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| Report of the Meeting of the WOAH Biological Standards Commission | Original: English (EN)9 to 13 September 2024Paris |

**USA EDITS IN BLUE FONT**

# **Annex 6. Chapter 1.1.7. Standards for High Throughput Sequencing, Bioinformatics and Computational Genomics**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 9–13 September 2024**

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Chapter 1.1.7.

STANDARDS FOR HIGH THROUGHPUT SEQUENCING, BIOINFORMATICS AND COMPUTATIONAL GENOMICS

…

**c. STANDARDS FOR THE USE OF HTS-BCG**

**3. Specimens and sample preparation**

Specimens should be collected and submitted to the testing laboratory in accordance with the standards communicated in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens.* The normal comprehensive information regarding the individual animal, the case or reason for sampling and the relevant epidemiological information should be recorded in the laboratory’s accessions processes, as for any submission to the laboratory.

As with other laboratory processes, ensuring the integrity of the specimen and the samples to be tested is critical. Nucleic acids, either DNA or RNA, need to be extracted from the samples. In some cases, host depletion or targeted enrichment strategies may be applied during extraction or library preparation to increase the ratio of pathogen to host nucleic acids ~~can be used to~~ and maximise the sensitivity of the technique. However, care must be taken to avoid biasing the outcome in the context of the intended purpose. Precautions to ensure the integrity and quality of nucleic acids must be followed similarly to any other molecular technique (e.g. polymerase chain reaction [PCR]) as already described in Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*. Those precautions are particularly relevant in HTS applications as they rely on nucleic acid fragment length and microorganism genome coverage for effective pathogen detection and identification, as opposed to PCR assays that amplify only a short fragment of nucleic acid. Once nucleic acids are extracted from the samples, they need to be further manipulated (e.g. reverse transcription of RNA into complementary DNA) in order to be used in HTS. Different technological platforms require specific sets of reagents in order to generate the final material (“libraries”) ready for sequencing. Commercial kits are available for this purpose.

HTS is an extremely sensitive technology and even few molecules of nucleic acid ~~could~~ can be detected. Hence, precautions to avoid cross-contamination~~s~~ must be followed as in the case of ~~many~~ other molecular techniques used to detect nucleic acids (e.g. PCR). Separation of work areas and decontamination of working spaces is an essential requirement to avoid cross contamination with nucleic acid ~~from other molecular investigations Separation of work areas from the possibility of cross contamination with nucleic acid from other molecular investigations is an essential requirement~~. In addition, HTS very frequently involves “multiplexing” of several samples in a single reaction. Individual samples are “tagged” during one of the stages of sample preparation by the use of short index sequences linked to nucleic acid molecules. Best practices should include index usage rotation ~~in benches and sequencers~~. Index sequences must be of sufficient quality and design to be relied on as a signature for the tagged library for HTS use in order to avoid artefacts during bioinformatics analysis of sequencing data obtained. Furthermore, index misassignment metrics including index hopping should be determined for each workflow and instrument, as well as instrument carryover, when applicable. When available, the amount of the target sequence should be determined by prior analyses to inform sample-batch processing.

**Category:** [addition, deletion, change, editorial, general]

Deletion

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Clarifying.

**Supporting evidence:**

Every application of HTS-BCG technology should include positive and negative controls appropriate to the investigation and that have been incorporated through the sample preparation processes of the sequencing run as well as the actual run on the technology platform. The use of unique molecules spiked in each sample is highly advisable to monitor intra-batch contamination, mainly in workflows using enrichment methods. Appropriate controls should be used to verify each step of the procedure including nucleic acid quality, library preparation, cross-contamination (including multiplexing) sensitivity and reproducibility.

As with any other diagnostic method, confirmation of results ~~would~~ may require resampling of the original specimen, which therefore has to be protected from cross-contamination and be stored appropriately. Validation of HTS results could also be achieved using a different diagnostic method.

**4. Generation of sequence data**

While HTS platforms differ widely in ~~their details~~ chemistry and protocols, basic principles of quality control relevant to the technology can be followed, and generic recommendations for acceptable quality metrics can be made. Suitable control measures might include the use of positive, negative and no-template controls run in replicates of the test, iteratively covering different locations in the multiwell plate, and a quality scoring system. Sequencing quality metrics provide suitable parameters for the validation and monitoring of platform performance. Most platforms offer the possibility to spike controls in reagents and to use the control’s QC metrics to monitor platform and reagent performance. Additional technology specific performance metrics can be used to monitor platform performance and to identify aberrant sequencing runs. Equally, maintenance of sample handling log files facilitates investigations in case of suspected contaminations that may lead to false positives.

Quality metrics for the evaluation of the analytical performance of HTS-based tests, include:

i) Depth of coverage. This indicates the number of sequence reads providing information about a given nucleotide position. When ongoing quality monitoring shows that the coverage depth at a given nucleotide is below the validated minimum coverage, confirmation should be provided using alternate methods (e.g. Sanger sequencing) or additional sequencing.

ii) Uniformity of coverage. This parameter describes how the depth of coverage is distributed over the test’s target region(s). Coverage across a pathogen genome is rarely uniform. However, deviations of uniformity of coverage from the validated range can potentially indicate errors in the testing process.

iii) GC bias. The GC content (relative abundance of G and C nucleotides) of a target region affects the efficiency of sequencing reactions and will affect the uniformity of coverage. ~~Where possible, the amount of GC bias in the test’s target region(s) should be determined during validation and monitored to evaluate test performance.~~

iv) Low complexity regions. Homopolymer regions and microsatellites in a target region (or close to a target region) may compromise accuracy. This should be determined during validation and if not possible to avoid, carefully assess performance.

v) Base call quality scores. These are platform-derived reflections of the signal-to noise ratio and reflect the probability that the base call was correct. An acceptable raw base call quality threshold should be established during validation, and incorporated in bioinformatics filters to eliminate poor quality data during analysis.

vi) Decline in signal intensity or read length. Depending on the exact application, HTS platform and chemistry, sequence reads have a typical distribution of read length and signal intensity. The expected signal intensity across reads (or read length distribution) should be established during validation and monitored for each run. Deviations in the distribution of read lengths may indicate problematic datasets. “Reads “trimming” is usually applied by removing low-quality bases or adapter sequences from raw reads, aiming therefore to balance read retention and read accuracy. The choice on the trimming threshold depends on the diagnostic question and the required read accuracy. For example, identifying a co-infection with two similar viral strains or detecting drug resistance at a sub-consensus level requires higher data quality.

vii) Mapping quality. This is a measure of uncertainty that a read is mapped properly to a genomic position within the target region. Acceptable values (e.g. proportion of reads mapping to the target) should be established during validation of bioinformatics workflows and the proportion of reads not mapping to the target can be monitored during each run.

viii) Internal controls. Most platforms offer the possibility to spike an internal control at very low frequency during the sequencing run. The quality metrics of those reads can be compared to previously reported quality metrics.

**5. Bioinformatics**

An absolute requirement for any laboratory intending to establish a HTS-BCG capability is the employment (or partnership) of specialised staff with bioinformatics skills. Even if platforms with supporting software for specific analyses in defined clinical situations ~~were to become~~ may be available, the use of such packages would not remove the responsibility of the laboratory to be able to competently analyse its own data.

The bioinformatic analysis assembling the pathogen genomic sequence from the raw data and the subsequent secondary analysis are the critical elements in HTS-BCG. Hence the approaches used must be transparent, with clear guidelines on how to record bioinformatic metadata, including a declaration of the software packages, software versions, and reference databases or sequences used should be a component of every report of sequence analysis. Software programs used for these analyses must be readily available (commercially or open access) in order to be evaluated by the international community.

As with any laboratory procedure, attention must be given to quality assurance. The test method should include criteria for acceptance or rejection of each run based on the satisfactory analyses of the controls and adequate reference material. Sequencing data must be documented to have satisfied minimum quality scores and coverage for each nucleotide of the assembled final consensus sequence obtained.

The appropriateness of chosen bioinformatics software for particular analyses can be evaluated through testing its performance against standard data sets containing data relating to agents expected to be present in the specimens to be tested and by comparing and benchmarking different bioinformatic tools.

**6. Data management**

The data generated from HTS-BCG operations are essential to reach the diagnosis or other scientific purpose of the investigation, such as agent characterisation, and are an integral component of the process. As such it is an essential requirement of laboratories to have policies, processes and supporting systems to curate, manage and store the data generated.

Different HTS technology platforms produce raw data in different formats and stage of pre-analysis, so it is necessary for laboratories to have policies and processes specific to the technology platform in use. Data management systems will include aspects of which data to keep, ~~and~~ the length of time for which they will be kept, and the back-up strategies to protect against accidental loss or deliberate erasure, and submission to national or international archives or databases. Metadata describing the generation and analysis of the sequence data is essential, so that the process itself can be analysed or repeated.

Where a sequence analysis leads to an output of animal health significance, especially one of trade or international significance, it is an absolute requirement that the data on which the analysis was performed be kept available for audit or confirmatory analysis for a period of time commensurate with the significance of the animal health finding. This is particularly important where the finding may be disputed. Failure to be able to produce the required data for independent analysis could be taken to invalidate the finding.

Sequence data should be stored in a manner in which there is a clear link to the metadata associated with the specimen that was the subject of the analysis. As is standard practice in laboratory investigations, such metadata includes information regarding the animal sampled, its ownership and location, and accompanying clinical and epidemiological information regarding the animal population.

Careful consideration should be given to platform ~~compatible~~ compatibility with integration of data from different sources namely sample, clinical and sequencing metadata.

**Category:** [addition, deletion, change, editorial, general]

Change.

**Proposed amended texts (or precise suggested deletion):**

compatibility

**Rationale:**

**Supporting evidence:**

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# **Annex 17. Chapter 3.1.21. Rinderpest (Infection with Rinderpest Virus)**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 9–13 September 2024**

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Chapter 3.1.21.

rinderpest
(infection with rinderpest virus)

**1. Detection and identification of the agent**

Any suspicion of rinderpest must be viewed as a potential threat to international biosecurity and must be rapidly confirmed or differentiated. RT-PCR is the most rapid and specific test. If RPV is confirmed, back-tracing measures must be immediately instigated. In addition, samples must be sent to a WOAH Reference Laboratory for rinderpest for final confirmation of the diagnosis, and the virus origin should be identified by sequencing and comparison with known RPV genomic data. If possible, the virus should be isolated (Anderson *et al.,* 1996), though this should only be attempted in a WOAH Reference Laboratory ~~an FAO-WOAH approved Rinderpest Holding Facility~~.

**1.1. Virus isolation**

RPV can be cultured from the leukocyte fraction of whole blood that has been collected into heparin or EDTA (ethylene diamine tetra-acetic acid) at final concentrations of 10 international units (IU)/ml and 0.5 mg/ml, respectively. Samples should be thoroughly mixed and transferred to the laboratory on ice, but never frozen. On average, the onset of viraemia slightly precedes the onset of pyrexia, and may continue for 1–2 days after pyrexia begins to wane. Consequently, animals showing a pyrexia are probably viraemic and therefore the best source of blood with which to attempt virus isolation. However, as occasional febrile animals may no longer be viraemic, samples from several febrile animals should be collected for submission. Virus can also be isolated from samples of the tonsil, spleen, prescapular or mesenteric lymph nodes of dead animals; these samples may be frozen for transportation. Transportation must be under biosecure conditions in compliance with international transport regulations described in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*, Chapter 1.1.3 *Transport of biological materials* and with the Guidelines for Rinderpest Virus Sequestration.

To isolate the virus from blood, uncoagulated blood is centrifuged at 2500 ***g*** for 15 minutes to produce a buffy coat layer at the boundary between the plasma and erythrocytes. This is removed as cleanly as possible, mixed in 20 ml physiological saline and recentrifuged in a washing procedure designed to remove any neutralising antibody present in the plasma. The resulting cell pellet is suspended in cell culture maintenance medium and 2 ml aliquots are distributed onto established monolayers of primary calf kidney, B95a marmoset lymphoblastoid, *Theileria*-transformed bovine T lymphoblast or African green monkey kidney (Vero) cells, preferably Vero cells expressing morbillivirus receptor SLAM. These cells may be cultured in roller tubes, culture flasks or multiwell plates.

Alternatively, 20% suspensions (w/v) of post-mortem tissue may be used. These should be made by macerating the solid tissues in serum-free culture maintenance medium using standard grinding or shearing techniques and inoculating monolayers as before. The release of virus from solid tissue can be achieved in several ways. Perhaps the easiest is with a pestle and mortar, but this technique requires the use of sterile sand as an abrasive. Alternatively, tissue may be ground without an abrasive using all-glass grinders, for example, a Ten Broeck grinder. Shearing techniques are equally applicable using laboratory blenders. Virus-containing suspensions are clarified by low-speed centrifugation. The volume of the inoculum is not critical; a working volume is between 1 and 2 ml. Commonly used antibiotics are penicillin and streptomycin in combination, ~~each~~ at a concentration of 100 IU/ml or 100 µg/ml, respectively. A similar broad-spectrum cover can be obtained using neomycin at 50 µg~~µl~~/ml. Amphotericin B should be included at 2.5 µg/ml.

The inoculum should be removed after 1–2 hours and replaced with fresh medium. Thereafter, the culture maintenance medium should be decanted and replaced every 2 or 3 days and the monolayer observed microscopically for the development of cytopathic effects (CPE). These are characterised by refractility, cell rounding, cell retraction with elongated cytoplasmic bridges (stellate cells) and/or syncytium formation. The speed with which the CPE develops varies by substrate and probably by strain of virus also. Up to 12 days should be allowed in primary cells, a week in Vero and 2–5 days in B95a cells or Vero cells expressing SLAM. Blind passages may be attempted before declaring an important sample negative. Isolates of virus can be ~~partially identified by the demonstration of morbillivirus-specific precipitinogens in infected cell debris~~ ~~or~~ completely identified by ~~either~~ RT-PCR using RPV-specific primers (see below) ~~or the demonstration of specific immunofluorescence using a RPV-specific monoclonal antibody~~.

**Category:** [addition, deletion, change, editorial, general]

Deletion.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Editorial correction.

**Supporting evidence:**

# **Annex 24. Chapter 3.4.13. Theileriosis in Cattle (infection with *Theileria annulata*,**

# ***T. orientalis* and *T. parva*)**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 9–13 September 2024**

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Chapter 3.4.13.

THEILERIOSIS in cattle
(Infection with *Theileria annulata*, *T. orientalis* and *T. parva*)

**A. introduction**

Theileriaeare obligate intracellular protozoan parasites that infect both wild and domestic *Bovidae* throughout much of the world. They are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts. There are a number of species of *Theileria* spp. that infect cattle; the ~~two~~ three most pathogenic and economically important are *T. parva,* ~~and~~ *T. annulata* and pathogenic strains of *Theileria* *orientalis*. *Theileria parva* occurs in 13 countries in sub-Saharan Africa causing three disease syndromes namely East Coast fever (ECF), Corridor disease (CD) and Zimbabwean theileriosis (January disease, JD). These syndromes differ in the origin of the parasite, ECF and JD being tick transmitted from cattle to cattle (cattle-derived) and CD from buffalo to cattle (buffalo-derived). ~~whilst~~ *Theileria annulata* (tropical/Mediterranean theileriosis) occurs in southern Europe as well as North Africa and Asia. Endemic regions of *T. annulata* and *T. parva* do not overlap extensively. *Theileria annulata* can occur in cattle, yaks, water buffalo and camels and is transmitted by ticks of the genus *Hyalomma*. Tropical theileriosis is more severe in European breeds, with a mortality rate of 40–90%, while the mortality rate in indigenous breeds of cattle from endemic areas can be as low as 3%. In Spain, *T. annulata* infections are mainly restricted to the southern and Mediterranean areas such as Menorca island, where the tick vector (*Hyalomma* sp.) is present. In northern Spain, reports of the presence of *Hyalomma* ticks are sporadic, as are associated *T. annulata* infections. However, tick distribution might change because of changes in climatic conditions.

*Theileria orientalis~~/buffeli~~* ~~complex is now thought to consist of two species~~ has at least two strains of economic importance, namely Chitose and Ikeda ~~–~~ *~~T. orientalis~~*~~, occurring in the far east, and~~ *~~T. buffeli~~* ~~having a global~~ distribution ~~(Gubbels~~ *~~et al~~*~~., 2000; Jeong~~ *~~et al.,~~* ~~2010)~~. Infection is generally subclinical; however, disease can occur in cattle depending on a number of epidemiological factors (including previous exposure to theileriae, stress or health status, and variations in ~~the species~~ strain pathogenicity, as reported recently in ~~Australia and New Zealand~~ Australasia ~~(Gebrekidan~~ *~~et al.,~~* ~~2015; McFadden~~ *~~et al~~*~~., 2011)~~. The main tick vector, *Haemaphysalis longicornis* accompanied by the Ikeda strain has spread from ~~to North the America~~ Australasia to North America (Hutcheson *et al.,* 2019).

**Category:** [addition, deletion, change, editorial, general]

Editorial.

**Proposed amended texts (or precise suggested deletion):**

Perhaps the sentence should read "from Australasia to North America".

**Rationale:**

Some words appear to be missing from the last sentence.

**Supporting evidence:**

*Theileria taurotragi* and *T. mutans* generally cause no disease or mild disease, and *T. velifera* is nonpathogenic. These last three parasites are found mainly in Africa, and overlap in their distributions, complicating the epidemiology of theileriosis in cattle. This has been further complicated by the finding of multiple related genotypes in cattle and buffalo, suggesting a genetically diverse population of *Theileria* circulating in bovids.

*~~Theileria lestoquardi,~~* ~~also transmitted by~~ *~~Hyalomma~~* ~~ticks, is the only species of economic significance infecting small ruminants and it also occurs in north Africa, the Mediterranean basin and Asia. In sheep and goats, the morbidity rate from~~ *~~T. lestoquardi~~* ~~can approach 100% with a mortality rate of 46–100% in the most susceptible breeds.~~ *~~Theileria uilenbergi~~* ~~and~~ *~~T. luwenshuni~~* ~~are pathogenic ovine piroplasms described in north-western China (People’s Rep. of), though~~ *~~Theileria~~* ~~parasites with similar sequences have been found in sheep in northern Spain and Turkey, but apparently with a low pathogenicity.~~ *~~Theileria luwenshuni~~* ~~has also been detected in sheep in the United Kingdom associated with clinical signs (Phipps~~ *~~et al~~*~~., 2016).~~

Some *T. parva* stocks produce a carrier state in recovered cattle, and studies using DNA markers for parasite strains have shown that *T. parva* carrier animals are a source of infection that can be transmitted naturally by ticks in the field ~~(Bishop~~ *~~et al~~*~~., 1992; Kariuki~~ *~~et al~~*~~., 1995; Marcotty~~ *~~et al~~*~~., 2002; Maritim~~ *~~et al~~*~~., 1989)~~. The severity of ECF may vary depending on factors such as the virulence of the parasite strain, sporozoite infection rates in ticks and genetic background of infected animals. Indigenous cattle in ECF-endemic areas are often observed to experience mild disease or subclinical infection, while introduced indigenous or exotic cattle usually develop severe disease.

The most practical and widely used method for the control of theileriosis is the chemical control of ticks with acaricides. However, tick control practices are not always fully effective for a number of reasons, including development of acaricide resistance, the high cost of acaricides, poor management of tick control, and illegal cattle movement in many countries. Vaccination using attenuated schizont-infected cell lines has been widely used for *T. annulata*, while for *T. parva* control, infection and treatment using tick-derived sporozoites and tetracycline is being implemented in a number of countries in eastern, central and southern Africa.

Chemotherapeutic agents such as parvaquone, buparvaquone and halofuginone are available to treat *T. parva* and *T. annulata* infections. Treatments with these agents rely on early detection of clinically affected animals and do not completely bring about eradication of theilerial infections, leading to the development of carrier states in their hosts.

~~The immune response to theileriae parasites is complicated. Cell-mediated immunity is thought to be the most important protective response in~~ *~~T. parva~~* ~~and~~ *~~T. annulata~~*~~. In~~ *~~T. parva,~~* ~~the principal protective responses are mediated through killing of infected cells by bovine major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes.~~ *~~Theileria annulata~~* ~~schizonts~~~~inhabit macrophages and B cells. Innate and adaptive immune responses cooperate to protect cattle against~~ *~~T. annulata~~* ~~theileriosis. Intracellular parasites are mostly affected by cell-mediated immunity. Infection of leukocytes with~~ *~~T. annulata~~* ~~activates the release of cytokines, initiating an immune response and helping to present parasite antigen to CD4~~~~+~~ ~~T cells. These cells produce interferon-γ (IFN-γ), which activates non-infected macrophages to synthesise tumour necrosis factor α (TNF-α) and nitric oxide (NO), which destroy schizont- and piroplasm-infected cells. CD8~~~~+~~ ~~T cells have recently been shown to recognise parasite antigens presented by the MHC and to kill infected leukocytes. B cells produce antibody that along with NO kill extracellular merozoites and intracellular piroplasms. On the other hand, overproduction of cytokines, in particular TNF-α, by macrophages generates many of the clinical signs and pathological lesions that characterise~~ *~~T. annulata~~* ~~theileriosis and the outcome of the infection depends upon the fine balance between protective and pathological properties of the immune system.~~

**b. DIAGNOSTIC TECHNIQUES**

Diagnosis of acute theileriosis is based on clinical signs, knowledge of disease, and vector distribution as well as examination of Giemsa-stained blood, lymph node and tissue impression smears. *Theileria parva* and *T. annulata* are diagnosed by the detection of schizonts in white blood cells or piroplasms in erythrocytes. The piroplasmic stage follows the schizont stage and, in both *T. parva* and *T. annulata*, it is usually less pathogenic and is thus often found in recovering or less acute cases. Infection with other *Theileria* parasites that also produce schizonts or piroplasms complicate the use of microscopy as diagnostic technique. ~~It is hoped that~~ A combination of serological ~~enzyme-linked immunosorbent assay (ELISA)~~ and polymerase chain reaction (PCR) ~~will~~ greatly enhance our present capacity to identify infected animals, thus making possible accurate surveys of *Theileria* species. ~~Eventually, the aim would be to develop these technologies for the diagnosis of all the vector-borne diseases.~~

Table 1. Test methods available for the diagnosis of theileriosis and their purpose

| Method | **Purpose** |
| --- | --- |
| Population freedom from infection(a) | Individual animal freedom from infection prior to movement(a) | Contribute to eradication policies | Confirmation of clinical cases(a) | Prevalence of infection – surveillance(a) | Immune status in individual animals or populations post-vaccination |
| Detection and identification of the agent(b) |
| Microscopic examination | – | ++ ~~+~~ | – | +++ | – | – |
| PCR | ++ | +++ | ++ | +++ | ++ | – |
| Detection of immune response |
| IFAT | + | ~~+++~~ ++ or + | ++ | – | +++ | – |
| ELISA | + | + | ++ | – | +++ | – |

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
(a)See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.
(b)A combination of agent identification methods applied on the same clinical sample is recommended. This is because parasitaemia may fluctuate below agent detection limits in carrier animals, while antibodies may still be detected using serology. Conversely, in early infection, or in cases where a carrier is not exposed to vector and parasite challenge, the antibody titre may fall below the detection limit, while still testing positive for the agent.

**Category:** [addition, deletion, change, editorial, general]

Change.

**Proposed amended texts (or precise suggested deletion):**

Pleased see text in blue above.

**Rationale:**

The IFAT's use in Appendix 1 lists the utility of IFAT for movement as + for both T. annulata and T. parva. Further, as described, the IFAT is not established at all for T. orientalis; so the most appropriate indication in the chart for IFAT should indeed be + at the most.

**Supporting evidence:**

Appendix 1, page 553.

**1. Detection and identification of the agent**

**1.1. Microscopic examination**

Multinucleate intralymphocytic and extracellular schizonts can be found in Giemsa-stained biopsy smears of lymph nodes, and are a characteristic diagnostic feature of acute infections with *T. parva* and *T. annulata.* Both intracellular and free-~~lyin~~g living schizonts may be detected, the latter having been released from parasitised cells during preparation of the smears. Schizonts are transitory in *T. mutans* and the *T. orientalis/buffeli* group, in which the piroplasm stage may be pathogenic. *Theileria taurotragi* schizonts are not readily detected in Giemsa-stained blood smears. A veil to the side of the piroplasm may ~~distinguish~~indicate *T. velifera*. The schizonts of *T. mutans*, if detected, are distinct from *T. parva,* having larger, flattened, and irregular nuclear particles. The piroplasms (intra-erythrocytic stage) of *T. parva, T. annulata* and *T. mutans* are similar, but those of *T. annulata* and *T. mutans* are generally larger and may be seen to divide. However, for practical purposes schizonts and piroplasms of different theilerias are very difficult to discriminate in Giemsa-stained smears.

**Category:** [addition, deletion, change, editorial, general]

Editorial.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Typographical error.

**Supporting evidence:**

# **Annex 33. Section 3.9. Suidae**

**MEETING OF THE BIOLOGICAL STANDARDS COMMISSION**

**Paris, 9–13 September 2024**

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section 3.9.

suidae

Chapter 3.9.1.

african swine fever
(infection with african swine fever virus)

**A. introduction**

The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF spread to eastern European countries extending westwards and reaching the European Union in 2014. Further westward and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar – were affected by the disease. In August 2018, the People’s Republic of China reported its first outbreak of ASF and further spread in Asia has occurred. ASF was identified on the island of Hispaniola (Haiti and the Dominican Republic) in 2021. See WAHIS (<https://wahis.woah.org/#/home>) for recent information on distribution at the country level.

ASF virus (ASFV) is a complex large, enveloped DNA virus with icosahedral morphology. It is currently classified as the only member of the *Asfaviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo *et al.,* 2018). More than a hundred infection-associated proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus genome. The complete genomes of several ASFV strains have been sequenced (Bishop *et al.,* 2015; Chapman *et al.,* 2011; de Villiers *et al.,* 2010; Portugal *et al.,* 2015). Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated by sequencing of the 3’ terminal end of the B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach *et al*., 2017; Boshoff *et al.,* 2007; Quembo *et al.* 2018). To distinguish subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo *et al.,* 2009; Lubisi *et al.,* 2005; Nix *et al.,* 2006) and in the intergenic region between the I73R and I329L genes, at the right end of the genome (Gallardo *et al.,* 2014), is undertaken. Several other gene regions such as the E183L encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track virus spread.

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act as reservoir hosts of ASFV in Africa (Costard *et al.,* 2013; Sánchez-Vizcaíno *et al.,* 2015).

The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed. Case fatality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce subclinical non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals that have recovered from either acute, subacute or chronic infections may potentially become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

~~As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease, particularly in subacute and chronic forms.~~

Vaccines should be prepared in accordance with Chapter 1.1.8 *Principles of veterinary vaccine production*. Current ASF modified live virus (MLVs) vaccines are based on the live virus that have been naturally attenuated or attenuated by targeted genetic recombination through cell cultures (Gladue & Borca, 2022). MLV production is based on a seed-lot system consistent with the *European Pharmacopoeia* (11th edition) and that has been validated with respect to virus identity, sterility, purity, stability potency, ~~stability, safety~~ and immunogenicity ~~(including spread), non-transmissibility, stability and immunogenicity~~. ASF MLV first generation vaccines ~~– defined as those for which peer-reviewed publications are in the public domain –~~ should meet or exceed the minimum standards as described below. ~~Paramount~~ Demonstration of acceptable safety and efficacy against the epidemiologically relevant ASFV field strain(s) circulating in areas where the vaccine is intended for use ~~are~~ is required. At the present time, a variety of mutants (Forth *et al.,* 2023) and recombinants (Zhao *et al.,* 2023) have emerged globally, and the prevalence of these strains is increasing. In addition, there is a risk that vaccine strains ~~will~~ might be transmitted to non-vaccinates and/or revert to virulence and/or recombine with circulating strains. These conditions should be taken into account in vaccine development. ~~acceptable efficacy should be shown against the B646L (p72) genotype II pandemic virus lineage currently circulating widely in domestic pigs and wild boar.~~

**Category:** [addition, deletion, change, editorial, general]

Addition.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

This was removed above ("non-transmissibility)" and not addressed in the inserted text. This is a major factor in evaluation of these vaccines and should be accounted for in the text.

**Supporting evidence:**

ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated animals (DIVA) by suitable methods (e.g. serology-based tests) are preferred. Minimum standards set out in this chapter include safety and efficacy testing in young pigs (4–10 weeks old) and safety testing in pregnant sows. Demonstration of MLV safety and efficacy in pigs at different growth stages, including ~~(suckling piglets, nursery pigs, fattening pigs), the safety in~~ breeding-age boars~~,~~ and gilts ~~and pregnant sows~~, ~~and onset and duration of protective immunity,~~ are also preferredbut are not required to meet the minimum standard. Additional data will likely be required by Regulatory Authorities if ~~these~~ specific categories are included in the indications for the vaccine. Details of the onset of immunity (the interval of time elapsed between vaccination and challenge if protection is confirmed) and the duration of immunity (last time-point at which vaccine-induced immunity has been demonstrated) ~~(the time point at which vaccine-induced immunity begins to decline and provides less protection)~~ are also required to meet minimum standards.

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.,* 2015). In regions where *Ornithodoros* ~~soft-bodied~~ ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (Costard *et al*., 2013).

ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al.*, 2009).

ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

**. . .**

**c. REQUIREMENTS FOR VACCINES[under review]**

~~At present there is no commercially available vaccine for ASF.~~ Commercially produced modified live virus vaccines are being evaluated and have ~~licensed~~ received regulatory approval for field use in some countries.

**1. Background**

The ASF p72 genotype II strains (ASFV Georgia 2007/1 lineage) (NCBI, 2020) are recognised to be the current highest global threat for domestic pig production worldwide (Penrith *et al.,* 2022). However, genotype I ~~attenuated~~ low virulent strains and genotype I/II recombinant strains have been reported to be circulating. In Africa, multiple genotypes are circulating.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of Veterinary Vaccine Production*. Varying additional requirements relating to quality (including purity and potency), safety, and efficacy will apply in particular countries or regions for manufacturers to comply with local regulatory requirements.

Wherever live, virulent ASFV or ASF MLVs are stored, handled and disposed, the appropriate biosecurity level, procedures and practices should be used. The ASF MLV vaccine production facility should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

An optimal ASF MLV first generation vaccine for the target host should have the following general characteristics (minimum standards):

• Safe: demonstrate absence of persistent fever as defined below (see Section 2.3.2) and clinical signs of acute or chronic ASF in vaccinated and in-contact animals, minimal and ideally no vaccine virus transmission, and absence of an increase in virulence (genetic and phenotypic stability);

• Efficacious: protects against mortality, reduces acute and other forms of disease (fever accompanied by the appearance of clinical signs caused by ASF) and reduces ~~vertical (boar semen and placental) and~~ levels of challenge virus viraemia and/or shedding ~~horizontal disease transmission~~;

**Category:** [addition, deletion, change, editorial, general]

Addition.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Viremia and shedding can be separate factors, and are not necessarily linked and should be evaluated separately.

**Supporting evidence:**

• Quality – purity: free from wild-type ASFV and extraneous microorganisms that could adversely affect the safety~~, potency~~ or efficacy of the product;

• Quality – ~~potent~~ stability: the ~~log~~~~10~~virus titre maintained throughout the vaccine shelf life that guarantees the efficacy demonstrated by the established minimum immunising (protective) dose;

• ~~Identity~~ Vaccine matching: based on the capacity to protect against the ASFV B646L (p72) genotype II pandemic strain or other p72 genotypes of recognised epidemiological importance.

Vaccine production should be carried out using a validated, controlled and consistent manufacturing process.

ASF MLV first generation vaccines must be safe (i.e. an acceptable safety profile) for non-target species and the environment in general.

Ideally, ASF MLV first generation vaccines that meet the minimum standards should also fulfil the following additional general characteristics: i) prevents acute and persistent ~~(carrier state)~~ disease; ii) prevents horizontal and vertical disease transmission; iii) induces rapid protective immunity (e.g. < ~~2 4~~ 2 weeks); and iv) confers stable, life-long immunity.

**Category:** [addition, deletion, change, editorial, general]

Change.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Reversion to 2 weeks is suggest as that is the ideal situation.

**Supporting evidence:**

The ideal is rapid induction of immunity, and cell-mediated immunity (most relevant for ASF) generally develops at 2 weeks in natural infections. No evidence in specific, but when identifying the ideal it is best to stick with the ideal situation which is the most rapid timeframe.

Furthermore, ASF MLV second and future generation vaccines should meet the minimum safety and efficacy standards as ASF MLV first generation vaccines, and ideally provide additional product profile benefits, including but not limited to: i) contain a negative marker allowing the differentiation of infected from vaccinated animals (DIVA) by reliable discriminatory tests such as serology-based tests; and ii) confer cross protection against circulating related strains and some ~~broad range of protection against~~ other p72 genotype field strains of varying virulence (low, moderate, and high).

The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV first generation vaccine candidate~~s that are safe and efficacious~~ against ASF viruses belonging to the ASFV p72 genotype II pandemic strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). More research is needed to determine whether these genotype II-specific MLVs can effectively protect against newly circulating variants of genotype II and recombinant strains.

Currently, two recombinant gene deleted MLV ~~recombinant~~ vaccines (ASFV-G-ΔI177L and ASFV-G-ΔMGF) have ~~been licensed~~ received regulatory approval ~~for field use~~ in Vietnam for use in domestic pigs ~~following supervised field testing to evaluate the safety and effectiveness of several vaccine batches~~.

There are numerous, promising ASF MLV vaccine candidates targeting the p72 genotype II pandemic strain under development, including:

• A naturally attenuated field strain (Lv17/WB/Rei1) (Barasona *et al.,* 2019) being developed as an oral bait vaccine for wild boar~~s~~;

• A laboratory thermo-attenuated field strain (ASFV-989) (Bourry *et al.,* 2022);

• Single gene-deleted, recombinant viruses (e.g. SY18ΔI226R, ASFV-G-ΔA137R) (Gladue *et al*., 2021; Zhang *et al*., 2021);

• Double gene-deleted, recombinant viruses (e.g. ASFV-G-Δ9GL/ΔUK; ASFV-SY18-∆CD2v/UK; Arm-ΔCD2v-ΔA238L) (O’Donnell *et al*., 2016; Pérez-Núñez *et al*., 2022; Teklue *et al*., 2020);

• Multiple gene-deleted, recombinant viruses (ASFV-G-ΔI177L/ΔLVR; ASFV-G-ΔMGF; ~~BA71ΔCD2;~~ HLJ/18-7GD; ASFVGZΔI177LΔCD2vΔMGF, Arm07ΔMGF) (Borca *et al*., 2021; Chen *et al*., 2020; Kitamura *et al.,* 2023; Liu *et al*., 2023; Monteagudo *et al*., 2017; O’Donnell *et al*., 2015).

Information regarding many of these MLV vaccine candidates can be found in a recent review publication (Brake, 2022).

Different DIVA strategies using serological methods (e.g. ELISA) or genome detection methods (e.g. differential real-time PCR) have been published for some of the ~~are not widely available for these~~ ASF MLV first generation vaccine candidates (Borca *et al.,* 2024; Velazquez-Salinas *et al.,* 2021). ~~Therefore~~ However, there is still room for improvement with respect to marker vaccines and their companion diagnostic tests. The field use of genetically modified ASFV strains with marker genes should be compliant with the Cartagena Protocol’s regulations for conserving biodiversity as set out in Chapter 1.1.8 Principles of Veterinary Vaccine Production, Section 7.2.3.2 Additional requirements for live rDNA products.

Inactivated (non-replicating) whole virus vaccines are not presently available and may be difficult to develop to meet minimum efficacy standards. Recombinant vectored, subunit vaccine candidates that can be produced in scalable vaccine platform expression systems and mRNA-based ASF vaccines are being evaluated in ongoing laboratory research, testing and evaluation in experimental challenge models*.* The publicly available *Center of Excellence for African Swine Fever Genomics* (ASFV Genomics, 2022[[1]](#footnote-2)*)* that provides the structural protein predictions for all 193 ASFV proteins may help accelerate ASF first and second generation vaccine research and development.

Any future use of vaccine candidates should be based on a thorough risk–benefit assessment considering all safety and efficacy features, as well as the potential vaccination scenario. Fit-for-purpose vaccine use scenarios matched to the intended use in a domestic pig-specific type of production system may require different vaccine product profiles or may influence the focus of essential versus ideal vaccine requirements. Prudent use of ASF MLVs as part of strict, controlled vaccination programmes, especially in the areas where ASF is not prevalent, should be implemented. Transmission of vaccine virus to non-vaccinates (domestic or wildlife) could be particularly problematic in areas where ASF is not known to be present.

It is important to know what genotypes of ASFV are circulating in a population before vaccination is introduced. Due to the potential risk of recombination events between circulating low and high virulent field strains with future ~~licensed~~ vaccine strains with regulatory approval, and the possibility of reversion to virulence of vaccine strains, strict pharmacovigilance post-vaccination using stringent reporting criteria (e.g. any fever is reportable – in contrast to the safety testing criteria below of 2 days of fever) is essential. Field pharmacovigilance data should be collected and analysed during vaccination campaigns using ASF MLV first generation vaccines post-~~licensing~~ regulatory approval. Active post-vaccination surveillance programmes for the detection of new ASF viruses that may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as revertant vaccine strains, should be implemented. It is also recommended that vaccine manufacturers carry out laboratory experiments to further evaluate the risk of vaccine virus recombination with field and vaccine strains.

As with any ~~MLV~~ vaccine, all ASF ~~MLV~~ vaccines should be used according to the label instructions, under the strict control of the country’s Regulatory Authority.

The minimum standards given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national, regional, and veterinary international medicinal product harmonised requirements. Minimum data requirements for ~~an authorisation~~ regulatory approval in exceptional circumstances (e.g. unexpected introduction of the virus, sudden outbreaks of the disease) should be considered where applicable.

**2. Outline of production and minimum requirements for vaccines**

**2.1. Characteristics of the seed virus**

**2.1.1. Biological characteristics of the master seed virus**

ASF MLVs are generally produced from ASFV field strains derived from naturally attenuated field isolates or using DNA homologous (genetically targeted) recombination techniques in cell cultures to delete one or more ASFV genes or gene families. These molecular techniques typically involve replacement of the targeted ASFV gene(s) with one or more positive, marker fluorescent (e.g. BFP, eGFP, mCherry) or enzyme-based (e.g. β-glucuronidase) ASFV promoter-reporter gene systems that allow the use of imaging microscopy or flow cytometry to visualise, select, and clone gene-deleted, recombinant, ASF MLVs. MLV production is carried out in cell cultures based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced based on their ease of growth in cell culture, virus yield (log10 infectious titre) and genetic stability over multiple cell passages. Preferably, a continuous well-characterised cell line (e.g. ZMAC-4; PIPEC; IPKM) (Borca *et al*., 2021; Masujin *et al*., 2021; Portugal *et al*., 2020) is used to produce a master cell bank (MCB) on which the MSV and MSV-derived working seed virus (WSV) can be produced. The exact source of the underlying ASFV isolate, the whole genome sequence, and the passage history must be recorded.

**2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)**

Only MSVs that have been established as sterile, pure (free of wild-type parental virus and free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*, and those listed by the appropriate ~~licensing~~ regulatory approval authorities) and immunogenic, should be used as the vaccine virus (WSV and vaccine batch production). ~~Live vaccines must be shown not to cause disease or other adverse effects in target animals in accordance with chapter 1.1.8, Section 7.1~~ *~~Safety tests~~* ~~(for live attenuated MSVs), that includes target animal safety tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission to other animals.~~

Identity of the MSV must be confirmed using appropriate methods (e.g. through the use of vaccine strain-specific whole genome detection methods such as next generation sequencing).

Demonstration of MSV stability over several cell passages is necessary, typically through at least five passages (e.g. MSV+5). For those MLV vaccines for which attenuation is linked to specific characteristics (gene deletion, gene mutations, etc.), genetic stability of attenuation throughout the production process should be confirmed by full genome sequencing and confirmation of the vaccine phenotype, for example, by confirming the virus titre obtained by growth in the cell line used for production ~~using suitable methods. Suitable techniques to demonstrate genetic stability may include but are not limited to: genome sequencing, biochemical, proteomic, genotypic (e.g. detection of genetic markers) and phenotypic strain characterisation~~. If final product yields (infectious titres) are relatively low, as is typically the case with ASFV, demonstration of stability is required for the maximum passage for use in the final product manufacturing as defined by the producer ~~genetic stability at a minimum of MSV+10 should be demonstrated to allow more flexibility in the outline of production. For example, if MSV+8 is the maximum passage for use in final product manufacturing, demonstration of genetic stability to at least MSV+10 is warranted~~.

**2.1.3. Validation as a vaccine strain**

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy. Live vaccines must be shown to not cause disease or other adverse effects in target animals in accordance with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission to other animals.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, consideration should also be given to minimising the risk of TSE transmission by ensuring that animal origin materials from TSE-relevant species, if no alternatives exist for vaccine virus propagation, comply with the measures on minimising the risk of transmission of TSE.

Ideally, the vaccine virus in the final product should generally not differ by more than five passages from the master seed lot.

ASF vaccines should be presented in a suitable pharmaceutical form (e.g. lyophilisate or liquid form).

**2.2. Method of manufacture**

**2.2.1. Procedure**

The MLV ~~virus~~ is used to infect swine primary cell cultures obtained from specific-pathogen free pigs, the requirements for which are defined in specific monographs (Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*, Section 2.4.2). It should be noted that each donor pig should be considered a different “master cell stock” and be tested for purity and extraneous agents to account for the risk of contamination during cell collection and processing. Similar considerations should apply to collections over time, and the herd health of the donor pigs should be closely monitored. Compared with primary cell cultures, use of a continuous cell line generally allows for more consistency, higher serial volumes in manufacturing and aligns better with a seed lot system. Thus, preferably a master cell bank based on an established, continuous cell line shown to support genetically stable ASFV replication and acceptable titres over several passages should be used.

Cell cultures shall comply with the requirements for cell cultures for production of veterinary vaccines in chapter 1.1.8. Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles, detergent lysis). The harvest can be further processed by filtration and other purification methods. A stabiliser or other excipients may be added as appropriate. The vaccine is homogenised to ensure a uniform batch/serial.

**2.2.2. Requirements for ingredients**

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

**2.2.3. In-process controls**

In-process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests. Biosafety precautions should align with the outcomes of a biosafety risk assessment and conform to local and national guidelines.

**2.2.4. Final product batch tests**

**i) Sterility**

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

**ii) Identity**

Appropriate methods such as specific genome detection methods (e.g. specific differential real-time PCR and/or full genome sequencing) should be used for confirmation of the identity of the vaccine virus and differentiation from the parent strain of the virus as a potential contaminant.

**iii) Purity**

Appropriate methods should be used to ensure that the final product batch does not contain any residual wild-type ASFV.

**iv) Safety**

Batch safety testing is to be carried out unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

**v) Batch/serial potency**

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the vaccine minimum immunising dose (MID) (minimum protective dose) and titre of the modified live vaccine in vitro. In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (Section C.2.3.3 *Efficacy requirements*, below).

**vi) Residual humidity/residual moisture**

The test should be carried out consistent with VICH[[2]](#footnote-3) GL26 (*Biologicals: Testing of Residual Moisture*, 2003[[3]](#footnote-4)). Required for MLV vaccines presented as lyophilisates ~~for suspension for injection~~.

**2.3. Requirements for ~~authorisation/registration/licensing~~ regulatory approval**

**2.3.1. Manufacturing process**

~~For regulatory approval of a vaccine,~~ All relevant details concerning history of the pre-MSV, preparation of MSV, manufacture of the vaccine and quality control testing (Sections C.2.1 *Characteristics of the seed* and C.2.2 *Method of manufacture*) should be submitted to the regulatory approval authorities.

Information shall be provided from preferably three or more ~~preferably~~ consecutive vaccine batches originating from the same MSV and representative of routine production~~, with a volume not less than 1/10, and more preferably with a volume not less than 1/3 of the typical industrial batch volume~~. The in-process controls are part of the manufacturing process.

Genetic stability of attenuation throughout the production process (i.e. to the maximum passage level to be used for vaccine production) should be confirmed by full genome sequencing and confirmation of virus phenotype (e.g. virus yield in cell line used for production).

**2.3.2. Safety requirements**

For the purpose of gaining regulatory approval, the following safety tests should be performed ~~satisfactorily.~~

As a minimum standard, vaccines should be tested for any pathogenic effects on healthy domestic pigs of the target age intended for use. In addition, due to risks from horizontal spread of the vaccine virus, vaccine safety testing should be carried out in pregnant sows as the population that is likely to be the most sensitive indicator of any adverse effects. Additional demonstration of MLV safety in pigs at different growth stages, including breeding-age boars and gilts ~~in breeding age gilts and pregnant sows~~ is preferred ~~but not required as a minimum standard~~. When the vaccine is recommended for use or may be used ~~If in the future a vaccine intended for use~~ in breeding animals ~~is developed~~, an evaluation of the impact of the vaccine on reproductive performance will be a standard safety requirement.

**i) Safety in young animals**

Carry out the test by each recommended route of administration using, in each case, piglets a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old.

The test is conducted using no fewer than eight healthy piglets, and preferably no fewer than ten healthy piglets.

To obtain individual mean baseline temperatures, the body temperature of each piglet should be measured on at least the 3 consecutive days preceding administration of the vaccine. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each piglet a quantity of the vaccine virus ~~equivalent to~~ not less than ten times the maximum virus titre ~~(e.g. 50% haemadsorption dose [HAD~~~~50~~~~], 50% tissue culture infective dose [TCID~~~~50~~~~], quantitative PCR, etc.) (maximum release dose)~~ likely to be contained in one dose of the vaccine.

~~To obtain individual and group mean baseline temperatures, the body temperature of each vaccinated piglet is measured on at least the 3 consecutive days preceding administration of the vaccine.~~

To confirm the presence or absence of fever accompanied by acute and chronic disease, ~~observe~~ measure body temperature and clinical signs in the piglets 4 hours after vaccination and then at least once daily for at least 45 days, preferably 60 days post-vaccination. On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be recorded. Carry out the daily observations for signs of acute and chronic disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.,* 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive ﬁndings).

At a minimum of 45 days post-vaccination, humanely euthanise all vaccinated piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes).

The vaccine is compliant ~~complies with the test~~ if:

* No piglet shows abnormal (local or systemic) reactions or notable signs of disease, or reaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;
* ~~The average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days.~~
* ~~On each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and~~ No individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding ~~3~~ 2 consecutive days that is attributable to ASFV infection. In cases where pigs exceed the temperature standard but show no behavioural changes or other clinical signs, regulators may determine vaccine safety without solely relying on temperature for non-compliance;
* No vaccinated pigs show notable signs of disease by gross pathology.

**ii) Safety test in pregnant sows and test for transplacental transmission**

There is limited ~~currently an absence of~~ published information on ASFV pathogenesis in ~~breeding-age gilts and in~~ pregnant sows associated with ASFV transplacental infection and fetus abortion/stillbirth. ~~If a label claim is pursued for use in breeding age gilts and sows, then~~ Due to risks from horizontal spread and contact transmission of the vaccine virus, a safety study in line with VICH GL44 (*Guidelines on Target Animal Safety for Veterinary Live and Inactivated Vaccines*, *Section 2.2. Reproductive Safety Test*, 2009[[4]](#footnote-5)) should be completed.

Carry out the test with vaccination by a recommended route using no fewer than eight healthy sows of similar age, between the 55th and 70th day of gestation, that do not have antibodies against ASFV and blood samples are negative on PCR. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each sow a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until farrowing. Blood samples should be taken from newborn piglets before ingestion of colostrum.

The test is invalid if the vaccinated sows do not seroconvert before farrowing.

The vaccine is compliant if:

* No pregnant sows show abnormalities in their gestation or in their piglets;
* No pregnant sows show notable signs of disease or dies from causes attributable to the vaccine;
* No vaccine virus or antibodies against ASFV are present in blood samples from newborn piglets.

**iii) Horizontal transmission**

The test is conducted using no fewer than 12 healthy piglets~~,~~ a minimum of ~~6~~ 4-weeks old and not older than 10-weeks ~~old~~. ~~and of the same origin, that~~ Piglets do not have antibodies against ASFV~~,~~ and blood samples are negative on real-time PCR. ~~All piglets are housed together from day 0 and the number of vaccinated animals is the same as the number of naïve, contact animals.~~

Carry out the test with vaccination by a recommended route, using vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

**Category:** [addition, deletion, change, editorial, general]

Addition.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Language copied from the section above and adapted to subsection iii) Horizontal transmission as a suggestion to have vaccination standards here to make the test valid.

**Supporting evidence:**

Co-mingle the piglets in the same pen or room so that equal numbers (at least six) can be ~~of~~ vaccinated and the same number can remain unvaccinated (naïve contact piglets) ~~and naïve, contact piglets from day 0 in the same pen or room~~.

To obtain individual mean baseline temperatures, the body temperature of each naive contact piglet should be measured on at least the 3 consecutive days preceding administration of the vaccine. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by ~~each~~ the recommended route of administration to no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the vaccine.

~~To obtain individual and group mean baseline temperatures, the body temperature of each naïve, contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated piglets.~~ The body temperature of each naïve, contact piglet is then measured daily for at least 45 days, preferably 60 days. ~~To confirm the presence or absence of fever accompanied by disease, observe the naïve, contact piglets daily for at least 45 days, preferably 60 days.~~ On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be recorded. ~~used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days.~~ Carry out ~~the~~ daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.,* 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive ﬁndings.

~~In addition,~~ Blood should be taken from the naïve contact piglets at least twice a week for the first 21 days post-vaccination and then on a weekly basis. From the blood samples, determine vaccine virus titres by quantitative virus isolation (HAD50/ml, TCID50/ml or other methods, e.g. titration using IPT or FAT detection). Real-time PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~infectious virus titres by quantitative virus isolation (e.g. HAD~~~~50~~~~/ml or TCID~~~~50~~~~/ml) and using a real-time PCR test~~.

~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used.~~

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 ~~days~~and carry out an appropriate test to detect vaccine virus induced antibodies.

**Category:** [addition, deletion, change, editorial, general]

Deletion.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Deletion of a duplicative word.

**Supporting evidence:**

At a minimum of 45 days, humanely euthanise all naïve, contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. Determine virus titres in all collected samples as described above. ~~by quantitative virus isolation (HAD~~~~50~~~~/mg or TCID~~~~50~~~~/mg) or other appropriate methods (e.g. IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above and real-time(RT)-PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~

The vaccine ~~complies with the test~~ is compliant if:

• No vaccinated or naïve contact piglet shows abnormal (local or systemic) reactions or notable signs of disease, reaches the predetermined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;

• ~~On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and~~ No individual naïve contact pig ~~should~~ shows a rise in temperature above baseline greater than 1.5°C for a period exceeding ~~3~~ 2 consecutive days that is attributable to ASFV infection. In cases where pigs exceed the temperature standard but show no behavioural changes or other clinical signs, regulators may determine vaccine safety without solely relying on temperature for non-compliance;

~~The average body temperature increase for all naïve, contact piglets (group mean) for the observation period does not exceed 1.5°C: above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days~~;

• No naïve, contact pig~~let~~ shows notable signs of disease by gross pathology ~~and no virus is detected in their blood or tissue samples~~;

* ~~No or a low percentage of contact piglets test both real-time PCR positive and seropositive No naïve contact pigs test positive for antibodies to the vaccine virus~~ No or a low percentage of naïve, contact pigs test positive to the vaccine virus and/or to antibodies against the vaccine virus.

**iv) ~~Post-vaccination kinetics~~ Dissemination ~~of viral replication~~ of the vaccine strain in the vaccinated** **animals ~~(MLV blood and tissue dissemination) study~~**

Prior to the reversion to virulence study (Section C2.3.2.v. below), a minimum of one study should be performed to determine the dissemination of the vaccine strain ~~the post-vaccination kinetics of virus replication~~ in the blood (viremia), tissues and viral shedding.

The test consists of the administration of the vaccine virus from the master seed lot to no fewer than eight ~~healthy piglets,~~ and preferably ten healthy ~~piglets~~ pigs. Pigs should be a minimum of ~~6~~ 4-weeks old and not older than 10-weeks. They should test negative for ~~old and of the same origin, that do not have~~ antibodies against ASFV~~,~~ and ~~blood samples are negative on real-time~~ by PCR.

Administer to each piglet, using the recommended route of administration most likely to result in spread (such as the intramuscular route or intranasal route), a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine.

Record daily body temperatures and observe inoculated animals daily for clinical disease for at least 45 days, preferably 60 days.

Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.* (2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive ﬁndings.

Collect blood samples from all the piglets at least two times per week from 3 days post-vaccination for the first 2 weeks, then weekly for the duration of the test. Determine vaccine virus titres in the samples by quantitative virus isolation (HAD50/ml or TCID50/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Real-time PCR may be used to detect positive samples but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Determine which blood timepoint(s) should be used in the design of the reversion to virulence study (Section C2.3.2.v. below), for example, specific blood sample(s) at specific timepoints that show the highest titres should be considered for selection and use in the reversion to virulence study.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) at least two times per week from 3-days post-vaccination for the first 2 weeks, then weekly for the duration of the test. ~~Test the swabs for the presence of vaccine virus.~~ Determine virus titres in all collected samples by quantitative virus isolation ~~(HAD~~~~50~~~~/ml or TCID~~~~50~~~~/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Euthanise at least two piglets on days 5, 7, 14, 21, and preferably on day 28 (±2 days at each timepoint) and collect spleen, lung, tonsil, kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation ~~(HAD~~~~50~~~~/mg or TCID~~~~50~~~~/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above ~~and using real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Determine which tissue(s) and timepoint(s) should be used to aid in the design of the reversion to virulence study (Section C.2.3.2.v), for example, specific tissues at specific timepoints which show the highest titres should be considered for selection and use in the reversion to virulence study.

**v) Reversion to virulence**

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[[5]](#footnote-6)).

The test for increase in virulence consists of the administration of the vaccine master seed virus to healthy piglets of an age (e.g. between ~~6~~ 4 weeks and 10 weeks old) suitable for recovery of the strain. ~~and of the same origin, that do not have~~ Piglets should test negative for antibodies against ASFV, and ~~blood samples that are negative on real-time~~ by PCR. This protocol is typically repeated five times.

To obtain individual mean baseline temperatures, the body temperature of each piglet should be measured on at least the 3 consecutive days preceding administration of the vaccine or passaged material.

*First passage* (*p1*)

Administer to no fewer than two piglets, and preferably no fewer than four piglets using the intended route of administration for the final product, a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs ~~and record daily body temperatures~~ using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.,* 2015a) and record daily body temperatures.

Based on results from at least one completed post-vaccination kinetics of viral replication (MLV ~~vaccine shed and spread (virus~~ blood and tissue dissemination study (Section C.2.3.2.iv above), collect an appropriate quantity of blood from each piglet on the predetermined ~~single~~ timepoint(s) (i.e. day ~~5~~ 3–13). Determine virus titres in individual bloodsamples by quantitative virus isolation (HAD50/ml or TCID50/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Real-time PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and by real-time PCR. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~. Identify the individual blood sample(s) with the highest infectious titre and reserve for the subsequent *in-vivo* passage (second pass, p2). If appropriate, blood samples with the highest infectious titres from different pigs should be pooled to prepare inoculum for further passages.

Based on results from at least one completed ~~vaccine virus~~ MLV blood and tissue ~~distribution~~ dissemination study (Section C.2.3.2.iv above), euthanise piglets on the predetermined timepoint (i.e. day 5, 7, 14, 21, or 28). Determine infectious virus titres in individual tissue samples by quantitative virus isolation ~~(HAD~~~~50~~~~/ml or TCID~~~~50~~~~/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above. ~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~ Identify individual tissuesample type(s) with the highest infectious titre~~.~~ ~~Pool the Use tissues with the highest titres~~ from different organs from ~~all~~ each animal~~s~~ ~~with the highest titres~~ and prepare ~~at least~~ a ~~10%~~ virus suspension ~~to obtain a virus titre within the range used for inoculation~~ in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage. Identify the individual tissue samples with the highest infectious titres from each animal and reserve for subsequent *in-vivo* passage (second pass, p2). If appropriate, tissue samples with the highest infectious titres from different pigs should be pooled to prepare inoculum for further passages.

~~Test each blood and tissue sample pool used for inoculation by PCR to confirm the absence of potential viral agent contaminants (i.e. CSFV, FMDV, PRRSV, PCV2). Blood and pooled tissue (p1) are used to inoculate 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine using the intended route of administration for the final product to each of at least two and ideally at least four further pigs of the same age and origin.~~

*~~Second pass~~* ~~(~~*~~p2~~*~~)~~

If no virus is found at passage 1 (p1), repeat the administration by the intended route ~~once~~ again with the same master seed vaccine virus used in p1 (see above) ~~pooled material (blood and pooled tissue, p1)~~ in another ten healthy piglets of the same age and origin. If no virus is found at this point ~~during this second passage (p2) at this point~~, end the process here.

*Second passage* (*p2*)

If ~~however~~ virus is found ~~in~~ at p1, carry out a second series of passages by administering an appropriate volume (e.g. 2 ml) of positive material from p1 ~~diluted to the maximum release dose likely to be contained in 1 dose of the vaccine~~ using the intended route of administration for the final product to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system ~~adding the values for multiple clinical signs (e.g. Gallardo~~ *~~et al.,~~* ~~2015a)~~, ~~and~~ record daily body temperatures, collect blood and tissue samples at the predetermined time points in the blood and tissue dissemination study (above) and determine infectious virus titres in individual blood and tissue samples as described for p1 above.

*~~Third and fourth pass~~* ~~(~~*~~p3 and p4~~*~~)~~

If no virus is found at ~~in~~ ~~(~~p2~~)~~, repeat the ~~intramuscular~~ administration by the intended route ~~once again~~ with the same ~~pooled~~ material (blood and/or ~~pooled~~ tissue) from, p~~2~~ 1) in another eight healthy piglets of the same age and origin. If no virus is found at this point, end the process here.

*Third and fourth passage* (*p3 and p4*)

If~~, however,~~ virus is found on p2, carry out further ~~this~~ passages ~~operation~~ no fewer than two additional times (p3 and p4) (to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin). Record clinical signs and body temperature daily, collect blood and tissue samples at the predetermined time points (see above) and determine infectious virus titres ~~verifying the presence of the virus at each passage~~ in individual blood and tissue samples as described for p1 above. ~~Observe inoculated animals daily for the appearance of at least two and preferably at least three clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo~~ *~~et al.,~~* ~~2015a) and record daily body temperatures.~~

*Fifth passage* (*p5*)

Administer an appropriate volume (e.g. 2 ml) of the blood and ~~pooled~~ tissue (p4) to each of at least eight healthy piglets of the same age and origin. Observe inoculated animals daily for at least 28 days post-inoculation for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system ~~adding the values for multiple clinical signs (e.g. Gallardo~~ *~~et al.,~~* ~~2015a)~~, ~~and~~ record daily body temperature and determine infectious virus titres in individual blood and tissue samples as described above.

If the fifth group of animals shows no evidence of an increase in virulence indicative of reversion to virulence during the observation period, further testing is not required. Otherwise, materials used for the first passage and the final passage should be used in a separate experiment using at least 8 animals per group to directly compare the clinical signs and other relevant parameters. This study should be done by the route of administration that was used for previous passages.

The vaccine is compliant ~~complies with the test~~ if:

• No piglet shows abnormal (local or systemic) reactions, or notable signs of disease, orreaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine; and

• There is no indication of increasing virulence (as monitored by daily body temperature increases above the baseline accompanied by clinical sign observations) of the maximally passaged virus compared with the master seed virus.

At a minimum, a safe MLV vaccine shall demonstrate ALL the following features (minimal standards):

* Absence of fever (~~on each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to will be recorded calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and~~ no individual pig should show a rise in temperature above baseline greater than 1.5°C ~~(defined as average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C~~ for a period exceeding ~~3~~ 2 days that is attributable to ASFV infection). In cases where pigs exceed the temperature standard but show no behavioural changes or other clinical signs, regulators may determine vaccine safety without solely relying on temperature for non-compliance;
* Absence of chronic and acute clinical signs and gross pathology over the entire test period ~~or minimal chronic mild clinical signs (defined as e.g. mild swollen joints with a low clinical score that resolve within 1 week)~~;
* Absence of abnormal (local or systemic) reactions;
* ~~Minimal (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no or a low percentage of contact piglets test both real-time PCR positive and seropositive) or no vaccine virus transmission (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no contact piglets test both real-time PCR positive and seropositive) over the entire test period~~ No or a low percentage of naïve, contact pigs test positive to the vaccine virus and/or to antibodies against the vaccine virus;
* Absence of an increase in virulence (genetic and phenotypic stability) (complies wit
* h the reversion to virulence test).

In addition, for regulatory approval, ASF MLV ~~the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for safety ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field safety studies generally ~~evaluation studies~~ may include measurement of body temperatures, observation of local or systemic reactions and, where appropriate, performance measurements such as ~~but are not limited to~~: environmental persistence (e.g. determination of virus recovery from bedding or other surfaces), assessment of immunosuppression, and negative impacts on performance.

**2.3.3. Efficacy requirements**

**i) Protective dose**

Vaccine efficacy is estimated in immunised animals directly, by evaluating protection against ~~their resistance to~~ live virus challenge. The test consists of a vaccination/challenge trial in piglets a minimum of ~~6~~ 4-weeks old and not more than 10-weeks old, free of antibodies to ASFV, and negative ~~blood samples~~ by real-time PCR. The test is conducted using no fewer than 15 and preferably no fewer than 24 vaccinated pigs, and no fewer than five non-vaccinated control piglets.

The test is conducted to determine the minimal immunising dose (MID) (also referred to as the minimal protective dose [MPD] or protective fraction); using at least three groups of no fewer than five and preferably not fewer than eight vaccinated piglets per group, and one additional group of no fewer than five non-vaccinated piglets of the same age and origin as controls. Use vaccine containing virus at the highest passage level that will be present in a batch of vaccine. Each group of piglets, except the control group, is immunised with a different vaccine virus content in the same vaccine volume. In at least one vaccinated group, piglets are immunised with a vaccine dose containing not more than the minimum virus titre ~~(minimum release dose)~~ likely to be contained in one dose of the vaccine as stated on the label.

The MID of the vaccine is calculated from the number of animals protected in each group using an appropriate statistical test, such as the Spearman–Kärber method.

A suitable challenge model should be developed based on anticipated field usage of the vaccine. As a baseline protocol, ~~Twenty-eight~~ 28 days (±2 days) after the single ~~injection~~ dose of vaccine (or if using two ~~injections~~ doses of the vaccine then 28 days [±2 days] following the second ~~injection~~ dose), challenge all the piglets by the intramuscular route. If previous studies have demonstrated acceptable efficacy using IM challenge, then a different challenge route (e.g. direct contact, oral or oronasal) may be used. Challenged, vaccinated piglets may be housed in one or more separate pens in the same room or in different rooms. Challenged, naïve controls can be housed in one or more rooms that are separate from challenged, vaccinated piglets.

Carry out the test using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiologic importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD50 ~~(~~or TCID50 ~~for non-HAD viruses)~~ challenge dose sufficient to cause death or meet the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

To obtain individual mean baseline temperatures, the body ~~rectal~~ temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the challenge virus. Body temperature is then measured, at the time of challenge, 4 hours after challenge, and then daily for the observation period of at least ~~28~~ 45 days, preferably ~~35~~ 60 days. ~~Observe the piglets at least daily for at least 28 days, preferably 35 days.~~ Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system (e.g. Gallardo *et al.*, 2015). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive ﬁndings.

**Category:** [addition, deletion, change, editorial, general]

Deletion.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

This was removed above and should be deleted here as well. Clinical signs should be evaluated independently.

**Supporting evidence:**

Collect oral, nasal, faecal swabs ~~anal~~ and blood samples from the vaccinated challenged piglets at least two times ~~once~~ per week from 3 days post-challenge for at least ~~28~~ 14 days, then weekly up to 35 days post-challenge and then every 14 days up to the end of the observation period ~~preferably 35 days~~. From the blood samples, determine infectious virus titres by quantitative virus isolation (HAD50/ml or TCID50/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Real-time PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

At the end of the test period, humanely euthanise all vaccinated challenged piglets. Conduct gross pathology (and histopathology if considered necessary) on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation ~~(HAD~~~~50~~~~/mg or TCID~~~~50~~~~/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above ~~and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

If using a highly virulent challenge virus, as described above, the test is invalid if fewer than 100% ~~the difference between in the number~~ of unvaccinated control piglets infected with the live challenge virus ~~and the number of vaccinated / challenged piglets vaccinated with the minimum release dose that~~ die or reach a humane endpoint ~~is not statistically significant~~.

The vaccine (or a specific vaccine virus dose if conducting a vaccine dose titration study) is compliant ~~complies with the test~~ if:

• No vaccinated challenged piglet dies or shows abnormal (local or systemic) reactions, or reaches the humane endpoint ~~or dies~~ from causes attributable to ASF;

• ~~On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 2.0°C for a period exceeding 2 consecutive days~~ T~~he average body temperature increase for all vaccinated challenged piglets (group mean) for the observation period does not exceed 2.0°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.0°C~~;

• The vaccinated challenged piglets display a reduction or absence of pyrexia, typical acute clinical signs or other forms of disease and gross pathology, and a reduction or absence of challenge virus levels in blood, swabs and tissues.

**~~ii) Assessment for horizontal transmission (challenge virus shed and spread study)~~**

~~The ASF basic reproduction number, R0, can be defined as the average number of secondary ASF disease cases caused by a single ASFV infectious pig during its entire infectious period in a fully susceptible population (Hayes~~ *~~et al~~*~~., 2021). In general, if the ASFV effective reproduction number Re=R0 × (S/N) (S= susceptible pigs; N= total number of pigs in a given population) is greater than 1.0, disease is predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by reducing the number of susceptible, naïve, contact pigs exposed to vaccinated, infected pigs.~~

~~To evaluate ASF vaccine impact on ASF disease transmission, the test consists of a vaccination/challenge trial in piglets a minimum of 6 4-weeks old and not older than 10-weeks old, free of antibodies to ASFV, and negative blood samples by real-time PCR.~~

~~The test is conducted using no fewer than 15 healthy piglets at a ratio comprising twice the number of vaccinated piglets to naïve piglets (e.g. ten vaccinated and five naïve). Use vaccine containing virus at the highest passage level that will be present in a batch of the vaccine.~~

~~The quantity of vaccine virus administered to each pig is equivalent to be not less than the minimum virus titre (minimum dose) likely to be contained in one dose of the vaccine as stated on the label. Following immunisation, vaccinated and naïve piglets should continue to be co-mingled.~~

~~Twenty-eight days (±2 days) after the single injection dose of vaccine (or if using two injections doses of the vaccine then 28 days [±2 days] following the second injection dose), temporarily separate [into different pen(s) or room(s)] all vaccinated piglets from naïve piglets. Challenge all vaccinated piglets by the intramuscular or other previously verified route. Carry out the challenge using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiological importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD~~~~50~~ ~~(or TCID~~~~50~~ ~~for non-HAD viruses challenge dose sufficient to cause death or met the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.~~

~~Approximately 18–24 hours later, re-introduce naïve piglets to vaccinated, challenged piglets and allow for direct nose to nose contact exposure with vaccinated, challenged piglets. Allow for continuous contact exposure by co-mingling both groups through the end of the study. If more than one pen or room is used for co-housing, following reintroduction initially maintain a ratio of 2:1 of challenged, vaccinated piglets to contact exposed, naïve piglets.~~

~~The rectal temperature of each contact piglet is measured on at least the 3 days preceding administration of the challenge virus to vaccinated pigs, immediately prior to direct contact exposure, 4 hours post-contact exposure, and then daily for at least 28, preferably 35 days and twice a week for at least 60 days. Observe all contact exposed piglets at least daily for at least 28 days, and then twice a week for at least 60 days preferably for at least 35 days.~~

~~Carry out the daily observations in each contact piglet for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo~~ *~~et al.,~~* ~~2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive ﬁndings.~~

~~In addition, blood should be taken from the naïve contact piglets at least twice a week from 3 days post-contact exposure for the duration collect blood samples from the contact piglets at least two times per week from 3 days post-contact for at least 14 days, then weekly up to 35 days post-contact exposure and then every 14 days up to the end of the test period. Determine virus titres in all collected samples by quantitative virus isolation (HAD~~~~50~~~~/mg or TCID~~~~50~~~~/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above From the blood samples, determine infectious challenge virus titres by quantitative virus isolation (HAD~~~~50~~~~/ml or TCID~~~~50~~~~/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used.~~

~~Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 (±2 days), and at the end of the test period, and carry out an appropriate test to detect vaccine virus antibodies.~~

~~Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) from all contact-exposed naïve piglets at least two times per week from 3-days post-contact exposure for the first 2 weeks, then weekly for the duration of the test and test swabs for the presence of challenge virus. Determine virus titres in all collected samples by quantitative virus isolation (HAD~~~~50~~~~/mg or TCID~~~~50~~~~/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above Determine virus titres in all collected samples by quantitative virus isolation (HAD~~~~50~~~~/ml or TCID~~~~50~~~~/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~

~~At the end of the test period, humanely euthanise all contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD~~~~50~~~~/mg or TCID~~~~50~~~~/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above Determine virus titres in all collected samples by quantitative virus isolation (HAD~~~~50~~~~/mg or TCID~~~~50~~~~/mg) and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~

~~The test is invalid if the vaccine fails to comply with the compliance criteria described for the protected dose test in vaccinated pigs (Section C.2.3.3.i above).~~

~~If the manufacturer claims that the vaccine induces sterilising immunity, the vaccine complies with the test for a reduction in horizontal disease transmission if all the following conditions are satisfied:~~

~~• No naïve, contact exposed piglet shows abnormal (local or systemic) reactions, reaches the defined humane endpoint or dies from causes attributable to ASF;~~

~~• No naïve, contact exposed piglet displays fever accompanied by typical signs of disease, including gross pathology.~~

~~• Naïve contact pigs show an absence of challenge virus in blood and tissues.~~

~~• No naïve contact pigs test positive for antibodies to the challenge virus.~~

~~Otherwise, the vaccine complies with the test for a reduction in horizontal disease transmission if:~~

* ~~Naïve contact pigs show a reduction or absence of challenge virus levels in blood and tissues.~~
* ~~None of or a reduced number of naïve contact exposed pigs test positive for antibodies to the challenge virus.~~

At a minimum, an efficacious MLV vaccine shall demonstrate ALL the following features (minimal standards):

• Protects against mortality;

• Reduces acute or other forms of disease (fever accompanied by a reduction of ~~typical~~ clinical and pathological signs of ~~acute~~ disease);

~~• Reduces levels of viral shedding and viraemia.~~

~~• Reduces horizontal disease transmission (no none of or a reduced number of naïve, contact exposed piglets shows abnormal [local or systemic] reactions, reaches the humane endpoint or dies from causes attributable to ASF, and displays fever accompanied by typical acute disease signs caused by ASF) and test positive for antibodies to the challenge virus.~~

* Reduces levels of viral shedding and viraemia.

In general, for regulatory approval, ASF MLV ~~addition, the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for efficacy ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field efficacy ~~evaluation~~ studies ~~may~~ generally include ~~but are not limited to: onset of immunity, duration of immunity, and impact on disease transmission~~ measurement of relevant efficacy parameters including but limited to mortality, clinical signs, impact on disease transmission, performance parameters.

**2.3.4. Duration of immunity**

Although not included in the guidance for ASF MLV first generation vaccines, manufacturers are ~~encouraged~~ in general required, as part of the ~~authorisation~~ regulatory approval procedure, to define and demonstrate the duration of immunity of a given vaccine ~~by evaluation of potency at the end of the claimed period of protection~~.

**Category:** [addition, deletion, change, editorial, general]

Change.

**Proposed amended texts (or precise suggested deletion):**

Please see text in blue above.

**Rationale:**

Suggest change to "in general required" same as in the Article below for consistency.

**Supporting evidence:**

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1. http://asfvgenomics.com. Accessed 4/4/2023. [↑](#footnote-ref-2)
2. VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products [↑](#footnote-ref-3)
3. https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl26-biologicals-testing-residual-moisture-step-7\_en.pdf [↑](#footnote-ref-4)
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