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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. 0062, 0030, 5.2.4., 5.2.5.), immunological veterinary medicinal products and materials of biological origin 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. 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 threatening the good manufacturing practice (GMP) status of the company's facilities.
- The limit of detection of the chosen test method(s).

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 is not complete and covers only cells and other substrates as well as suitable test methods for porcine, bovine, feline and canine extraneous agents. Furthermore only a few companies contributed to this collection of data. The annex can be updated whenever necessary, in particular to take into account new extraneous agents or additional 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 relevant test method(s) mentioned 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 unless justified (i.e. the absence of use of primary cells for testing of primates extraneous viruses is acceptable). 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 the 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 the Ph. Eur. 5.2.4. Cell cultures for the production of veterinary vaccines.

3. Viral detection methods

Testing for potential infectious viruses can be shown by various means.

The use of cell cultures, intended to be inoculated with components of cell or virus seeds to allow appropriate detection of extraneous agents is one possibility. Once the cell culturing step is complete suitable tests for the detection of the extraneous agents should be performed on the cell monolayers.

The following listed methods for detection of extraneous agents using cell cultures 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 cytopathic effects (CPE)
- Detection of haemadsorption
- Detection of haemagglutination
- Detection by immunostaining (IS)
- Detection by ELISA

Suitable tests or methods for the detection of extraneous agents include also the use of molecular methods, e.g. nucleic acid amplification techniques (NAT). For the detection of selected agents, a suitable method can be applied either in cells after the amplification procedure, or directly on the cells/virus seeds, provided the sensitivity or the specificity have been proven. For virus seeds neutralisation is not necessary.

The use of embryonated eggs intended to be inoculated with seeds to allow appropriate detection of extraneous agents is a further possibility. When embryonated eggs are used for detection of extraneous agents, viruses can be cultivated in various parts of eggs like chorioallantoic membrane, allantoic cavity, amniotic sac and yolk sac. After incubation viral growth and multiplication in the embryonated egg is indicated by the death of the embryo, by embryo cell damage, or by the formation of typical pocks or lesions on the egg membranes.

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:

- Cells and other substrates, 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.
- 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	
Swine herpesvirus - Porcine cytomegalovirus	PLM	CPE, IS	Does not grow in cells other than

Porcine			
			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	

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	

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, a 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	

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, a 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	
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.