CHAPTER 2.1.2.
AUJESZKY’S DISEASE

SUMMARY

Aujeszky’s disease, also known as pseudorabies, is caused by an alphaherpesvirus that infects the central nervous system and other organs, such as the respiratory tract, in a variety of mammals except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks old, which die from encephalitis). The disease is controlled by containment of infected herds and by the use of vaccines and/or removal of latently infected animals.

A diagnosis of Aujeszky’s disease is established by detecting the agent (virus isolation, polymerase chain reaction [PCR]), as well as by detecting a serological response in the live animal.

Identification of the agent: Isolation of Aujeszky’s disease virus can be made by inoculating a tissue homogenate, for example of brain and tonsil or material collected from the nose/throat, into a susceptible cell line such as porcine kidney (PK-15) or SK6, or primary or secondary kidney cells. The specificity of the cytopathic effect is verified by immunofluorescence, immunoperoxidase or neutralisation with specific antiserum. The viral DNA can also be identified using PCR; this can be accomplished using the real-time PCR techniques.

Serological tests: Aujeszky’s disease antibodies are demonstrated by virus neutralisation, latex agglutination or enzyme-linked immunosorbent assay (ELISA). A number of ELISA kits are commercially available world-wide. An OIE international standard serum defines the lower limit of sensitivity for routine testing by laboratories that undertake the serological diagnosis of Aujeszky’s disease.

Since about 1990, it has become possible to distinguish between antibodies resulting from natural infection and those from vaccination by use of gene-deleted vaccines.

Requirements for vaccines and diagnostic biologicals: Vaccines, either gene-deleted attenuated or inactivated virus vaccines should prevent or at least limit the excretion of virus from the infected pigs. More recently, these conventional vaccines have been supplemented by rDNA-derived gene-deleted or naturally deleted live Aujeszky’s disease virus vaccines. The virus used in these new vaccines, sometimes referred to as marker vaccines, lacks a specific glycoprotein (gG, gE, or gC).

A. INTRODUCTION

Aujeszky’s disease, also known as pseudorabies, is caused by an alphaherpesvirus, a member of the family Herpesviridae. The virus infects the central nervous system and other organs, such as the respiratory tract, a variety of mammals except humans and the tailless apes. It is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks old, which die from encephalitis). The disease is controlled by containment of infected herds and by the use of vaccines and/or removal of latently infected animals.

Whereas isolation of the Aujeszky’s disease virus will assist in a provisional diagnosis in the case of lethal forms of Aujeszky’s disease or clinical disease in pigs, other techniques and serological tests are required for diagnosis of latent infections. Many affected animals, however, except pigs, do not live long enough to produce any marked serological response.
B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Virus isolation

The diagnosis of Aujeszky's disease can be confirmed by isolating PRV from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil swabs from living pigs, or from samples from dead pigs or following the presentation of clinical signs such as encephalitis in herbivores or carnivores. For post-mortem isolation of PRV, samples of brain, tonsil, lung and spleen are the preferred specimens. In cattle, infection is usually characterised by a pruritus, in which case a sample of the corresponding section of the spinal cord may be required in order to isolate the virus. In latently infected pigs, the trigeminal ganglion is the most consistent site for virus isolation, although latent virus is usually non-infective unless reactivated, making it difficult to recover in culture.

The samples are homogenised in normal saline or cell culture medium with antibiotics and the resulting suspension is clarified by low speed centrifugation at 900 \( g \) for 10 minutes. The supernatant fluid is used to inoculate any sensitive cell culture system. Numerous types of cell line or primary cell cultures are sensitive to PRV, but a porcine kidney cell line (PK-15) is generally employed. The overlay medium for the cultures should contain antibiotics (such as: 200 IU/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml polymyxin; and 3 µg/ml fungizone).

PRV induces a cytopathic effect (CPE) that usually appears within 24–72 hours, but cell cultures may be incubated for 5–6 days. The monolayer develops accumulations of birefringent cells, followed by complete detachment of the cell sheet. Syncytia also develop, the appearance and size of which are variable. In the absence of any obvious CPE, it is advisable to make one blind passage into further cultures. Additional evidence may be obtained by staining infected cover-slip cultures with haematoxylin and eosin to demonstrate the characteristic herpesviral acidophilic intranuclear inclusions with margination of the chromatin. The virus identity should be confirmed by immunofluorescence, immunoperoxidase, or neutralisation using specific antiserum.

The isolation of PRV makes it possible to confirm Aujeszky's disease, but failure to isolate does not guarantee freedom from infection.

b) Identification of virus by the polymerase chain reaction

The polymerase chain reaction (PCR) can be used to identify PRV genomes in secretions or organ samples. Many individual laboratories have established effective protocols, but there is as yet no internationally agreed standardised approach.

The PCR is based on the selective amplification of a specific part of the genome using two primers located at each end of the selected sequence. In a first step, the complete DNA may be isolated using standard procedures (e.g. proteinase K digestion and phenol–chloroform extraction) or commercially available DNA extraction kits. Using cycles of DNA denaturation to give single-stranded DNA templates, hybridisation of the primers, and synthesis of complementary sequences using a thermostable DNA polymerase, the target sequence can be amplified up to 10^6-fold. The primers must be designed to amplify a sequence conserved among PRV strains, for example parts of the gB or gD genes, which code for essential glycoproteins, have been used (10, 29).

The amplified product may be identified from its molecular weight as determined by migration in agarose gel, with further confirmation where possible by Southern hybridisation using a complementary probe. Recent techniques involve liquid hybridisation using enzyme-labelled probes, which give a colour reaction after incubation with the appropriate substrate. More recent techniques include the use of fluorescent probes linked to an exonuclease action and real-time monitoring of the evolution of product, enabling simultaneous amplification and confirmation of the template DNA thus increasing the rapidity and specificity of the PCR assays.

In all cases, the main advantage of PCR, when compared with conventional virus isolation techniques, is its rapidity; with most modern equipment, the entire process of identification and confirmation can be completed within one day. However, because of the nature of the test, many precautions need to be taken to avoid contamination of samples with extraneous DNA from previous tests or from general environmental contamination in the laboratory (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials). This may limit the value of the test for many laboratories unless care is taken to avoid DNA carry-over contamination.
2. Serological tests

Any serological technique used should be sufficiently sensitive to give a positive result with the OIE International Standard Reference Serum. This serum can be obtained from the OIE Reference Laboratory for Aujeszky's Disease in France (see Table given in Part 3 of this Terrestrial Manual). For international trade purposes, the test should be sensitive enough to detect the standard serum diluted 1/2.

Virus neutralisation (VN) has been recognised as the reference method for serology (4, 28), but for general diagnostic purposes it has been widely replaced by the enzyme-linked immunosorbent assay (ELISA) because of its suitability for large-scale testing (2, 12, 16, 18). The tests can be performed on a variety of matrices (e.g. serum, whole blood, milk) but the preferred matrix is serum.

A latex agglutination test has also been developed and can be used for screening for antibodies. Kits for the test are commercially available.

a) Virus neutralisation (a prescribed test for international trade)

VN in cell culture can be performed in several ways, which vary according to the length of incubation of the virus/serum mixtures (e.g. 1 hour at 37°C or 24 hours at 4°C), and the presence or absence of complement. Most laboratories use a reaction period of 1 hour at 37°C in the absence of complement, because this is easy and rapid. However, the sensitivity can be improved by increasing the incubation period to 24 hours at 4°C, which facilitates the detection of antibody levels 10–15 times lower than in the 1-hour method. For international trade purposes, the test method should be validated as being sensitive enough to detect the OIE Standard Reference Serum diluted 1/2.

VN cannot be used to differentiate antibodies of vaccinal origin from those caused by natural infection. It is one of the two tests available that complies with the requirement in the OIE Terrestrial Animal Health Code chapter when it refers to 'a diagnostic test to the whole virus'.

Cells: Cells susceptible to infection with PRV are used; they may be cell lines (e.g. PK-15, SK6, MDBK), or primary or secondary cell cultures.

Cell culture medium: The medium depends on the type of cells. For example, the medium for PK-15 cells is Eagle’s minimal essential medium (MEM) + 10% fetal bovine serum and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin, or alternatively, 50 µg/ml gentamycin).

Maintenance of the cells: The cells are cultured in cell culture vessels of, for example, 75 cm². They are trypsinised once or twice per week. For weekly trypsinisation, the cells are cultured in 50 ml of medium, with a multiplication rate of 5. For two trypsinisations a week, the cells are cultured in 30 ml of medium, with a multiplication rate of 3.

For trypsinisation, the growth medium is removed once the cell sheet is complete. The cell sheet is washed with about 5 ml of recently thawed trypsin/ethylene diamine tetra-acetic acid (EDTA) (0.25%) in an isotonic buffer. The washing fluid is discarded and the preparation is washed again, retaining only a few drops of trypsin. The container is placed in an incubator at 37°C for 5–10 minutes until the cells have become detached. Once the sheet is detached and the cells are well separated, they are suspended in 90 ml of growth medium, and this suspension is distributed into three 75 cm² cell culture bottles.

Virus: A suitable strain of PRV, such as the Kojnok strain, or NIA-3 strain, is stored at a temperature of –70°C or below, or in freeze-dried form at 4°C.

Preparation of stock virus suspension: The culture fluid is removed from a cell culture bottle containing a complete cell sheet. About 1 ml of stock virus suspension of known titre (about $10^7$ TCID$_{50}$/ml [50% tissue culture infective dose]) is added, and the bottle is incubated at 37°C for 1 hour. Then, 30 ml of culture medium is added and the bottle is again incubated at 37°C. The bottle is examined frequently until there is about 75% cell destruction (after about 36–48 hours). It is then frozen at a temperature of –20°C or lower in order to disrupt the cells.

The bottle is then thawed and shaken vigorously. Medium is collected and centrifuged at 1500 g for 15 minutes. The supernatant fluid is divided into portions (of about 0.5 ml) in small tubes that are labelled (date and virus reference) before being stored at a temperature of –70°C or lower until required.

Titration of the stock virus suspension: Titration of the stock suspension is performed by the method of Reed & Muench or that of Kärber, and the titre is expressed per 50 µl and per ml.
The VN test requires an internal quality control serum with a known titre of neutralising antibody to PRV (it must be calibrated against an international standard serum or a secondary standard prepared from that serum), and a negative control serum (from a specific antibody free pig, e.g. from an official Aujeszky’s disease free herd). The test sera themselves should be of good quality. Serum should be separated from the coagulum without delay, thereby preventing toxicity.

There are qualitative and quantitative procedures for VN, both of which are described below.

- **Qualitative technique**
  1. Complement in the serum samples is destroyed by heating in a water bath at 56°C for 30 minutes.
  2. Each undiluted serum is placed in three wells, at 50 µl per well, of a 96-well cell-culture grade microtitre plate.
  3. 50 µl of virus suspension containing 100 TCID50 (or 2 × 10³ TCID50/ml), obtained by diluting stock virus suspension of known titre with MEM, is added to each well.
  4. The plate is shaken and placed in an incubator for 1 hour at 37°C (CO2 optional).
  5. 150 µl of cell suspension containing about 150,000 cells/ml is added to each well.
  6. The plate is covered (for incubation in CO2), or a plastic sheet is sealed carefully around the edges of the plate (for incubation in air). The plate is shaken lightly to obtain an even distribution of cells at the bottom of the wells, and placed in the incubator at 37°C (CO2 optional).

- **Controls:** Each set of plates must include the following controls:
  - **Virus control:** This is to verify the amount of virus actually used for the test. The virus dose used for virus neutralisation (target titre 100 TCID50/50 µl) is diluted with MEM at 1/10, 1/100 and 1/1000. Of each dilution, 50 µl is placed in at least eight wells, to which 50 µl of medium is added before the wells are incubated for 1 hour at 37°C. The cell suspension is added in the same way as for the sera under test.
  - **Cell control:** 150 µl cell suspension and 100 µl MEM are placed in each of at least two wells.
  - **Positive serum control:** A serum of known PRV neutralising antibody titre is used. Five dilutions are prepared in the same way as for the sera under test: a dilution corresponding to the serum titre, two-fold and four-fold dilutions, and 1/2 and 1/4 dilutions (equivalent to T, T/2, T/4, 2T and 4T, where T is the serum titre, i.e. undiluted serum for the qualitative test). To 50 µl of positive control sample dilutions, add 50 µl of virus suspension containing 100 TCID50/50 µl. The cells are incubated and the cell suspension is added in the same way as for the sera under test.
  - **Serum control:** This is to verify the absence of a toxic effect of the sera on the cells. Wells containing 50 µl of each serum are incubated for 1 hour at 37°C in the presence of 50 µl of medium. Then, 150 µl of cell suspension is added in the same way as for the sera under test.
  - **Negative serum control:** This is done in the same way as for sera under test.

- **Reading the results:** An inverted-image microscope (×100) is used to examine the wells for toxic effects and CPEs after 48 and 72 hours. The controls must give the following results if the tests are to be considered valid:
  - **Virus control:** The titre of the viral suspension should be between 30 and 300 TCID50/50 µl.
  - **Cell control:** The cell sheet must be intact.
  - **Positive serum control:** The titre obtained must be equal to the predicted titre, within one dilution.
  - **Serum control:** Examination for a CPE should take into account a possible toxic effect on cells.
  - **Negative serum control:** A CPE should be present.

- **Interpretation of the results:** This test is capable of detecting the presence or absence of neutralising antibody to PRV. It is incapable of distinguishing vaccinated animals from infected animals.

The technique described (VN for 1 hour at 37°C) can give false-negative and false-positive results. The sensitivity can be increased (leading to fewer false negatives) by adopting a method based on neutralisation involving 24 hours of contact between virus and serum at 4°C, before the addition of cells.
A qualitative technique such as this one, which employs undiluted serum (1/2 final dilution), can give a false-positive result in certain cases due to nonspecific neutralisation of the virus. This problem can be addressed by carrying out a confirmatory test using the quantitative technique (see below).

### Quantitative technique

This is similar to the qualitative procedure, but each serum is used both undiluted and in a series of dilutions. Depending on the desired precision, the purpose of testing and the expected titre, two wells are used for each dilution of serum, and a greater or smaller range of dilutions. Ideally, the procedure may be described for a range of dilutions reaching an initial maximum of 1/256.

i) Complement in the serum samples is destroyed by heating in a water bath at 56°C for 30 minutes.

ii) 50 µl of MEM is added to wells A3 to A6 of a 96-well cell-culture grade microtitre plate.

iii) 50 µl of undiluted serum is added to wells A1 to A3, and continued for wells in rows B, C, etc., with other serum samples.

iv) Using a multichannel pipette, the contents of wells in row 3 are mixed, then 50 µl is transferred to row 4, and so on to row 6 or further to a predetermined row, using the same nozzles. The 50 µl portions remaining after the last row is discarded.

v) Controls are set up as described for the qualitative technique.

vi) 50 µl of MEM is added to row 1 instead of virus: this is a control row of sera. Viral suspension is deposited in the wells of the other rows. Subsequent manipulations are the same as described for the qualitative technique.

vii) **Reading the results:** The neutralising titre of a serum is expressed by the denominator of the highest initial dilution that brings about complete neutralisation of the CPE of the virus in 50% of the wells. Neutralisation at any dilution (even undiluted, equivalent to a final dilution of 1/2) is considered to be positive. If the serum shows neutralisation only when undiluted (with growth of virus and CPE at the 1/2 and subsequent dilutions), it would be advisable to apply alternative tests (ELISA or latex agglutination) to provide confirmation of the result, or to request another sampling of the animal, at least 8 days after the first.

b) **Enzyme-linked immunosorbent assay (a prescribed test for international trade)**

The sensitivity of the ELISA is generally superior to that of the VN test using 1-hour neutralisation without complement. Some weak positive sera are more readily detected by VN tests using 24-hour neutralisation, while others are more readily detectable by ELISA.

ELISA kits, which are available commercially, use indirect or competitive techniques for measuring antibody levels. They differ in their mode of preparation of antigen, conjugate, or substrate, in the period of incubation and in the interpretation of the results. Their general advantage is that they enable the rapid processing of large numbers of samples. This can also be automated and the results analysed by computer. Some of these kits make it possible to differentiate between vaccinated and naturally infected animals when used with a ‘matching’ vaccine (6, 21, 22). Alternatively, non-commercial ELISA protocols may be adopted (2, 18) provided they are shown to detect the OIE International Standard Reference Serum as positive at a dilution of 1/2 (the minimum sensitivity for international trade purposes). It is recommended to use a kit or in-house assay that has been validated to this standard by external quality control tests by an independent laboratory. A suitable test protocol for whole virus antibodies is presented below (18).

### Preparation of antigen

i) A cell line sensitive to PRV is used, such as PK-15 or fetal pig testis. It must be free from extraneous viruses, such as bovine viral diarrhoea virus. The cells should be split and seeded into fresh 75 cm² flasks the day before inoculation. A suitable medium such as MEM, without serum, is used to overlay the cultures.

ii) Virus inoculated, and control uninoculated flasks are processed in parallel throughout. A suitable well characterised strain of PRV is used, e.g. Kojnock strain. When a confluent cell monolayer has developed (approximately 24 hours after seeding), it is inoculated with 10⁸ TCID₅₀ PRV in 5 ml medium; and 5 ml medium (without virus) is placed in control flasks. The cultures are left for adsorption for 30 minutes at 37°C, and then overlaid with 20 ml medium.

iii) When CPE is just beginning, the supernatant medium is discarded and 4 ml KCl (4 mM solution) and glass beads are added. The flasks are shaken gently to detach cells.

iv) Cells are washed by centrifuging three times at 770 g in 4 mM KCl. The pellet is resuspended in 4 mM KCl with 0.2% Triton X-100 (1 ml per flask) by applying 60 strokes with a glass homogeniser.
v) The cell homogenate is layered on to 0.25 mM sucrose in 4 mM KCl and centrifuged for 10 minutes at 770 g.

vi) The pellet is resuspended in antigen-diluting buffer, pH 9.6 (0.1 M Tris, 2 mM EDTA, 0.15 mM NaCl) at 1/50 the volume of the original culture medium. It may then be stored in small aliquots at –70°C. Antigen is stable in this form for 2 years.

Coating microtitre plates

i) Virus antigen and control (no virus) antigen are diluted in diluting buffer, pH 9.6 (see above) to a dilution predetermined in checkerboard titrations.

ii) 200 µl of antigen is dispensed into each well of 96-well ELISA-grade plates, coating alternate rows with PRV positive and control antigen. Incubation is for 18 hours at 4°C.

iii) The plates are washed three times with washing solution (Tween 20, 0.5 ml/litre).

iv) Coated plates are stored at –20°C or –70°C. They are stable for several months.

Test procedure

i) Test serum samples are diluted 1/30 in PBS/Tween buffer, pH 7.2 (137 mM NaCl, 9.5 mM phosphate buffer, 0.5 ml/litre Tween 20).

ii) Diluted samples are added to virus and control antigen coated wells, and incubated at 37°C for 30 minutes.

iii) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

iv) Protein A/peroxidase conjugate is added to all wells at a predetermined dilution in PBS/Tween buffer, pH 7.2 (see above), with added bovine serum albumen fraction V (10 g/litre), and the plates are incubated at 37°C for 30 minutes.

v) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

vi) A suitable chromogen/substrate mixture, such as tetra methyl benzidine (TMB)/hydrogen peroxide, is added to each plate.

vii) The reaction is stopped with 2 M sulphuric acid. The absorbance is read at 492 nm.

The test must be fully validated using known positive and negative sera, and calibrated against the OIE International Standard Reference Serum. All tests must include positive and negative internal controls, including a weak positive that, when diluted at the appropriate dilution for the test, has equivalent activity to a 1/2 dilution of the OIE International Standard Reference Serum. For further details see reference 18 and Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases. Commercial ELISA kits also have to be validated in the setting in which they are going to be used.

As well as testing sera, the ELISA can be adapted to test filter paper disks that have been moistened with a small quantity of blood obtained by puncturing a superficial vein. This technique makes it convenient to collect blood samples from large numbers of pigs (3, 19). The disks are air-dried before shipment to the laboratory.

Requirements for the detection of gE antibodies by ELISA in pigs destined for slaughter, that are to be introduced into zones free from Aujeszky’s disease, have been defined by several control authorities. The OIE Terrestrial Animal Health Code specifies circumstances in which gE-specific tests may be used. The gE ELISAs can also be adapted to test blood on filter paper disks.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Aujeszky’s disease may be controlled by the use of vaccines containing either modified live or inactivated virus antigens. In addition, these conventional vaccines have been supplemented by recombinant DNA-derived gene-deleted or naturally deleted live PRV vaccines. These vaccines, sometimes referred to as marker vaccines, are made with a virus that lacks a specific glycoprotein (most commonly gE-, although gG- or gC-deleted vaccines have also been described1). At least one commercially available vaccine has dual deletions. These gene-deleted marker vaccines have the advantage over conventional whole virus vaccines that it is possible to distinguish noninfected vaccinated animals from those with field infection. This is done by testing for the antibodies directed against the protein coded for by the deleted gene, which will be absent in noninfected marker-vaccinated pigs but present in field-infected pigs. Therefore, in countries with infected pigs, where the eradication of Aujeszky’s disease is

1 The nomenclature for the genes changed several years ago, but the old designation is still in the literature. The old and the new nomenclature is: g11 – gB; g111 = gC; gp50 = gD; g1 = gE; gX = gG; gp63 = gl.
planned, these marker vaccines are the vaccines of choice. Standards applicable to the manufacture of live and inactivated virus vaccines are described. For marker vaccines, the tests should include demonstrable absence of a serological response in vaccinated pigs to the protein coded for by the deleted gene, and in addition a demonstrable response to the same protein in vaccinated pigs that become infected by field virus.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1. **Seed management**

   a) **Characteristics of the seed**

   Vaccines are made using a seed-lot system in which a master seed virus (MSV) is prepared from a suitable strain of Aujeszky’s disease virus. A number of strains are used for vaccine manufacture. The antigen in an inactivated vaccine can be one of a number of wild-type strains, or the naturally deleted Bucharest virus, or rDNA-derived gene-deleted virus. Modified live conventional vaccines and rDNA-engineered vaccines use numerous strains, such as Bartha (8, 9, 11, 15, 17, 23, 27), or are derived from Aujeszky’s original isolate or from other field isolates, such as the NIA-3 strain.

   It is recommended that for differentiating between infected and vaccinated animals, deleted strains should be used.

   A virus identity test (using either a fluorescent antibody test, neutralisation test, [constant serum/decreasing virus method], or any other suitable identity test) must be conducted on the MSV.

   The MSV must be shown to be free from mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, porcine parvovirus, cytopathic and noncytopathic ovine and bovine pestiviruses and other extraneous agents, such as circovirus, as determined by culturing and by fluorescent antibody procedures or others, such as PCR.

   b) **Method of culture**

   Most of the cell lines used to propagate PRV are continuous lines, such as the PK-15 line. A master cell stock (MCS) is established at a specified passage level. The MCS and the highest passage level (MCS × n) intended for use in the preparation of a biological product is specified in an Outline of Production. Both MCS and MCS × n are monitored by a variety of procedures to characterise the cell line and to ensure freedom from adventitious agents. The extraneous agents to be detected are generally defined in monographs and/or guidelines (e.g. European Pharmacopoeia, US Code of Federal Regulations, EU guidelines, etc.). In general, the type of agents to be looked for is founded on a risk analysis depending on the history of the viral strain and cells on which the vaccinal strain was isolated and on which it is cultivated. The MCS must be monitored for species of origin. A minimum of 50 mitotic cells should be examined at both the MCS and MCS × n passage levels. The modal number in the MCS × n must not exceed 15% of the modal number of the MCS. Any marker chromosomes in the MCS must also be present in the highest cell passage.

   If there is evidence that the cell line may induce malignancies in the species for which the product is intended, the cell line is tested for tumorigenicity and oncogenicity.

   c) **Validation as a vaccine**

   i) **Purity**

   The MSV must be shown to be free from mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, porcine parvovirus, cytopathic and noncytopathic ovine and bovine pestiviruses and other extraneous agents, such as circovirus, as determined by culturing and by fluorescent antibody procedures or others, such as PCR.

   ii) **Stability tests**

   Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.
iii) **Safety tests**

Local and general reactions must be examined. When a live vaccine is used, it is necessary to differentiate the exact safety properties of the vaccinal strain from those of the finished product if this includes an adjuvant.

Objective and quantifiable criteria to detect and measure adverse reactions should be used; these would include temperature changes, weight gain, litter size, reproductive performance, etc., of vaccinated and control groups. The tests must be performed by administering the vaccine to the pigs in the recommended dose and by each recommended route of administration.

In general, safety is tested initially under experimental conditions. When the results of these preliminary tests are known, it is necessary to increase the number of animals vaccinated in order to evaluate the safety of the vaccine under practical conditions.

o **Laboratory testing**

All tests must be carried out on pigs that do not have antibodies against Aujeszky's disease virus or against a subunit of the virus.

a. **General effects**

1. **Live vaccines**

Intranasal tests and vaccination of 3–5-day-old piglets are very useful for ascertaining the degree of safety of a strain. At least five piglets should be used.

It is also essential to assess the properties of a vaccine, especially live ones, in the target animals under normal conditions of use and at the youngest age intended for vaccination, e.g. fattening pigs, which are generally vaccinated when they are between 9 and 12 weeks old, and pregnant sows when this use of the vaccine is claimed by the manufacturer and is authorised. No clinical signs, including significant thermal reactions (data have to be recorded before vaccination and 6 hours, 24 hours and 48 hours later, then on a daily basis during the observation period), should be observed after vaccination. These assays have to be performed on at least ten vaccinated pigs, with unvaccinated pigs as controls.

Reversion to virulence following serial passage must be examined. Primary vaccination is done by the intranasal route. Series of at least five passages in piglets are made. No fewer than two fully susceptible animals must be used for each passage.

The object of these assays is to test the genetic stability of live vaccine strains. The tests appear to be less necessary when a genetically modified live strain is concerned, especially if it is produced by gene deletion.

It is recommended to test for possible excretion of the vaccine strain. For this purpose, no fewer than 14 piglets, 3–4 weeks old each receive one dose of vaccine by the recommended route and at the recommended site (except for vaccines administered by the intranasal route). Four unvaccinated piglets are kept as controls. Suitably sensitive tests for the virus are carried out individually on the nasal and oral secretions of vaccinated and in-contact pigs as follows: nasal and oral swabs are collected daily from 1 day before vaccination to 10 days after vaccination. Vaccine strains that are isolated from the nasal/oral secretion collected from pigs in which the vaccine was administered by the parenteral route are not recommended.

The ability of the Aujeszky's disease vaccine strain to spread from a vaccinated pig to unvaccinated ones (lateral spread) must be tested by using the recommended route of administration that presents the greatest risk of spreading (except for vaccines administered by the intranasal route). A repetition of the assays (four times) is necessary as this phenomenon is difficult to detect. Four piglets should be used each time for vaccination and placed in contact, 1 day later, with two unvaccinated piglets. It may also be necessary to examine the spread of the strain to non target species that may be susceptible to the vaccine strain.

Live attenuated vaccine strains are tested with regard to their general effects by administering to 5–10-day-old piglets ten times the field dose. This administration of an overdose makes it possible to detect reactions not produced under normal conditions of use. Such reactions may be produced inadvertently when large numbers of animals are vaccinated. If vaccines are administered by the intranasal route, the manufacturer has to indicate clearly that the vaccine will spread from vaccinated pigs to unvaccinated ones.

2. **Inactivated vaccines**

It is essential to test inactivated vaccines in the target animals under normal conditions of use for fattening pigs and for sows when this use is claimed by the manufacturer and authorised (26). As described previously, it is fundamental to use objective and quantifiable criteria to detect and to measure
adverse reactions, such as temperature changes, weight performance, litter size, reproductive performance, etc., on vaccinated and control groups. The tests must be performed by administering the vaccine in the recommended dose and by each recommended route of administration to the pigs for which it is intended.

Pigs or sows are usually observed until there is no further evidence of vaccine reaction. The period of observation must not be fewer than 14 days from the day of administration. This period has to be extended when, for example, the vaccine is used in pregnant sows and it is necessary to assess the possible effects of the vaccine on reproductive performance. In this case, the period of observation lasts the full duration of the pregnancy.

Control authorities generally request vaccination with a double dose so that adverse reactions, which may be at the limit of detection when a single dose is administered, are more likely to be detected.

b. Local reactions

Local reactions are often associated with the use of inactivated vaccines, as these side-effects can be induced by adjuvants, particularly oil adjuvants (24). However, some Aujeszky’s disease live vaccines are mixed with different adjuvants, which modify what has been observed in the past.

Local reactions are mainly inflammatory and can be more or less complicated (necrotic or supplicative), depending on the nature of the adjuvants used and the aseptic conditions of the vaccination. Oil adjuvants can induce a variety of effects including muscular degeneration, granuloma, fibrosis and abscessation. In addition to the nature of the oil used (the intensity of the reaction is reduced when metabolisable oils are used in the vaccine), the type of emulsion used (water/oil, oil/water, water/oil/water) induces these reactions to a greater or lesser extent. In consequence, it is necessary to observe the site of injection not only from the outside, but also by dissection after slaughter, especially for growing and finishing pigs.

Field testing

Field trials are necessary to assess the safety of an Aujeszky’s disease vaccine in a large number of pigs or sows. In Europe (7), tests must be carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). At least three groups of no fewer than 20 animals each are used with corresponding groups of no fewer than 10 controls. The rectal temperature of each animal is measured at the time of vaccination, and 24 and 48 hours later. At slaughter, the injection site must be examined for local reactions. If the vaccine is intended to be used in sows, reproductive performances have to be recorded. Field trials are supplemented by laboratory studies of efficacy correlated to vaccine potency.

iv) Efficacy tests

Laboratory trials

All tests must be carried out on pigs that do not have antibodies against Aujeszky’s disease virus or against a subunit of the virus, except that some tests may be done using maternally immune animals.

a. Assessment of passive immunity

To test the efficacy of vaccines, it is important to mimic the natural infection conditions (1). PRV infection gives rise to important losses of young piglets from nonimmune sows. Thus, when vaccinating sows, the main goal is to protect the young piglets through passive immunity conferred by the colostrum ingested immediately after birth, with the secondary objective of preventing abortion.

To measure this passive immunity and the protection induced by vaccinating the sows, experimental models have been established. The sows are vaccinated according to the vaccinal protocol during pregnancy. When the piglets are, for example, 6–10 days old they are given an intranasal challenge exposure with a virulent PRV strain. It is preferable to use a strain titrated in median lethal doses (LD50). Pigs should be inoculated by the nasal route, 10^2 pig LD50 per pig in 1 ml. The efficacy of the vaccine is assessed by comparing clinical signs, but also and more importantly, mortality in piglets from unvaccinated dams with that observed in piglets from vaccinated sows.

Piglets from vaccinated sows can be found to have 80% protection against mortality compared with those from the control sows. In order for the results to be significant, it is recommended that eight vaccinated sows and four control sows be used (subject to satisfactory numbers of piglets from each sow).

b. Assessment of active immunity

1. Clinical protection

Several criteria can be considered when measuring active immunity induced by vaccinating pigs. Generally, pigs are vaccinated at the beginning of the growing period, i.e. when they are between 9 and
12 weeks old. Laboratory trials are performed by challenging pigs at the end of the finishing period, when they weigh between 80 and 90 kg.

In general, at least three criteria, such as rectal temperatures, weight losses and clinical signs, along with mortality, are used to measure the clinical protection of pigs after vaccination and challenge (5). The antibody titres have little predictive value for the efficacy of the vaccines. Weight loss compared between the vaccinated and control groups is the most reproducible and reliable parameter when the challenge conditions are well standardised. The measure of the difference in weight gain or loss between the two groups of pigs and, in the interval of time between challenge (day 0 and day 7), has a very good predictive value for the efficacy of the vaccines (14). Significant results can be obtained when weight performances are compared between one group of at least eight vaccinated pigs and another group of eight unvaccinated control pigs

For challenge, it is usually preferable to use a high titre of a virulent strain, as this makes it possible to obtain a more marked difference between vaccinated and control pigs. On the basis of previous work, a challenge dose with at least $10^6$ TCID$_{50}$/ml virulent strain having undergone not more that three passages on primary cells can be sufficient, but a higher titre ($10^{7.5}$ TCID$_{50}$/ml) is recommended. The oro-nasal route should be used to challenge the pigs by introducing the virulent strain in an appropriately high volume ($\geq 4$ ml).

This method of evaluating the efficacy of PRV vaccines is now well tested and has made it possible to establish an objective index for determining the efficacy of a vaccine. This index, which compares the relative weight losses between vaccinated and control pigs, can also be used for potency testing batches before release and for batch efficacy testing. However, the value of the cut-off index will be different as the conditions of the assay will not be identical. The influence of passively acquired, maternally derived antibodies on the efficacy of a vaccine must be evaluated adequately.

2. Virulent virus excretion

Additionally, it is desirable that vaccines should prevent or at least limit viral excretion from infected pigs (13, 20, 25). When a control programme against Aujeszky’s disease is based on large-scale vaccination, it is essential to choose the vaccines or the vaccinal scheme that best limits the replication of virulent virus in infected pigs. Several assays have been performed to compare vaccines on that basis.

Generally, the pigs are vaccinated and challenged at different periods. It is better, but more time-consuming, to infect pigs at the end of the finishing period. To measure the virus excretion, nasal swabs (taken at 10 cm depth in the nostrils) are taken daily from each pig from the day before challenge to at least 12 days after challenge. The swabs can be weighed before the sampling and immediately after to calculate the exact weight of collected mucus. Medium is then added to each tube containing a swab. The virus is titrated from the frozen and thawed medium.

Different indexes can be used to express the quantity of virulent virus excreted by pigs, taking into consideration the duration and the level of viral excretion, and the number of pigs excreting virulent virus.

3. Duration of immunity

It is recommended that any claims regarding the onset and duration of immunity should be supported by data from trials. Assessment of duration of immunity can be based on challenge trials or, as far as it is possible, on immunological and serological tests.

- Field trials

In general terms, it is extremely difficult to assess vaccine efficacy in animal populations. In order to do this, it would be necessary to vaccinate the animals in the absence of the pathogen that the vaccine protects against, then to await the moment of infection and to compare the effects of infection in vaccinated animals (or the offspring of vaccinated dams) with the effects in the unvaccinated animals of the same age, in the same building and in the same batch as the vaccinated animals (or those protected passively). As all these conditions are difficult to achieve in the field, field trials are certainly more appropriate to safety testing than to efficacy testing.

2. Method of manufacture

Only MSV that has been established as pure, safe and immunogenic may be used as seed for a vaccine product. Cells from the MCS are propagated in a variety of growth media. All batches of vaccine must be from the first to the twentieth passage of MCS.
3. In-process control

It is necessary to carry out tests at each critical step of the manufacturing process. The control tests are also carried out on intermediate products with a view to verifying the consistency of the production process and the final product.

4. Batch control

It is essential to differentiate the tests that are carried out on a routine basis to release batches of final product from those that are performed to define the biological properties of a vaccine. The trials carried out for batch release are not the same as the ones carried out once only to determine the safety and efficacy of a vaccine. The batch release controls are always short-term trials, as inexpensive as possible, and not always carried out in pigs. Their purpose is mainly to attest to the reproducibility of the quality of the finished product, which has to be in compliance with the quality initially defined in the application for marketing authorisation.

a) Sterility and purity tests

Tests must be carried out for sterility and freedom from contamination (see Chapter 1.1.9).

Each batch of PRV vaccines must be tested for freedom from extraneous viruses. Using a minimum amount of a monospecific antiserum, the live vaccinal strain is neutralised and inoculated into cell cultures known to be sensitive to viruses pathogenic for pigs. No CPE and no haemadsorbing agents should be detected. The vaccines have to be free from pestiviruses.

b) Inactivation

For inactivated vaccines, inactivation must be checked using two passages in the same type of cell culture as used in the production of the vaccine. Tests can be carried out by vaccinating susceptible animals such as rabbits.

c) Identity

Where necessary, a specific test for virus identification should be carried out.

d) Safety

Safety of live vaccines is tested by administering ten doses of the reconstituted vaccine by the route stated on the leaflet to each of at least two piglets of the minimum age recommended for vaccination that are free from PRV antibodies. Two piglets of the same origin and age are kept as controls. No abnormal local or systemic reaction should occur. The weight curve of the vaccinated piglets must not differ significantly from that of the controls.

For inactivated vaccines, safety is tested by injecting two doses into piglets under the same conditions as described previously.

e) Potency

The potency of the vaccine must be demonstrated using a suitable method, the results of which have to be correlated with the efficacy tests described previously.

In this kind of test, the most difficult point is to determine an acceptability threshold for using or rejecting the batch according to the results that are obtained.

Virus content tests should be carried out using each of at least three containers. The virus titre of the vaccine must be determined and must normally not be higher than 1/10 of the dose at which the vaccine has been shown to be safe, and not lower than the minimum release titre.

f) Preservatives

If no preservative is included in the final product, the manufacturer must demonstrate that the product remains acceptable for its recommended period of use after opening the vial.

The efficacy of preservatives in multidose containers must be demonstrated. The concentration of the preservative in the final filled vaccine and its persistence throughout shelf life must be checked.
g) **Precautions (hazards)**

All information about possible adverse reactions induced by the vaccine must be indicated. Any putative risk for human health if the user is accidentally given a small quantity of the product has to be indicated. The manufacturer should indicate all the conditions of use of the vaccine: mixing, reconstitution, storage, asepsis, length of needle, route of administration and health status of the vaccinated animals.

5. **Tests on the final product**

a) **Safety**

Every batch of vaccine must be tested for safety, as described in Section C.4.d.

b) **Potency**

Every batch of vaccine must be tested for potency, as described in Section C.4.e.

**REFERENCES**


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NB: There are OIE Reference Laboratories for Aujeszky’s disease (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).