 Addition of adjuvant

The inactivated bacterial solution shall be adjusted in concentration as necessary, and an oil-based adjuvant shall be added. This shall serve as the bulk material.

The bulk material shall be tested as specified in 3.4.

2.3.2 SE bulk material

2.3.2.1 Cultivation

Production seed bacteria shall be inoculated in a medium and cultured. The resulting product shall serve as cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.2.2 Inactivation

Formalin shall be added to the cultured bacterial medium to inactivate the bacteria, then washed with PBS and concentrated to serve as an inactivated bacterial solution.

The inactivated bacterial solution shall be tested as specified in 3.3.

2.3.2.3 Addition of adjuvant

The inactivated bacterial solution shall be adjusted in concentration as necessary, and an oil-based adjuvant shall be added. This shall serve as the bulk material.

The bulk material shall be tested as specified in 3.4.

2.3.3 ST bulk material

2.3.3.1 Cultivation

Production seed bacteria shall be inoculated in a medium and cultured. The resulting product shall serve as cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.3.2 Inactivation

Formalin shall be added to the cultured bacterial medium to inactivate the bacteria, then washed with PBS and concentrated to serve as an inactivated bacterial solution.

The inactivated bacterial solution shall be tested as specified in 3.3.

2.3.3.3 Addition of adjuvant

The inactivated bacterial solution shall be adjusted in concentration as necessary, and an oil-based adjuvant shall be added. This shall serve as the bulk material.

The bulk material shall be tested as specified in 3.4.

2.4 Final bulk

These bulk materials shall be mixed, and this mixture shall serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers to serve as the final product.

The final product shall be tested as specified in 3.5.

3 Test methods

3.1 Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

The tests given in 1.4.2.4.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

The test given in the Sterility Test 1 of the General Tests must not show any bacterial growth other than *Salmonella* unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.1.3 Tests on production seed bacteria

3.1.3.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.2 Tests on cultured bacterial medium

3.2.1 Test for freedom from contaminant microorganisms

The test given in 3.1.1.2 shall apply.

3.2.2 Viable count test

3.2.2.1 Materials

3.2.2.1.1 Test materials

The test article shall be serially diluted ten-fold with aqueous saline and the dilution at each level shall serve as a test material.

3.2.2.1.2 Medium

Agar medium approved as suitable shall be used.

3.2.2.2 Test procedures

A 1 mL portion of the test material shall be inoculated onto at least two plates using the mixed dilution pour plate culture method and incubated at 37°C for 24 hours. After incubation, the number of colonies that have developed shall be counted.

3.2.2.3 Judgment

The viable count shall be calculated from the colony counts in dilutions at each level.

The viable count shall be at least 107.9 or higher per mL for SI, 108.2 or higher per mL for SE, and 107.6 or higher per mL for ST.

3.3 Tests on inactivated bacterial solution

3.3.1 Inactivation test

3.3.1.1 Materials

3.3.1.1.1 Test materials

The test article shall be used as the test material.

3.3.1.1.2 Medium

A broth medium and agar medium approved as suitable shall be used.

3.3.1.2 Test procedures

A 1 mL portion of the test material shall be inoculated onto 100 mL of broth medium and cultured at 37°C for 24 hours. A 0.1 mL portion of the cultured broth shall be inoculated onto at least two agar medium plates and incubated at 37°C for 24 hours.

3.3.1.3 Judgment

No bacterial growth must be observed on any of agar plates.

3.3.2 Total count test

3.3.2.1 Materials

3.3.2.1.1 Test materials

The test article or one diluted with PBS approved as suitable shall be used as the test material.

3.3.2.2 Test procedures

The absorbance of the test material shall be measured using a spectrophotometer.

3.3.2.3 Judgment

When calculated using the standard curve, the absorbance in the test material and the factor for diluting the total count in the test article shall be at least 108.2 or higher per mL for SI, at least 108.5 or higher per mL for SE and at least 107.9 or higher per mL for ST

3.4 Tests on bulk material

3.4.1 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.5 Tests on final product

3.5.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be homogeneous suspension with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.5.2 Sterility test

The test given in the Sterility Test of the General Tests shall apply.

3.5.3 Test for formalin content

The test sample processed by a method approved as suitable shall be used as the test material. When the test given in the Test for Formalin Content of the General Tests apply, the formalin content must be not more than 0.2 vol%.

3.5.4 Safety test

3.5.4.1 Materials

3.5.4.1.1 Injection materials

The test sample shall be used as an injection material.

3.5.4.1.2 Test animals

The chickens aged five to seven weeks derived from embryonated eggs specified in 1.1 in the Materials for Live Vaccine Production shall be used.

3.5.4.2 Test procedures

A group of ten test animals shall be used as the test group and three test animals as the control group.

A dose of the injection material for a chicken shall be injected subcutaneously into the dorsal skin of the test animals, and both the test and control groups shall be observed for four weeks. On the last day of the test, the injection site shall be autopsied.

3.5.4.3 Judgment

No clinical abnormalities shall be observed in the test or control group during the observation period. In addition, no significant abnormalities should be observed at the injection site upon autopsy.

3.5.5 Potency test

3.5.5.1 SI infection potency test

3.5.5.1.1 Materials

The animals used in the test specified in 3.5.4 shall be used.

3.5.5.1.2 Antigen for enzyme-linked immunosorbent assay (hereinafter “ELISA” within this section)

Recombinant SI flagellar antigen (Note 2) shall be used.

3.5.5.1.3 Test procedures

ELISA shall be performed on sera obtained from each animal in the test and control groups on the last day of the test specified in 3.5.4.

Sera from the test and control groups, the SI reference positive serum (Note 3) and the reference negative serum (Note 4) shall be diluted 400-fold with the solution for dilution and washing (Note 5) and 100 μL each of these dilutions shall be added to four wells each on a recombinant SI flagellar antigen adsorbing plate (Note 6). Each plate shall have three wells for the solution for dilution and washing only and these wells shall be handled as blank wells. After incubating at 37°C for one hour, the plates shall be washed with the solution for dilution and washing. Next, 100 μL of enzyme-labeled antibody (Note 7) shall be added to each well, followed by incubation at 37°C for 1 hour. The plate shall then be washed with the solution for dilution and washing. A 100 μL portion of substrate solution (Note 8) shall be added to each well. The plates shall be shielded from light and allowed to react at room temperature for 15 minutes, and 50 μL of the quenching solution (Note 9) shall be added to stop the reaction. The absorbance of each well shall be measured at a wavelength of 492 nm.

3.5.5.1.4 Judgment

The absorbance of each well shall be the mean absorbance of the blank wells subtracted from the absorbance of each well. The absorbance of the four wells for each serum shall be compared, and the mean absorbance of two wells excluding the highest and lowest values shall be the absorbance of each serum. The absorbance of each serum in the test and control groups divided by the absorbance of the SI reference positive serum shall be the ELISA antibody titer for each serum.

The mean ELISA antibody titer in the test group shall be 0.25 or higher, whereas the mean in the control group shall be not more than 0.1. Furthermore, the absorbance shall be between 0.8 and 1.2 in the SI reference positive serum and not more than 0.1 in the reference negative serum.

3.5.5.2 SE infection potency test

3.5.5.2.1 Materials

The animals used in the test specified in 3.5.4 shall be used.

3.5.5.2.2 ELISA antigen

Purified SE flagellar antigen (Note 10) shall be used.

3.5.5.2.3 Test procedures

ELISA shall be performed on sera obtained from each animal in the test and control groups on the last day of the test specified in 3.5.4.

Sera from the test and control groups, the SE reference positive serum (Note 11) and the reference negative serum shall be diluted 800-fold with the solution for dilution and washing, and 100 μL of each of these dilutions shall be added to each of four wells on a purified SE flagellar antigen adsorbing plate (Note 12). Each plate shall have three wells for the solution for dilution and washing only, and these wells shall be handled as blank wells. After incubating at 37°C for one hour, the plates shall be washed with the solution for dilution and washing. Next, 100 μL of enzyme-labeled antibody shall be added to each well, followed by incubation at 37°C for 1 hour. The plate shall then be washed with the solution for dilution and washing. A 100 μL portion of substrate solution shall be added to each well. The plates shall be shielded from light and allowed to react at room temperature for 15 minutes, and 50 μL of the quenching solution shall be added to stop the reaction. The absorbance of each well shall be measured at a wavelength of 492 nm.

3.5.5.2.4 Judgment

The absorbance of each well shall be the mean absorbance of the blank wells subtracted from the absorbance of each well. The absorbance of the four wells for each serum shall be compared, and the mean absorbance of two wells excluding the highest and lowest values shall be the absorbance of each serum. The absorbance of each serum in the test and control groups divided by the absorbance of the SE reference positive serum shall be the ELISA antibody titer for each serum.

The mean ELISA antibody titer in the test group shall be 0.25 or higher, whereas the mean in the control group shall be not more than 0.1. Furthermore, the absorbance shall be between 0.8 and 1.2 in the SE reference positive serum and not more than 0.1 in the reference negative serum.

3.5.5.3 ST infection potency test

3.5.5.3.1 Materials

The animals used in the test specified in 3.5.4 shall be used.

3.5.5.3.2 ELISA antigen

Recombinant ST flagellar antigen (Note 13) shall be used.

3.5.5.3.3 Test procedures

ELISA shall be performed on sera obtained from each animal in the test and control groups on the last day of the test specified in 3.5.4.

Sera from the test and control groups, the ST reference positive serum (Note 14) and the reference negative serum shall be diluted 400-fold with the solution for dilution and washing, and 100 μL of each of these dilutions shall be added to each of four wells on a recombinant ST flagellar antigen adsorbing plate (Note 15). Each plate shall have three wells for the solution for dilution and washing only and these wells shall be handled as blank wells. After incubating at 37°C for one hour, the plates shall be washed with the solution for dilution and washing. Next, 100 μL of enzyme-labeled antibody shall be added to each well, followed by incubation at 37°C for 1 hour. The plate shall then be washed with the solution for dilution and washing. A 100 μL portion of substrate solution shall be added to each well. The plates shall be shielded from light and allowed to react at room temperature for 15 minutes, and 50 μL of the quenching solution shall be added to stop the reaction. The absorbance of each well shall be measured at a wavelength of 492 nm.

3.5.5.3.4 Judgment

The absorbance of each well shall be the mean absorbance of the blank wells subtracted from the absorbance of each well. The absorbance of the four wells for each serum shall be compared, and the mean absorbance of two wells excluding the highest and lowest values shall be the absorbance of each serum. The absorbance of each serum in the test and control groups divided by the absorbance of the ST reference positive serum shall be the ELISA antibody titer for each serum.

The mean ELISA antibody titer in the test group shall be 0.2 or higher, whereas the mean in the control group shall be not more than 0.1. Furthermore, the absorbance shall be between 0.8 and 1.2 in the ST reference positive serum and not more than 0.1 in the reference negative serum.

4 Storage and expiry date

The expiry date shall be three years after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.

5. Others

5.1 Notes for descriptions in package inserts

1 Specify that when using this vaccine to chickens, the local Livestock Health Service Center should be consulted for instructions and approximately 1% of the chickens shall remain identified as uninjected.

2 Chickens treated with this vaccine can be tested positive in an antibody testing for Pullorum disease

3 In conjunction with use of this vaccine, comprehensive hygiene control measures shall be implemented in accordance with the government-established comprehensive countermeasure guidelines for chicken egg Salmonella.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 1 | Phosphate-buffered saline |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.0 g | |
|  | Potassium chloride | 0.2 g | |
|  | Potassium dihydrogen phosphate | 0.3 g | |
|  | Disodium hydrogen phosphate | 1.15 g | |
|  | Water | Residual quantity | |

Adjust the pH to 6.8 - 7.4.

Note 2 Recombinant SI flagellar antigen

*E. coli* transformed with a plasmid inserted with a portion of *fliC* gene of SI I-178 strain shall be ultrasonicated, flagellar antigens shall be purified by affinity chromatography and dialyzed in PBS. This shall be stored not more than -80°C. When performing SDS-polyacrylamide gel electrophoresis, a specific band between 35 and 40 kDa shall be observed. Adjust the antigen with carbonate buffer solution (Note 16) to ensure that, when the ELISA is performed in a test as specified in 3.5.5.1, the absorbance shall be between 0.8 and 1.2 for the SI reference positive serum , not more than 0.1 for the reference negative serum, and the protein content during use shall be between 0.01 and 0.06 μg per well.

Note 3 SI reference positive serum

Serum obtained from chickens immunized with the SI I-178 strain, derived from embryonated eggs that meet the specifications in 1.1 of the Materials for Live Vaccine Production. The absorbance shall be between 0.8 to 1.2 when ELISA is performed in a test as specified in 3.5.5.1, not more than 0.25 in 3.5.5.2 and not more than 0.2 in 3.5.5.3. Store frozen at -20°C or lower.

Note 4 Reference negative serum

Serum obtained from chickens derived from embryonated eggs that meet the specifications in 1.1 of the Materials for Live Vaccine Production. The absorbance shall be not more than 0.1 in all of the ELISA test specified in 3.5.5.1, 3.5.5.2 or 3.5.5.3. Store frozen at -20°C or lower.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 5 | Solution for dilution and washing |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium chloride | 8.0 g | |
|  | Potassium chloride | 0.2 g | |
|  | Disodium hydrogen phosphate | 1.15 g | |
|  | Potassium dihydrogen phosphate | 0.3 g | |
|  | Water | Residual quantity | |

After adjusting the pH to 7.2, add 0.5 mL of polysorbate 20.

Note 6 Recombinant SI flagellar antigen adsorbing plate

Dilute recombinant SI flagellar antigen with carbonate buffer solution and dispense 100 μL of the dilution into each well of a 96-well plate. Allow the plate to react at 37°C for one hour. Then, add 200 μL of the solution for dilution and washing supplemented with 1 w/v% skim milk (Note 17) to each well, allow the plate to react at 37°C for another hour, and then wash the plate with the solution for dilution and washing.

Note 7 Enzyme-labeled antibody

Peroxidase labeled anti-chicken IgG (H+L) antibody. This is adjusted with either the solution for dilution and washing or the one supplemented with 1 w/v% skim milk to ensure that the absorbance shall be between 0.8 to 1.2 for the SI, SE and ST reference positive sera and not more than 0.1 for the negative sera when performing ELISA test specified in 3.5.5.1, 3.5.5.2 and 3.5.5.3.

Note 8 Substrate solution

This is prepared by dissolving 10 mg of σ-phenylenediamine dihydrochloride in 10 mL of citrate-phosphate buffer solution (Note 18) while being shielded from light and adding 10 μL of hydrogen peroxidase solution just before using.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 9 | Quenching solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Oxalic acid dihydrate | 28.02 g | |
|  | Water | Residual quantity | |

Note 10 Purified SE flagellar antigen

After adding hydrochloric acid to the culture medium of the SE E-926 strain, precipitate the flagellar antigen with ammonium sulfate and dialyze it with PBS. Store it at -80°C or lower. Adjust the antigen with carbonate buffer solution to ensure that, when the ELISA is performed in a test as specified in 3.5.5.2, the absorbance shall be between 0.8 and 1.2 for the SE reference positive serum, not more than 0.1 for the reference negative serum, and the protein content during use shall be between 0.01 and 0.2 μg per well.

Note 11 SE reference positive serum

Serum obtained from chickens immunized with the SE E-926 strain, derived from embryonated eggs that meet the specifications in 1.1 of the Materials for Live Vaccine Production. The absorbance shall be not more than 0.25 when ELISA is performed in a test as specified in 3.5.5.1, between 0.8 and 1.2 in 3.5.5.2 and not more than 0.2 in 3.5.5.3. Store frozen at -20°C or lower.

Note 12 Purified SE flagellar antigen adsorbing plate

Dilute purified SE flagellar antigen with carbonate buffer solution and dispense 100 μL of the dilution into each well of a 96-well plate. Allow the plate to react at 37°C for one hour. Then, add 200 μL of the solution for dilution and washing supplemented with 1 w/v% skim milk to each well, allow the plate to react at 37°C for another hour, and then wash the plate with the solution for dilution and washing.

Note 13 Recombinant ST flagellar antigen

*E. coli* transformed with a plasmid inserted with a portion of *fliC* gene of ST T-023 strain shall be ultrasonicated, flagellar antigens shall be purified by affinity chromatography and dialyzed in PBS. This shall be stored at -80°C or lower. When performing SDS-polyacrylamide gel electrophoresis, a specific band between 35 and 40 kDa shall be observed. Adjust the antigen with carbonate buffer solution to ensure that, when the ELISA is performed in a test as specified in 3.5.5.3, the absorbance shall be between 0.8 and 1.2 for the ST reference positive serum, not more than 0.1 for the reference negative serum, and the protein content during use shall be between 0.01 and 0.06 μg per well.

Note 14 ST reference positive serum

Serum obtained from chickens immunized with the ST T-023 strain, derived from embryonated eggs that meet the specifications in 1.1 of the Materials for Live Vaccine Production. The absorbance shall not exceed 0.25 when ELISA is performed as in a test specified in 3.5.5.1 and 3.5.5.2 while it shall be between 0.8 to 1.2 in 3.5.5.3. Store frozen at -20°C or lower.

Note 15 Recombinant ST flagellar antigen adsorbing plate

Dilute recombinant ST flagellar antigen with carbonate buffer solution and dispense 100 μL of the dilution into each well of a 96-well plate. Allow the plate to react at 37°C for one hour. Then, add 200 μL of the solution for dilution and washing supplemented with 1 w/v% skim milk to each well, allow the plate to react at 37°C for another hour, and then wash the plate with the solution for dilution and washing.

|  |  |  |  |
| --- | --- | --- | --- |
| Note 16 | Carbonate buffer solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Sodium carbonate | 1.59 g | |
|  | Sodium hydrogen carbonate | 2.93 g | |
|  | Water | Residual quantity | |

Adjust the pH to 9.6.

Note 17 Solution for dilution and washing supplemented with 1 w/v% skim milk

To the solution for dilution and washing, dissolve skim milk to achieve concentration of 1 w/v%

|  |  |  |  |
| --- | --- | --- | --- |
| Note 18 | Citrate-phosphate buffer solution |  |  |
|  | 1,000 mL consists of the following: |  |  |
|  | Citric acid monohydrate | 10.3 g | |
|  | Disodium hydrogen phosphate | 14.5 g | |
|  | Water | Residual quantity | |

Adjust the pH to 5.0.

**Poultry Colibacillosis Vaccine, Live, Seed**

1 Definition

Poultry Colibacillosis Vaccine, Live, Seed is a freeze-dried vaccine of culture bacteria medium of *Escherichia coli* (serotype O78) with a partial gene deletion that codes cyclic AMP receptor proteins (*crp* gene) and meets the Seed Lot Specifications.

2 Production methods

2.1 Bacterial strain used for production

2.1.1 Name

*Escherichia coli* AESN1331 strain (serotype O78) or strain approved as equivalent thereof

2.1.2 Properties

This bacterial strain has *crp* gene deletion. No clinical symptoms are observed in chickens sprayed with this strain.

2.1.3 Master seed bacteria

2.1.3.1 Preparing, storing, and maximum passage number allowed for obtaining final products

The master seed bacteria shall be propagated in a medium approved as suitable, prepared in consecutive processes, and dispensed into storage containers.

The dispensed master seed bacteria shall be added with a stabilizer approved as suitable and given a specific manufacturing number or manufacturing code and stored freeze-dried at 10°C or lower.

The master seed bacteria shall be tested as specified in 3.1.1.

The master seed bacteria shall not be passaged for purposes other than production of vaccines. The master seed bacteria shall not be passaged more than 10 times to obtain the final product.

2.1.4 Working seed bacteria

2.1.4.1 Propagation, passage and storage

The working seed bacteria shall be propagated and passaged in a medium approved as suitable.

The working seed bacteria shall be stored frozen at -60°C or lower or be stored freeze-dried at 10°C or lower.

The working seed bacteria shall be tested as specified in 3.1.2.

2.1.5 Production seed bacteria

2.1.5.1 Propagation and storage

The production seed bacteria shall be propagated in a medium approved as suitable.

Production seed bacteria shall not be stored.

2.2 Materials for production

2.2.1 Medium

Agar and broth media approved as suitable for production shall be used.

2.3 Bulk material

2.3.1 Cultivation

Colonies of production seed bacteria that were cultivated on agar plates shall be inoculated into a broth medium for culturing. This broth medium shall be further inoculated into a broth medium, and the resulting broth medium shall serve as the cultured bacterial medium.

The cultured bacterial medium shall be tested as specified in 3.2.

2.3.2 Bulk material

The bacteria collected from centrifuging the cultured bacterial medium shall be resuspended in a broth medium, and their concentration adjusted. This preparation shall then serve as the bulk material.

The bulk material shall be tested as specified in 3.3.

2.4 Final bulk

A stabilizer approved as appropriate shall be added to the bulk material and mixed to serve as the final bulk.

2.5 Final product

The final bulk shall be dispensed into small containers and freeze-dried to serve as the final product.

The final product shall be tested as specified in 3.4.

3 Test methods

3.1 Tests on bacterial strain used for production

3.1.1 Tests on master seed bacteria

3.1.1.1 Identification test

The tests given in 1.4.2.2.1.1 in the Seed Lot Specifications shall apply.

3.1.1.2 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than the production strain.

3.1.1.3 Target animal immunogenicity test

The test given in the Target Animal Immunogenicity Test of the General Tests shall apply.

3.1.1.4 Target animal safety test

The test given in the Target Animal Safety Test of the General Tests shall apply.

3.1.1.5 Test for absence of reversion virulence

The test given in the Test for Absence of Reversion to Virulence of the General Tests shall apply.

3.1.1.6 Test for Stability Confirmation of Recombinant Gene

The test given in the Test for Stability Confirmation of Recombinant Gene of the General Tests shall apply.

3.1.2 Tests on working seed bacteria

3.1.2.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than the production strain.

3.2 Tests on cultured bacterial medium

3.2.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than the production strain.

3.2.2 Viable count test

3.2.2.1 Materials

3.2.2.1.1 Test materials

The test article shall be serially diluted ten-fold with a diluent approved as suitable and each level dilution shall serve as a test material.

3.2.2.1.2 Medium

An agar medium approved as suitable shall be used.

3.2.2.2 Test procedures

A 0.1 mL portion of the test material shall be inoculated onto at least two plates and incubated at 37°C for 24 hours. After incubation, the number of colonies that have developed shall be counted.

3.2.2.3 Judgment

The number of colonies shall be counted from plate medium containing between 30 and 300 colonies. The viable count of the test article shall be 2.5 × 108 CFU or higher per mLwhen calculated from the mean colony counts, dilution factor and quantity of inoculum.

3.3 Tests on bulk material

3.3.1 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than the production strain.

3.3.2 Viable count test

When the test is performed as specified in 3.2.2, the viable count shall be 5.0 × 109 to 5.0 × 1011 CFU or higher per mL.

3.4 Tests on final product

3.4.1 Properties test

When the test is performed as specified in the Properties Test of the General Tests, the final product shall be dried substances with a specific color. The dissolved product shall be a liquid with a specific color, and no foreign matter and foreign odor shall be observed. The property of product per small container shall be uniform.

3.4.2 Vacuum degree test

The test given in the Vacuum Degree Test of the General Tests shall apply.

3.4.3 Test for moisture content

The test given in the Test for Moisture Content of the General Tests shall apply.

3.4.4 Test for freedom from contaminant microorganisms

The test given in the Sterility Test of the General Tests must not show any bacterial growth other than the production strain.

3.4.5 Viable count test

The test sample shall be dissolved in 300 mL of sterile saline for 1,000 doses, and when the test is performed as specified in 3.2.2, the viable count shall be 3.3 × 107 to 3.3 × 109 CFU or higher per mL.

3.4.6 Safety test

3.4.6.1 Materials

3.4.6.1.1 Inoculation material

The test sample dissolved in 300 mL of sterile saline for 1,000 doses shall serve as the inoculation material.

3.4.6.1.2 Test animals

Chickens aged 4 days derived from embryonated eggs that meet 1.1 in the Materials for Live Vaccine Production shall be used.

3.4.6.2 Test procedures

Both the test and control groups shall consist of at least ten animals.

The inoculation material intended for one dose shall be sprayed onto twice with a four-week interval in the test group, and both the test and control groups shall be observed for two more weeks following the second administration.

3.4.6.3 Judgment

No clinical abnormalities shall be observed in the test and control groups during the observation period.

3.4.7 Potency test

3.4.7.1 Materials

3.4.7.1.1 Test animals

The animals used in the test specified in 3.4.6 shall be used.

3.4.7.1.2 Bacterial solution for challenge

*Escherichia coli* J46 strain (serotype O78) or a strain with equivalent virulence thereof shall be cultured and appropriately diluted with sterile saline to serve as the bacterial solution for challenge.

3.4.7.2 Test procedures

On the final day of the test specified in 3.4.6, a 0.5 mL of the bacterial solution for challenge shall be injected intravenously under the wing of animals in both the test and control groups for the challenge, and the animals shall be observed for seven days.

3.4.7.3 Judgment

The survival rate of the test group must be 70% or higher. In this case, the mortality rate of the control group must be 80% or higher.

4 Storage and expiry date

The expiry date shall be three years and three months after the manufacturing unless otherwise specified by the Minister of Agriculture, Forestry and Fisheries.