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CVMP reflection paper on methods found suitable within the EU for demonstrating freedom from extraneous agents of the seeds used for the production of immunological veterinary medicinal products

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1. Introduction

According to Directive 2001/82/EC and relevant European Pharmacopoeia (Ph. Eur.) monographs (i.e. Ph. Eur. 0062, 0030, 5.2.4., 5.2.5.), veterinary immunological medicinal products and biological origin materials used in their production should be demonstrated to be free from contamination with extraneous agents.

The suitability of cells and methods used for the detection of extraneous agents is an essential prerequisite. In this context, the following points need to be considered:

- The capacity of the test method(s) to detect the relevant extraneous agents. This includes parallel testing of negative controls and positive controls with a specified content. The agents used as positive controls may be those to be tested or other suitable agents, but in any case they must be carefully chosen to:
    - ensure fitness of cells to appropriately grow the extraneous agents,
    - ensure the proficiency of the control techniques used,
    - avoid handling of very harmful/dangerous viruses,
    - avoid threatening the GMP status of the company's facilities.
- The limit of detection of the chosen test method(s). However, the establishment of the limit of detection is not necessary if passaging/amplification steps are implemented in such a way that a virus initially present at a very low level will de facto become detectable after several amplification runs.

Based on available data from already EU-assessed and EU-approved seeds examples of suitable cells and methods for testing for freedom from a range of extraneous agents are shown in the annex to this reflection paper. The details given in the annex are however not intended to be a technical manual for lab technicians, giving the full details of the tests in order to be reproducible by any other lab technician. Each testing laboratory must demonstrate their suitability to perform the relevant test.

The annex of the document needs to be updated whenever necessary, in particular to take into account new extraneous agents to be considered, as well as cells and techniques for which sufficient experience and/or validation data become available to justify their inclusion into the annex below.

2. Preparation of material for testing

2.1. Extraneous agents testing of virus seeds

If the material (e.g. vaccine virus) would interfere with the conduct and sensitivity of a test for extraneous viruses, a sample of the material is treated with a minimum amount of monoclonal or polyclonal antibodies so that the material is neutralised as far as possible. The final material-serum blend must, if possible, contain at least the vaccine virus content equivalent to 10 doses of vaccine per ml.

The relevant test method(s) described in the annex may be implemented. The applicant is free to select a combination of cell systems in such a way that with a minimum of different cell systems, all extraneous agents required to be tested for are included, taking into account that primary cells from the species of origin of the seed must be included in all cases. Each chosen cell has to be used during the whole testing procedure for passaging/amplification of the test sample.

For the testing of virus seeds, methods and requirements described in Ph. Eur. 0062 Vaccines for veterinary use apply. Cell cultures are observed at regular intervals until day 28, when the detection phase is started.

2.2. Extraneous agents testing of cell seeds

For the testing of cell seeds (cell banks), methods and requirements are described in Ph. Eur. 5.2.4. Cell cultures for the production of veterinary vaccines. The detection phase is described in section 3 below.

3. Viral detection methods

The following methods for detection of extraneous agents are examples of methods that have been found suitable. Such detection methods for specific extraneous agents are mentioned in column 3 of the tables in the annex.

- Detection of CPE

  At least 2 cell cultures on a surface of at least 6 cm^2 screened under the microscope and/or stained with (May-Grünwald)-Giemsa. Inclusions, giant cells or other abnormalities attributable to a virus replication are examined over the whole surface of the cell layers.

- Detection of haemadsorption

  At least 4 cell monolayers of at least 70 cm^2 each are washed several times with PBS or saline and a mixed suspension of 0.2% (v/v) each of human O, chicken, and guinea pig erythrocytes in saline or PBS is added to cover the surface of the monolayers evenly. Separate cultures are incubated for at least 25-30 min (25-30 min and/or one hour) at (a) 2-8°Cand (b) at 20-25°C. The monolayers are regularly inspected for haemadsorption.

- Detection of haemagglutination (possible confirmation of the presence of Canine Parainfluenza 2 virus, Canine parvovirus and Feline panleucopenia virus)

  A sample is put in haemagglutination microplates (conical bottom). An equivalent volume (e.g. 50 µl) of red cells suspension in physiological buffer (of appropriate species) is added to each well. After an incubation period of at least 30 minutes at 4°C allowing agglutination of positive control, the microplates are observed for complete haemagglutination (absence of red pellet).

- Detection by immunostaining (IS)

  After the 28 days amplification passage of the test sample, a total cell area of at least 6 cm^2 is fixed with cold acetone and the cells are subjected to a suitable immunostaining (direct or indirect, e.g. immunofluorescence, immunoperoxidase), using specific immunosera or monoclonal antibodies. At least one uninoculated negative control and at least one positive control are included for each extraneous virus to be tested for with this technique.

- Detection by ELISA

  After one further amplification in appropriate cells (inoculation of 28-day amplification suspension [≥ 0.1 ml] onto at least 1 established culture of cells used for virus culture or into 0.8 ml of cells in suspension [≥ 100 000 cells/ml]), or directly after the 28 days amplification passages of the test sample, instead of testing the cells, the culture supernatant may be tested

by an appropriate ELISA shown to be specific for the extraneous virus to be tested for. At least one negative and one positive antigen control should be included in the test.

- Detection by molecular methods (PCRs)

For the detection of selected agents, suitably validated molecular methods can be applied either in cells after the amplification procedure, or directly on the cells/virus seeds or materials of animal origin, provided the sensitivity has been proven. For virus seeds neutralisation is not necessary.

- Detection using embryonated eggs

When embryonated eggs are used for detection of influenza virus, the following test method may be used without validation:

Inoculation of the product on test:

Inoculate at least 10 embryonated eggs (9-11 days old) with 0.2 ml of the test sample per egg into the allantoic cavity. The eggs are incubated at 33-37°C. The death of any embryo within 24h of inoculation is considered as non-specific mortality and the egg is discarded. The test is invalid if fewer than 60% of the eggs survive. After 3-4 days of incubation, the eggs are chilled by overnight refrigeration, the allantoic fluid of each egg is harvested and equal quantities are pooled to carry out a second passage: the pool is inoculated into the allantoïc cavity of another 10 embryonated eggs (9-11 days old) with 0.2 ml/egg. After 3-4 days of incubation, the eggs are chilled by overnight refrigeration, and the allantoic fluid is harvested and pooled for the haemagglutination test.

Detection by Haemagglutination:

A drop (about 100 µl) of the allantoic fluid is located on a clean microplate cover lid (or a similar smooth transparent or white surface) in horizontal position. A drop (around 100 µl) of 2-5 % chicken erythrocytes in saline is added. The lid is horizontally shaken for 60 sec. (e.g. on a microplate shaker). Then the mixture is kept for at least 30 min at room temperature and regularly inspected for haemagglutination.

When embryonated eggs are used for detection of poxvirus, the following test methods may be used:

Swine poxvirus - inoculation of the product on test:

Inoculate 10 embryonated eggs (9-11 days old) with 0.2 ml of the test sample per egg onto the chorio-allantoic membrane. The eggs are incubated at 33-37°C. The death of any embryo within 24h of inoculation is considered as non-specific mortality and the egg is discarded. The test is invalid if fewer than 60% of the eggs survive. After 7 days of incubation, the eggs are chilled by overnight refrigeration and candle the chorioallantoic membranes are removed and inspected and look for the absence of pox lesion and/or for the normal appearance of the embryos.

Cowpox virus - inoculation of the product on test:

Inoculate 10 embryonated eggs (6-7 or 9-11 days old with 0.2 ml of the test sample per egg by intravitelline route. The eggs are incubated at 37°C. The death of any embryo within 24h of inoculation is considered as non-specific mortality and the egg is

discarded. The test is invalid if fewer than 60% of the eggs survive. After 7 days, carry out a second passage in the same conditions. After a further 7 days of incubation, candle the eggs and look for the absence of lesion and/or for the normal appearance of the embryos.

4. References

- Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products.
- European Pharmacopoeia 0062 Vaccines for veterinary use.
- European Pharmacopoeia 0030 Immunosera for veterinary use.
- European Pharmacopoeia 5.2.4 Cell cultures for the production of veterinary vaccines.
- European Pharmacopoeia 5.2.5 Substances of animal origin for the production of veterinary vaccines.

Annex - Detection of extraneous agents

Based on available data from already EU-assessed and EU-approved seeds, the cells, as listed in column 2 of the tables below, are considered suitable to check seeds for the absence of the extraneous agents listed correspondingly in column 1. Further, suitable methods of the detection of extraneous agents are mentioned in the corresponding column 3.

Porcine			
1. Extraneous agent(s)	**2. Suitable culture substrates for amplification**	**3. Suitable methods of detection**	**Remarks**
Bovine viral diarrhoea virus	BEL, BHK-21, BT, CK, EBK, EBTr, FBLP, FBT10, IPB3, MDBK, PK-15, SCP	CPE for cytopathic strains	
		IS for non-cytopathic and cytopathic strains	
Classical swine fever virus	IPB3, PK, PK-15	ELISA, IS	
Encephalomyocarditis virus	BHK-21, PK, SK, ST, Vero	CPE	
Foot-and-mouth disease virus	BHK-21, CTY, IB-RS-2, IPB3, MDBK, PK	CPE, ELISA	
Influenza virus	embryonated eggs	HAg	
	MDCK	IS	
Porcine adenovirus	PK, PK-15, SK, ST	CPE	
	FSK, MA104	IS	
Porcine circovirus, type-1 and type-2	CCL-33, PK, PK-15, PS, SK, ST	IS	
Porcine coronavirus - Transmissible Gastroenteritis Coronavirus/Porcine Respiratory Corona Virus	PK, PK-15, ST	CPE	
Porcine coronavirus - Porcine Epidemic Diarrhea Virus	Vero	IS	Requires trypsin to grow in cell culture. Therefore, no need for testing when trypsin is not used.
Porcine enterovirus (incl. SVDV)	BHK-21, PK, PK-15, SK, ST	CPE	
Porcine parvovirus	MA104, PK, PK-15, SK, ST	IS	
Porcine reproductive respiratory syndrome virus	MA104, PAM, PLM	IS	EU strains do not grow in cells other than macrophages.
Porcine rotavirus	MA104	IS	Requires trypsin to grow in cell culture. Therefore, no need for testing when trypsin is not used.
Rabies virus	BHK-21, BSR, BT, DK , EBK, FK, FLK, FSK, MA104, MDCK, MDBK, ST, Vero	IS	
Swine herpesvirus - Aujeszky's disease virus	BEL, BSR, CK, CrFK, DK, FEA, FK, FLK, IPB3, MDCK, MDBK, PBEK, PEK, PK, PK-15, SK, ST, Vero	CPE, IS	
	MA104	IS	

Porcine			
Swine herpesvirus - Porcine cytomegalovirus	PLM	CPE, IS	Does not grow in cells other than macrophages.
Swinepox virus	PK, PK-15, SK, ST	CPE	At least 5 passages are needed
	embryonated eggs	embryo lesions (pock on CA membrane)	
Vesicular stomatitis virus	BHK-21, PK, PK-15	CPE	
	embryonated eggs	embryo death	

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Bovine			
1. Extraneous agent(s)	**2. Suitable culture substrates for amplification**	**3. Suitable methods of detection**	**Remarks**
Akabane virus	BEL, CK, FLK, MDBK	CPE	
	Vero	IS	
Alcelaphine herpesvirus (= malignant catarrhal fever virus – African form)	BEL, CK, FLK, MDBK	CPE	
Bluetongue virus	BHK-21	ELISA, IS	
	embryonated eggs	embryo death	
	BK, BT, FBLP, FK, Vero	IS	
Bovine adenovirus (subgroup 1)	BEL, CK, CT, FBTy, FLK, IPB3, PBEK, MDBK	CPE, HAd, IS	
	BT, EBK, FBLP	IS	
Bovine coronavirus	CK, FLK, MDBK, PBEK, PK-15, SKP	CPE, HAd, IS	
	BT, EBK	IS	
Bovine enterovirus	BHK-21, CK, Vero	CPE, IS	
	BT, EBK	IS	
Bovine herpesvirus	CK, EBTr, FLK, IPB3, MDBK, PBEK, SKP	CPE	
	BT, EBK, FLK	IS	
Bovine leukemia virus	BHK-21, CK, FBL, FLK, IPB3, MDBK	IP, IS	
Bovine papilloma virus	this virus does not grow in cell culture		
Bovine papular stomatitis virus	CK, FBTy, MDBK, PBEK	CPE	
Bovine parainfluenza virus 3	BEL, CK, EBTr, FLK, IPB3, MDBK, PBEK	CPE, HAd, IS	
	Vero	CPE, HAd	
	BT, EBK	IS	
Bovine parvovirus	CK, EBTr, FLK, IPB3, MDBK, PBEK	CPE, HAd, IS	
	FBT-10	CPE	
	BT, EBK, FBLP	IS	
Bovine respiratory syncytial virus	BEL, BFDL, BHK-21, CK, MDBK, IPB3, Vero	CPE, IS	
	BT, EBK, FBLP	IS	
Bovine rhinovirus	CK	CPE	
Bovine viral diarrhoea virus	BEL, BHK-21, BT, CK, EBK, EBTr, FBLP, FBT10, IPB3, MDBK, PK-15, SCP	CPE for cytopathic strains IS for non-cytopathic and cytopathic strains	
Cowpox virus	BEL, CK, CrFK, EBTr, FEF, FK, FLK, MDBK, PBEK, Vero	CPE, IS	
	embryonated eggs	embryo lesions (pock on CA membrane)	
	BSR, FEA	IS	
Epizootic haemorrhagic disease virus	BHK-21, MDBK, Vero	IS	
	embryonated eggs	embryo death	
Foot-and-mouth disease virus	BHK-21, CTY, IB-RS-2, IPB3, MDBK, PK	CPE, ELISA	
Jena virus (Norwalk-like)	this virus does not grow in cell culture		
Lumpy skin disease virus	CK, IPB3, MDBK, PBEK, Vero	CPE	

Bovine			
Ovine herpesvirus 2 (= malignant catharral fever virus – European type)	this virus does not grow in cell culture		
Pseudocowpox virus	BHK-21, CK, MDBK	CPE	
Rabies virus	BHK-21, BSR, BT, DK, EBK, FK, FLK, FSK, MA104, MDCK, MDBK, ST, Vero	IS	
Reovirus	BEL, CK, MDBK	CPE, IS	
	BT, DK, FBLP, FK, Vero	IS	
Rinderpest virus	CK, MDBK, Vero	CPE	
Rotavirus	BT, CK, EBK, MDBK	IS	Requires trypsin to grow in cell culture. Therefore, no need for testing when trypsin is not used.
Swine herpesvirus 1 (= Aujeszky's disease virus)	BEL, BSR, CK, CrFK, DK, FEA, FK, FLK, IPB3, MDCK, MDBK, PBEK, PEK, PK, PK-15, SK, ST, Vero	CPE, IS	
	MA104	IS	
Vesicular stomatitis virus	BEL, BHK-21, CK, CTY, IB-RS-2, MDBK, PK, Vero	CPE, IS, ELISA	
	FBLP	IS	
	embryonated eggs	embryo death	

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Canine			
1. Extraneous agent(s)	**2. Suitable culture substrates for amplification**	**3. Suitable methods of detection**	**Remarks**
Canid herpesvirus	DK, MDCK	CPE	
Canine adenovirus	DK, MDCK	CPE, HAd	
Canine coronavirus	A-72, CrFK, DK, IRC, MDCK	CPE	
Canine distemper virus	A-72, DK, MDCK, Vero	CPE	
Canine oral papilloma virus	No known cell culture replication		
Canine Parainfluenza 2 virus	DK, MDCK	CPE (+ HAg), IS	Additionally, an haemagglutination test may be performed to improve reading of the CPE.
	CrFK, Vero	CPE (+HAg)	
Canine parvovirus	CrFK, DK, FEF, IRC, MDCK	CPE (+ HAg)	Additionally, an haemagglutination test may be performed to improve reading of the CPE.
	CrFK	IS	
Rabies virus	BHK-21, BSR, BT, DK, EBK, FK, FLK, FSK, MA104, MDBK, MDCK, ST, Vero	IS	
Swine herpesvirus 1 (= Aujeszky's disease virus)	BEL, BSR, CK, CrFK, DK, FEA, FK, FLK, IPB3, MDCK, MDBK, PBEK, PEK, PK, PK-15, SK, ST, Vero	CPE, IS	
	MA104	IS	

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Feline			
1. Extraneous agent(s)	**2. Suitable culture substrates for amplification**	**3. Suitable methods of detection**	**Remarks**
Cowpox virus	BEL, CK, CrFK, EBTr, FEF, FK, FLK, MDBK, PBEK, Vero	CPE, IS	
	BSR, FEA	IS	
	embryonated eggs	embryo death	
Feline endogenous retrovirus	HEK293	RT-PCR for RD114 virus : sense primer: 5'-ccattcctgccattgatcatta-3' antisense primer: 5'-ggtgattcccagtccagctagt-3'	
Feline calicivirus	CrFK, FEF, FK, IRC	CPE, IS	
	FEA	CPE	
Feline coronavirus	CrFK, FEF, FK, IRC	CPE, IS	Type-II feline coronaviruses induce CPE on various feline cell lines. Type-I feline coronaviruses only replicate in feline macrophages.
Feline foamy virus (feline syncytia forming virus)	FEA, FEF	CPE	
	CrFK, IRC	IS	
Feline herpesvirus 1	CrFK, FEA, FEF, FK, IRC	CPE	
Feline immunodeficiency virus	MYA-1, Q-201	ELISA	
Feline leukemia virus	CrFK, FEF	ELISA	
	CrFK, IRC	IS	
	C81, FEA, QN-10	CPE	S+L- cells are transformed by infection with FeLV or replication-competent FeSV.
Feline panleucopenia virus	CrFK, FK, IRC	CPE (+ HAg)	Additionally, an haemagglutination test may be performed to improve reading of the CPE.
	CrFK	IS	
Feline sarcoma virus	FEA, QN-10	CPE	S+L- cells are transformed by infection with FeLV or replication-competent FeSV.
	CrFK	ELISA	
Rabies virus	BHK-21, BSR, BT, DK, EBK, FK, FLK, FSK, MA104, MDCK, MDBK, ST, Vero	IS	

Feline			
Swine herpesvirus 1 (= Aujeszky's disease virus)	BEL, BSR, CK, CrFK, DK, FEA, FK, FLK, IPB3, MDCK, MDBK, PBEK, PEK, PK, PK-15, SK, ST, Vero	CPE	
	MA104	IS	

A-72 = canine fibroblast cell line; BEL = bovine embryo lung cell line; BFDL = bovine fetal diploid lung cell line; BHK-21 = baby hamster kidney cell line; BT = bovine turbinate cell line; C81 = feline S+L- fibroblast cell line; CCL-33 = porcine kidney cell line; CK= primary calf kidney cell; CrFK = Crandell-Rees feline kidney cell line; CT = primary calf testis cell; CTY = calf thyroid cell line; DK = primary dog kidney cell; EBK = embryonic bovine kidney primary cell; EBTr = embryonic bovine trachea cell line; FBL = foetal bovine lung cell; FBT10 = ; FBTy = primary fetal bovine thyroid cell; FEA = feline embryo fibroblast cell line; FEF = primary feline embryo fibroblast; FK = primary feline kidney cell; FLK = foetal lamb kidney cell; FSK = primary fetal swine kidney cell; HEK293 = human embryonic kidney cell line; IB-RS-2 = porcine kidney cell line; IPB3 = bovine lung cell line; IRC = cat kidney cell line; L929 = murine fibrosarcoma cell line; MA104 = monkey african green kidney cell line; MDBK = Madin-Darby bovine kidney cell line; MDCK = Madin-Darby canine kidney cell line; MYA-1 = feline lymphoid cell line; PAM = porcine alveolar macrophage; PBEK = primary bovine embryo kidney cell; PEK = pig embryo kidney cell line; PK = primary kidney cell; PK-15 = porcine kidney cell line; PLM = porcine lung macrophage; PS = porcine kidney cells; Q-201 = feline S+L- lymphoid cell line; QN-10 = feline S+L- fibroblast cell line; SCP = sheep choroid plexus cell line; SK = primary swine kidney cell; SKP = sheep kidney primary cell; ST = swine testis cell line; Vero = african green monkey kidney cell line.

CPE= cytopathic effect; ELISA = enzyme linked immuno assay; HAd = haemadsorption assay; HAg = haemagglutination test; RT-PCR = reverse-transcriptase polymerase chain reaction; IP = immunoprecipitation; IS = immunostaining assay.