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ANNEX 2

DRAFT EU COMMENTS

ON THE PROPOSED CHANGES TO THE

OIE MANUAL OF DIAGNOSTIC TESTS AND VACCINES FOR TERRESTRIAL ANIMALS

PRESENTED FOR COMMENTS IN OCTOBER 2019

DRAFT EU COMMENTS
On the proposed changes to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals

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2.1.2. Biotechnology advances in the diagnosis of infectious diseases

General comment:

The EU can support the revised chapter.

We would like to suggest that if there is a subsequent rewrite- it may be helpful to say which nucleic acid based methods are used more frequently now and why. For example nested PCR was used a number of years ago- but has been mostly superseded by real time methods, because it's very sensitive and subject to contamination in a routine lab setting.

The sequencing section (5) seems to be written from the lab angle. It would be helpful to mention:

- cost of sequencing are reducing and mobile technologies being developed

- talk about analytics - as the majority of the challenge lies here- for example:

- The use of high throughput sequencing:

Generates a lot of data- there are questions about storage and computing capacity to analyse in a timely and cost effective manner;

There is a considerable amount of discussion about how data should be shared and protected (Nagoya and Data protection – GDPR in Europe);

Requires specialised bioinformatics to analyse and make accessible to vets/practitioners;

The use of these approaches as part of an epidemiological investigation is already happening and has potential to revolutionise this area.

Specific comments:

LINE 25: DNA amplification platforms are rapid. We suggest deleting 'relatively'.

LINE 28: We believe that the quality of the sample is key and therefore it should be mentioned.

LINE 96: in the table Nested PCR should include in the 'Disadvantage' column that it is not recommended for diagnostics.

We do not believe cost for Real-time PCR is a disadvantage any longer.

For Multiplex real-time PCR, in the 'Disadvantages' column it should also include that it is 'very difficult to validate if more than 2 or 3 targets are included'.

LINE 164: in bullet point states 'i) A much simpler procedure than PCR'. We consider that this is not correct, as the high number of primers makes the developement often more complex than for PCR. Ultrafast real-time PCR is not much slower than for example LAMP.

LINE 196: for clarity, add '(NGS)', after next-generation sequencing.

LINE 225: table 2, for High throughput sequencing (HTS) also known as next-generation sequencing (NGS) and massive parallel sequencing (MPS), in the 'Details' column we suggest adding 2 extra bullet points for Microbiome detection and Transcriptome detection.

LINE 227: we disagree with the text stating that 'Sanger sequencing can be expensive' because a single run of around 1000bp, as a commercial sequencing run, costs less than 3 euros.

LINE 254: we consider that the error rate of these instruments is around 5-20%, the maximum figure of 50% is too high.

LINE 278: you may wish to include a more recent reference: Loeffler 4.0: Diagnostic Metagenomics, Höper D, Wylezich C, Beer M. Adv Virus Res. 2017;99:17-37. doi: 10.1016/bs.aivir.2017.08.001. Epub 2017 Sep 21. Review. PMID: 29029726

LINE 289: we suggest adding an extra reference: A Versatile Sample Processing Workflow for Metagenomic Pathogen Detection, Wylezich C, Papa A, Beer M, Höper D. Sci Rep. 2018 Aug 30;8(1):13108. doi: 10.1038/s41598-018-31496-1. PMID: 30166611

3.1.7 Epizootic haemorrhagic disease (infection with epizootic haemorrhagic disease virus)

General comment:

The EU can support the revised text. We suggest using VP7 or VP-7 consistently in text, the authors change between the two.

Specific comments:

LINE 21: there is a reference missing that describes one if the two new putative serotypes. The reference required is *Wright, I. M. 2013: Serological and Genetic Characterisation of Putative New Serotypes of Bluetongue Virus and Epizootic Haemorrhagic Disease Virus Isolated From an Alpaca. North-West University, South Africa.* This also needs to be referenced on LINE 74.

LINE 154: Pirbright (UK) used to supply reagents for the EHDV antigen capture ELISA, but they n longer do, this sentence should be removed. This is also mentioned in LINES 176-180.

LINE 125 and 126: please, correct the citation *Eschembauer et al*. as *Eschbaumer et al*.

3.1.10 Japanese encephalitis

General comment:

The EU in general can support the revised text.

We wish to highlight that the most recent reference is from 2012 and this is for a book chapter, but most are much older. Overall references should be updated to reflect developments and reviews from the past 10 years.

We are including a few more contemporaneous scientific references:

Veterinary Review

Mansfield et al., (2017). Japanese encephalitis virus infection, diagnosis and control in domestic animals. Vet Microbiol 201, 85-92.

JEV in mosquitoes

Oliveira et al., (2018). Assessment of data on vector and host competence for Japanese encephalitis virus: A systematic review of the literature. Prev Vet Med 154, 71-89.

Phylogeny of JEV

Schuh et al., (2014). Dynamics of the emergence and establishment of a newly dominant genotype of Japanese encephalitis virus throughout Asia. J Virol 88, 4522-4532.

Specific comments:

LINE 60: Table 1, RT-PCR is most rapid/sensitive/specific method commonly used for detection of viruses. Real-time RT-PCR should be given higher rating for “confirmation of clinical cases” - +++.

LINES 70-71: states that “The definitive diagnosis of JE in horses, or in cases of reproductive failure in sows, depends on the isolation or detection of the causal virus in neurological specimens.” But, encephalitis is not a common feature in swine. We suggest revising this phrase.

LINE 421: A commercial ELISA for veterinary use in JE should be a priority and more information in section B2.4 is needed.

LINE 472: Section C2.1.3 required re-editing to include a reference for single serotype of JEV (Wills et al Vaccine 1992, 10, 861-861 / Tsarev et al Vaccine 2000 S2, 36-43).

3.1.11 Leishmaniosis

General Comment:

The EU can support the revised chapter.

We suggest mentioning that for long time, the dog has been considered the main reservoir of *L. infantum*, but in the recent outbreak of visceral leishmaniasis in Spain, hares (*Lepus grana*tensis) and rabbits (*Oryctolagus cuniculus*) were described as a reservoir (Molina et al., 2012; Jiménez et al., 2014).

Specific Comments:

LINE 74: We would suggest adding onychogryphosis as one of the typical external signs recorded in infected dogs (Ciaramella et al. 1997- The Veterinary record 141(21): 593-43.

LINE 111: editorial typo, the word ‘be’ is repeated twice.

3.1.15 Paratuberculosis (Johne's disease

General comment:

The EU can support the revised chapter.

Specific comments:

LINE 21: PCR should be mentioned here as well especially as PCR is described as the first or equal choice of diagnostic techniques in Table 1 (except in the case of clinical cases).

LINE 53: The disease has also been reported in donkeys (Stief et al., 2012). This should be added.

Reference: Stief B, Möbius P, Türk H, Hörügel U, Arnold C, Pöhle D: [Paratuberculosis in a Miniature Donkey (Equus Asinus F. Asinus)]. Berl Munch Tierarztl Wochenschr, 125 (2012), 38-44. [Article in German]

LINE 104: Table 1: Culture is as suitable for the confirmation of freedom from infection as PCR. PCR is quicker and may detect viable but non-culturable bacteria, but generally, it is less sensitive than culture. Please insert "+" in both cells of the table.

3.3.4 Avian influenza (infection with high pathogenicity avian influenza viruses)

General comment:

The EU in general can support the revised chapter. We have included specific comments below.

We have observed that major modifications in the terminology have been introduced during the revision in line with the proposed changes of the relevant chapters of the Terrestrial Code: “high pathogenicity AI” is now the official denomination instead of highly pathogenic AI, and definitions for “LPAI” and “influenza A” have been amended. However, these changes have not been consistently made across the text: in many in phrases ‘highly pathogenic AI’ is still there (although a couple of these are quotes in the text and may therefore remain as they are) and should be changed to “high pathogenicity AI”, wherever possible. These changes have not been included in the specific comments.

The same applies also to “influenza A (virus)” vs “influenza virus”: the former should be consistently used in the text (since there is a specific definition) instead of the latter. This is included in the specific comment in line 112.

We would also like to suggest that revisions of the chapters on AI and ND in this Terrestrial Manual should be reviewed in parallel. It is really important that some sections of the text, for example those dealing with virus isolation in embryonated eggs, including processing of samples, and with HA/HI test, where the methods are similar and in some cases identical for both pathogens with the only exception of the specific antigens or antisera used for HI or the description of cross-reactions observed.

Specific comments:

LINE 2: To be consistent with the definitions, the title should read: “HIGH PATHOGENICITY AVIAN INFLUENZA (INFECTION WITH HIGH PATHOGENICITY AVIAN INFLUENZA VIRUSES)”.

LINE 7: Taxonomy of the family Orthomyxoviridae has been updated. The highlighted sentences should read:

“Influenza A is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus Alphainfluenzavirus. There are four influenza genera – Alpha, Beta, Gamma and Deltainflunzavirus; only influenza A viruses are known to infect birds.”

For the last sentence, although it may be borderline regarding taxonomy, we wish to mention that only the 4 influenza virus genera sensu stricto are mentioned: there are actually 7 defined genera in the family Orthomyxoviridae…

LINE 50-51: we suggest the introduction of the abbreviation LPAI for future reference, in line 69"Low pathogenicity avian influenza (LPAI) in poultry is notifiable […]".

Low pathogenicity avian influenza in poultry is notifiable if accompanied by a sudden and unexpected increase in virulence (emerging disease) or has proven natural transmission to humans associated with severe consequences. In these disease scenarios there should be formal monitoring in relevant poultry populations by national authorities. The occurrence of H5 and H7 low pathogenicity avian influenza viruses should be monitored as some have the potential to mutate into high pathogenicity avian influenza viruses.

LINE 54: As written it is not clear to which viruses exactly refer to when it comes to LPAI viruses that "*suddenly show unexpected increase in virulence or has proven natural transmission to humans associated with severe consequences"* are notifiable. Can we include all ortomixoviruses type A?
Given that even viruses that do not belong to the H5 or H7 subtypes have proven natural transmission to humans (i.e. H9 strains): how should these be considered? Furthermore, other no H5/H7 strains (i.e. the recent H3N1 virus responsible for losses in Belgium and France this year) can be responsible for emerging disease with severe consequences for the poultry sector.
WE believe that the text could be improved by removing any doubt about such issues.
Furthermore, what are the objective elements that can make these viruses fall into this category (viruses that "*may have potential to exert a negative impact on animal and public health*"). Should the severity of the losses (direct and indirect) in poultry and the spread of these strains be of a certain type? Are any specific molecular markers required?

LINE 55: This sentence does not look coherent with the text itself when reading line 295, and when the requirement for LPAI monitoring are also mentioned in the Terrestrial Code to apply for HPAI-free status. If this is the case, the abstract should probably read:

"The occurrence of H5 and H7 low pathogenicity avian influenza viruses has to be monitored as some have the potential to mutate into high pathogenicity avian influenza viruses."

If line 55 is maintained then line 295 has to be changed accordingly.

LINE 84: this comments also refers to appendix 3.3.4.1 and line 1094. Although BSL corresponds to group 4 pathogens, it is mentioned here, while the text in lines 1095-1098 actually refers to BSL corresponding to group 3 pathogens. We believe that these references need some clarification.

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LINE 87: see line 7 => "Alphainfluenzavirus"

LINE 110: The definition of HPAI differs from the rest of the text. This should read:

"HPAI as an infection by any influenza A virus that meets the definition of high pathogenicity,"

LINE 112: Influenza A is defined here, and is coherent with the rest of the definitions. However, in many instances the phrase "influenza virus" is used in place of "influenza A virus". We have not highlighted these in the text but we suggest a comprehensive review so that all occurrences are modified to read "influenza A virus", unless a compelling reason exists.

LINE 127: we believe that the reference to section B.2 is wrong, and should be "section B.1.1.1".

LINES 134-135: we believe the following phrase could be written with a different structure, the other way round, to read better, e.g.: "In order to avoid accidental spread of viruses that cause influenza A, laboratories should define and implement adequate levels of biosecurity and biosafety to handle these viruses."

LINES 150-188: Since virus isolation in embryonated SPF/SAN hen eggs is a technique used for both AI and ND diagnosis, most of these paragraphs should be compared and harmonised with the corresponding revised text for ND. We have noticed at least 2 discrepancies that must be resolved:

- Temperature requirement < 25°C for pre-analytical treatment of samples mentioned for ND but not for AI,
- Temperature range for egg incubation is discordant, but should probably be 35-39°C to accommodate the requirement for AI,
- Minimum number of eggs for testing (3 for AI, 5 for ND),
- Volume of inoculum (0.2 ml?) is not mentioned in the chapter for AI.

We may have missed other discrepancies but we believe it will be better to have a single text for the pre-analytical treatment of samples and for the inoculation, incubation and handling of eggs.
For a more comprehensive review of the discrepant or alternative formulations between both chapters, please refer to the comments on the chapter for Newcastle disease.

LINE 185: we believe that the reference to section B.3.2 is wrong, and should be "section B.1.2.2".

LINE 190: we believe that hhe reference to section B.3.1 is wrong, and should be either deleted or it may need to be replaced by "section B.2.2".

LINES 203-204: in line 488 BPL is also mentioned for the inactivation of the influenza A antigen. For this reason we believe that it should be added: “following by treatment with 0.1% formalin or 1% betapropiolactone”.

LINE 213: The abbreviation RBCs is not spelt out here, but only in line 512. The meaning of RBC should be included here and later removed from line 512 “the agglutination of red blood cells (RBCs)”.

LINE 214: we suggest comparing with the revised text for ND “There are currently 20 recognised serotypes of avian paramyxoviruses (Miller et al., 2010).” The reference to the 2010 article will also have to be revised accordingly.

LINES 285-288: Typo: a closing bracket is missing at the end of line 288 “(see the table that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence, which can be found on the OFFLU site at: http://www.offlu.net/fileadmin/home/en/resource-centre/pdf/Influenza_A_Cleavage_Sites.pdf).”

LINE 295: Please, see line 55 (in the abstract). Both texts have to be made coherent and be either a strict requirement or a recommendation (“are / has to be” vs “should”).

LINE 296-301:Low pathogenicity avian influenza in poultry is notifiable if accompanied by a sudden and unexpected increase in virulence (emerging disease) or has proven natural transmission to humans associated with severe consequences. These viruses should be monitored. ~~For the purposes of the Terrestrial Code, HPAI and H5/H7 LPAI in poultry are termed “avian influenza” and are notifiable. Non-H5/H7 influenza A (i.e. H1–4, H6 and H8–16) are not “avian influenza” and are not notifiable~~.

According to the text highlighted in yellow above, most H9N2 infections in Asia/Africa have to be notifiable.

LINE 289: To avoid any misinterpretation, it could be specified: “*Multi–basic cleavage sites containing several basic amino acids in the critical position of the cleavage site of the haemagglutinin molecule (HA0) currently remains the identifying sign of HPAI strains; therefore, the isolate being tested to confirm this characteristic will be considered to be HPAI even if the sequence is unusual and different from that observed so far”.* It being understood that any new isolate should also be tested in vivo by IVPI.

LINE 297: It may be advisable to say “….as H5/H7 LPAI and are to be (or must be) monitored”.

LINE 307: we believe that there is an omission: “This can be done by extraction of the specimen and direct sequencing […]”.

LINE 326: It may be necessary to rethink about what to do in these cases. What should be the cases for a non-H5 / H7 virus (such as an H10 or an H9) with an IVPI> 1.2 not inducing death or signs of disease when inoculated intranasally (or when circulating in poultry without serious consequences) and without a site sequence of hemagglutinin cleavage compatible with HPAI virus, are they notifiable or not?
In this case, would it be useful to introduce an in vivo test performed with a natural infection to discriminate a true HPAI from a laboratory phenomenon?

LINE 353: we do not consider all these tests working in 15 minutes.

LINE 436: Typo: “For example, Hoffman et al. (2007) have described a real-time RT-PCR test […]”.

LINE 488: Please, see lines 203-204. WEW believe that both statements should be the same “0.1% formalin or 1% betapropiolactone”.

LINES 503-510: we suggest rewording as follows: ‘Tests are usually carried out using 15 ml of gel of 1,25% (w/v) agarose or purified type II agar and 8% (w/v) in distilled water, poured in a 90 mm Petri dish (to have about 3 mm thick agar gel), and incubated in a humidified chamber. By using a template and cutter, wells of approximately 5 mm in diameter at a distance of about 3 mm from each are cut into the agar. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. Each well should have reagent added to fill the well, corresponding to the top of the meniscus with the top of the gel, but do not over fill. Approximately 25-30 µl of each reagent should be required per well, but this depends on thickness of the gel, with thicker gels requiring an additional volume of reagent’.

LINES 508-576: we believe that the paragraph related to the HA/HI test should be harmonised with the revised text proposed for ND: U-shaped plates are now mentioned for ND. Practical experience shows that when U-shaped plates are compared to V-shaped plates, it is the quality and the nature of the plastic itself what probably makes the difference in terms of clarity of the test. For this reason some organisations use U-plates and this is why for example it is explicitly indicated in the corresponding French AFNOR standardised methods NF U47-011 / NF U47-036-1 / NF U47-036-2.

LINE 1094: See also line 84. We recommend that the BSL requirements/ recommendations should be clarified here and in the following lines.

3.3.5 Avian mycoplasmosis (*Mycoplasma gallisepticum, M. synoviae*)

General comment:

The EU can support the revised chapter.

Specific comments:

LINE 11: we suggest rewording to read ‘It can result in ~~loss of~~ significant…’

LINE 59: we suggest deleting ‘usually’ to read: ‘…organisms in an oil emulsion are licensed in several countries. ~~Usually t~~ They should be ~~are~~ administered parenterally to pullets at 12–16 weeks of age~~, usually subcutaneously in the~~…’

LINE 74: when the text talks about ‘important impacts’, we suggest including those, for example: inability to find food, starvation or death.

LINE 140: footnote b, we suggest including that it can also be done on samples.

LINE 188: when ‘pig serum’ is mentioned, we suggest including that it should be free of antibiotic residues that inhibit mycoplasma growth.

LINE 191: Thallium can also have a slight inhibitory effect on the growth of Mycoplasma species.

LINE 454: PCR also works on DNA extracts from clinical samples

LINE 571: No comment has been made about the use of immunoblotting as a confirmatory test. Reference: D. de B. Welchman, H. L. Ainsworth, T. K. Jensen, M. Boye, S. A. King, M. S. Koylass, A. M. Whatmore, R. J. Manvell, R. D. Ayling & J. R. Dalton (2013) Demonstration of Ornithobacterium rhinotracheale in pheasants (Phasianus colchicus) with pneumonia and airsacculitis, Avian Pathology, 42:2, 171-178, DOI: 10.1080/03079457.2013.778387

3.3.14 Newcastle disease (infection with Newcastle disease virus)

General comment:

Some revisions of the chapters on AI (3.3.4) and on this one for the Terrestrial Manual should be reviewed in parallel: it is really important that some portions of the text (those dealing with virus isolation in embryonated eggs, including processing of samples, and with HA/HI tests) be as identical as possible, given some of the described methods are essentially the same for both pathogens (except for the specific antigens or antisera used for HI or some description of cross-reactions observed).

Given a large part of the revision is about the molecular methods: the corresponding part has been much expanded, making in some instances the information difficult to extract concerning the most relevant methods described. We fully appreciate the effort taken by the experts to produce this update (and the difficulty to produce an up-to-date text), but a more clear statement about the existing published methods, that have been comprehensively tested for their fitness for purpose, would be an important clarification for a correct implementation of a relevant laboratory diagnostic pipeline.

Specific comments:

LINE 6: should be set in italic type to conform with the rest of the paragraph = "also known as Newcastle disease virus (NDV)".

LINE 7: should be set in Roman type = "Orthoavulavirus".

LINE 8: Abreviations for the different types of avian paramyxoviruses should use the same spelling with a hyphen in all the document = "APMV-1 to APMV-20".
The same correction APMV1 => APMV-1 should also be applied in lines 354, 373, 525, 543 and 553 (highlighted in the main text).

LINES 15-16: Concerning swabs from the upper respiratory tract, tracheal or oropharyngeal swabs are mentioned in the chapter for ND, whereas only oropharyngeal swabs are explicitly mentioned in the chapter for AI. It would be better to use the same terminology (and maybe retaining "tracheal or oropharyngeal swabs") in both texts.

LINE 18: The denomination "embryonated fowl eggs" / "embryonated hens' eggs" (in line 46) / "embryonated chicken eggs" is variable within the text. Since the phrase "embryonated chicken eggs" is constantly used in the chapter for AI, it would be better to use this last option only.
Duration of egg passages has also been changed in the main text: "The eggs are incubated at 37°C for 2–7 days".

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LINES 21-23: As mentioned in lines 187-191, APMV-12 should be added to the list. The paragraph should also be complemented with a mention of the possible alternative use of molecular techniques for the detection of the APMV-1 genome instead of the HI test. Lines 192-196 mention this possibility for some unspecified RT-PCR techniques, with some warning about careful fitness for purpose testing of the method: however, using the real-time RT-PCR methods referenced in lines 415-426 (Sutton 2019 and Kim 2008) would overcome the defects in inclusivity mentioned at the end of paragraph 1.3 (analytical sensitivity is not an issue here since positive allantoic fluids are high titer samples).
The end of the paragraph should therefore read: ”[…], particularly APMV-3 and APMV-7. Real-time reverse-transcription polymerase chain reaction (RT-PCR) on the positive allantoic fluids could also be used as an alternative method for initial APMV-1 identification (provided the method was determined as fit for this purpose).”

LINES 24-26: An explicit mention of the main criteria of the definition for a NDV would be important to add here in the summary “Any APMV-1 with an ICPI ≥ 0.7, or with multiple basic amino acids at the C-terminus of the F2 protein and phenylalanine at residue 117 of its F protein, is considered as a virulent NDV strain.”

LINE 35: Since inclusivity of the screening test is as important an issue as sensitivity, this could read “Confirmation of detection using highly sensitive and inclusive screening real-time RT-PCR […]”.

LINE 36-37: The end of the sentence could be rephrased “[…] by F gene sequencing to determine the virus virulence (the F gene proteolytic cleavage site) and virus genotype.”

LINES 30-38: Given the more comprehensive introduction and description of recent molecular tests for APMV-1 diagnosis, an explicit reference to the screening real-time RT-PCR methods highlighted in lines 415-426 for their extensive validation / field usage (Sutton 2019 and Kim 2008) would be helpful.

LINE 101: Table 1. Some of the grades indicated are really surprising, and need either justification or re-evaluation.
Virus isolation is now considered as suitable in very limited circumstances for individual animal freedom testing before movement: this is not coherent with the current status of the test (as prescribed test for international trade in the current version of the chapter), contradicts lines 263-265 in the main text and is nearly opposite to the grade given to the same method for AI diagnosis. A head-to-head comparison of the tables for AI and ND concerning virus isolation would be important to mitigate their indications.
Given some of the warnings mentioned in the text about the possible lack of sensitivity / inclusivity of some real-time RT-PCR techniques, the +++ grade in the table should probably be qualified and restricted to specified protocols, which should be made explicit in the table.
The relative grades for HI / ELISA for serological surveillance / control o indicated in the table are also opposite to what is mentioned in the text as common practice in lines 439-445.

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My general advice is to try and make the indications of table 1 coherent with the text itself and with some of the indications given for AI, when this is relevant (for example, for virus isolation).

LINES 107-151: Unless otherwise indicated, all highlighted phrases correspond to parts of the text that need comparing with the corresponding method described for AI (processing of samples for virus isolation, inoculation and collection of allantoic fluids). The goal would be here to have a single text, unless there is a strong reason for alternative techniques.

1.1. Samples for virus isolation

When investigations of ND are the result of severe disease and high mortality in poultry flocks, it is usual to attempt virus isolation from recently dead birds or moribund birds that have been killed humanely. Samples from dead birds should consist of oro-nasal swabs, as well as samples collected from lung, kidneys, intestine (including contents), caecal tonsils, spleen, brain, liver and heart tissues. These may be collected separately or as a pool, although brain and intestinal samples are usually processed separately from other samples.

Samples from live birds should include both tracheal or oropharyngeal and cloacal swabs, the latter should be visibly coated with faecal material. Swabbing may harm small, delicate birds, but the collection of fresh faeces may serve as an adequate alternative. Further the type of swabs used may affect test sensitivity or validity with thin wire or plastic shafted swabs preferred.

Where opportunities for obtaining samples are limited, it is important that cloacal swabs (or faeces), tracheal (or oropharyngeal) swabs or tracheal tissue be examined as well as organs or tissues that are grossly affected or associated with the clinical disease. Samples should be taken in the early stages of the disease.

The samples should be placed in isotonic phosphate buffered saline (PBS), pH 7.0–7.4, containing antibiotics. Protein-based media, e.g. brain–heart infusion (BHI) or tris-buffered tryptose broth (TBTB), have also been used and may give added stability to the virus, especially during shipping. The antibiotics can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml); streptomycin (2 mg/ml); gentamycin (50 µg/ml); and mycostatin (1000 units/ml) for tissues and tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to readjust the concentrated stock solution to pH 7.0–7.4 before adding it to the sample. If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely ~~minced~~ homogenised tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. When immediate processing is impracticable, samples may be stored at 4°C for up to 4 days.

1.2. Virus isolation ~~(the prescribed test for international trade)~~

The supernatant fluids of faeces or tissue suspensions and swabs, obtained through clarification by centrifugation at 1000 ***g*** for about 10 minutes at a temperature not exceeding 25°C, are inoculated in 0.2 ml volumes into the allantoic cavity of each of at least five embryonated SPF fowl eggs of 9–11 days incubation. If SPF eggs are not available, at least NDV antibody negative eggs are required. After inoculation, these are incubated at 35–37°C for ~~4~~ 2–7 days. To accelerate the final isolation, it is possible to carry out two passages at ~~a~~ 1- to 3-day intervals, obtaining results comparable to two passages at ~~4~~ 2–7-day intervals (Alexander & Senne, 2008a). Eggs containing dead or dying embryos as they arise, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for a minimum of 4 hours or overnight (and checked for embryo death before proceeding) and the allantoic fluids tested for haemagglutination (HA) activity. Fluids that give a negative reaction should be passed into at least one further batch of eggs. Routine checks for contamination should be conducted by streaking samples in Luria Broth agar plates and reading these at 24 and 48 hours of incubation against a light source. Contaminated samples can be treated by incubation with increased antibiotic concentrations for 2–4 hours (gentamicin, penicillin g, and amphotericin b solutions at final concentrations to a maximum of 1 mg/ml, 10,000 U/ml, and 20 µg/ml, respectively). Samples heavily contaminated by bacteria that cannot be removed by centrifugation or controlled by antibiotics can be

> filtrated through 0.45 and 0.2 micron sterile filters. Filtration should be used only when other methods fail because aggregation may significantly reduce virus titre.

LINES 141-142: The requirement for the minimum duration of chilling is now strong. However, based on the standard AFNOR method NF U47-210 (isolation of hemagglutinating avian viruses by embryonated egg inoculation), we routinely perform the chilling step for 2 hours at 2-8 °C and have not observed any evidence that the embryos were still alive after this step. The indication given in the revised text could relaxed accordingly, and some precise indication given for checking embryonic death, if the sentence is maintained.

LINE 170: we propose to rephrase the sentence for clarity "Comparing virus isolation rates for APMV-1, in different in ovo models inoculated with real-time reverse-transcription polymerase chain reaction (RT-PCR)-positive wild bird surveillance samples, […]".

LINE 239: we suggest rephrasing the sentence: "It would appear that the F0 molecules of viruses virulent for chickens can be cleaved by a host protease or proteases found in a wide range of cells and tissues, and thus allow the virus to spread throughout the host damaging vital organs, […]".

LINES 314-315: In order not to risk failing to detect a dangerous strain in poultry holdings that use live vaccines, or in the case of mixed infection with virulent and avirulent strains, we would suggest changing the sentence "*Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test*" adopting the following that is the result of a brainstorming between AI-ND EURL (also AI OIE Ref. labs) and other two OIE labs (APHA, UK and FLI, Germany) "*Failure to detect viruses with multiple basic amino acids at the F0 cleavage site using molecular techniques does not necessarily confirm the absence of a virulent virus. In case a primer mismatch for a virulent virus detection is assumed, and/or in the event that a mixed population of virulent and a-virulent viruses is suspected on the basis of clinical disease indicators or of the diagnostic tests performed, further samplings and analyses are required using approved methods that might include virus isolation followed by in-vivo assessment of virulence (ICPI), use of specific PCRs demonstrated to reliably detect virulent virus or combination of methods*"

LINE 362: we suggest rephrasing the last part of the sentence: "Increasingly, given the difficulties in shipping biological materials containing infectious substances, FTA (finders technology association) cards have been used […]".

LINE 370: see comment for line 35 above "A molecular testing algorithm for NDV should ideally comprise a screening assay of high sensitivity and inclusivity […]"

LINES 394-406: we consider that most of the paragraph is redundant with lines 369-373. Given the expansion and complexity of paragraph 1.9, all these should be summarized as a single paragraph before line 374.

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LINES 415-426: Modify class 1 and class 2 to “class I” and “class II”: figures should be in roman not arabic (they are spelt as roman numerals elsewhere in the text).

LINES 446-493: Apart from lines 454-455 discussed below, I have not noticed major variations between HA/HI methods described in this chapter, compared with the corresponding part for avian influenza. However, as stated above for parts 1.1 and 1.2 (virus isolation), harmonized texts should be given between both chapters (as far as benchwork description is concerned, since obviously details about possible cross-reactions in HI will vary).
Concerning the mention of U-Plates in lines 454-455, I would not be as definite as the proposed text about assigning observed differences only to the shape of the wells. Actually, in our experience with U-shaped plates compared to V-shaped plates, it is the quality and the nature of the plastic itself that probably makes the difference in terms of clarity of the test: we routinely use U-plates and this is explicitly indicated in the corresponding French AFNOR standardized methods NF U47-011 / NF U47-036-1 / NF U47-036-2.
This use of U-shaped plates should also be added to the corresponding AI chapter for HA/HI testing.
There is also no discussion about the choice of the reference antigen for HI testing, although this may induce a systematic shift in HI titers: this is however mentioned later in lines 610-617 in the context of post-vaccination serological control. Some part of this discussion should be added earlier, in section B.2.1: for example, lines 613-616 are quite general and appropriate “Furthermore, reference antigens produced with historic strains may reduce the sensitivity of HI assay when used for the detection of antibodies against ND viruses currently circulating. For this reason it is important to investigate the antigenic relationships between the antigen used in the laboratory and current circulating viruses.”

LINE 478: The procedure described after line 478 is too long and not adequate to manage a large numbers of samples. A more handy alternative should be suggested as for HI for Avian influenza. Please refer to: Terregino C. & Capua I. (2009). Conventional diagnosis of avian influenza. In: Avian Influenza and Newcastle Disease, Capua I., ed. AD Springer Milan, Milan, Italy.

LINES 558 and 688: we suggest replacing beta-propiolactone by its abbreviation “BPL” in line 688 (or supress the abbreviation in line 558).

LINE 730: reference to section C.4.c does not seem to correspond to any item in the present chapter. We suggest that you check and modify or clarify the reference accordingly.

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3.4.5 Bovine spongiform encephalopathy

General Comments:

The EU in general can support the revised chapter. We have included specific comments below.

Specific Comments:

LINE 6 "… atypical forms …". We suggest that the names of both forms are included in brackets: "… (H- and L-type) …"

LINE 9: we believe it is not yet known, if "Scrapie" will be Chapter 3.7.11 in the new version of the Manual.

LINE 32-34 "… Atypical forms … intensive surveillance for C-type." WE suggest that these two sentences should be switched to the end of line 12.

LINE 97-99: Since 2003, reports of variant pathology or molecular characteristics in aged cattle from many countries have indicated agent strain variation (Biacabe et al., 2004; Casalone et al., 2004). It would make sense to indicate clearly Atypical BSE here. For example by amending "the so called Atypical BSE".

LINE 115: we believe that it is not certain if "Biosafety and Biosecurity" will be Chapter 1.1.4 in the new version of the Manual

LINE 138-139: In the absence of in-vitro methods for isolation of the causative agent, disease can be confirmed by demonstrating characteristic TSE-specific vacuolation by histopathology at several different levels of the brain.

It could be wise to include a sentence indicating that histopathological lesions are not necessarily seen in the beginning of the disease. Pathogenesis studies showed that in most cases an up to moderate accumulation of PrPSc precedes the first detectable lesions. Therefore following sentence could be amended in line 139: However, it has to be bear in mind that an absence of histopathologcal lesions does not exclude a PrP^{Sc} accumulation.

LINES 345 & 350: PrP^{Sc}

LINE 349-362: This paragraph addresses matters of IHC and would be better placed in Chapter 1.2.2.1, combined with the text therein.

LINE 458: "TSE-LAB-NET" has to be replaced by "**TSEglobalNet**"

LINE 495: Section "1.2.5" has to be replaced by Section "**1.2.2.3**"

Footnote 8: http://www.tse-lab-net.eu/test.html has to be replaced by https://science.vla.gov.uk/tseglobalnet/test.html

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Footnote 9: http://www.tse-lab-net.eu/documents/tse-oie-guide.pdf has to be replaced by https://science.vla.gov.uk/tseglobalnet/documents/tse-oie-guide.pdf

LINE 588: we believe it is not yet known, if Table of OIE-RL will be in Part 4 in the new version of the Manual.

3.4.8 *Contagious bovine pleuropneumonia* (*infection with* **Mycoplasma mycoides** *subsp.* **mycoides**)

General comment:

We suggest that the introduction includes a statement about why antibiotics are not used to treat/control CBPP. Several references indicate that antibiotics may be effective.

The use of antibiotics for treatment of CBPP is forbidden, but farmers use them and are willing to pay anything to treat cattle acutely infected with CBPP. Obviously, some salutary effects have been observed by the farmers – which is why they continue to use antibiotics despite official sanctions. FAO.

Huebschle OJ, Ayling RD, Godinho K, Lukhele O, Tjipura-Zaire G, Rowan TG, Nicholas RA.(2006). Danofloxacin (Advocin) reduces the spread of contagious bovine pleuropneumonia to healthy in-contact cattle. Res Vet Sci. 2006 Dec;81(3):304-9.

Suggested sentence: Although several researchers indicate that antibiotic treatment of CBPP may be effective (Huebschle et al., 2006), the treatment of CBPP with antibiotics is forbidden, as it may induce carrier disease status and facilitate the spread of the disease.

Specific comments:

LINE 173: Table 2 needs to be completed.

LINE 181: Section 1.4.1, the centrifugation g and times are different, it will be appropriate to be consistent or state either are suitable. 8000 g for 1 minute or 1500 g for 5 minutes

LINE 412: Section 2.3.2.(iii), the reversible staining solution, it would be good to have examples: Ponceau S or similar

LINE 555: Section 2.2.2, there is typo: it should read Streptomycin~~e~~-sensitivity. We also suggest that the concentration of streptomycin should be stated.

LINE 573: Section 2.2.4. iv, we suggest deleting the word ‘highly’.

3.4.12 *Lumpy skin disease*

General comment:

The EU can in general support this revised chapter.

Specific Comments

LINE 18: we believe the words “vacuolar changes with” is wrong in context with capripoxvirus assembly and should be reworded; we suggest: ‘histological key lesions consist of epidermal intracytoplasmic inclusion bodies and dermal vasculitis’**.**

LINES 27-28: “Neither of these viruses, however, causes generalised infection and both are uncommon in cattle.” – which viruses are meant by “neither and both”?, WE suggest rewording: ‘Neither parapox nor orthopox, however, cause generalised infection; besides, orthopox infections are uncommon in cattle**’.** (note: parapox is not uncommon in cattle).

LINE 106: Table 1, electron microscopy should not be deleted as electron microscopy can differentiate in half an hour between parapox- and capripoxvirus and helps in this way with the differential diagnosis of PCR.

LINE 125: “…of neutral buffered 10% formal saline”, it is more common and easier to understand the following wording: ‘…of 10% neutral buffered formalin’.

LINE 126: we suggest adding the words concerning biorisk aspects: ‘Tissues in formalin have no special transportation requirements concerning biorisk aspect**’**. Note: From biorisk aspect we consider that the text is correct but transport rules also include rules for carcinogenous hazardous chemicals.

LINE 172: Please add “or TEM”, then reading “PCR or TEM me be used…”(see also Chapter: Camelpoxvirus) – it is faster than histology

LINE 232: we suggest adding two citations: Menasherow et al. 2014, Vidanovic et al. 2019.

LINE 233: we do not think that electron microscopy should be deleted (see line 106): Title should be “**Transmission electron microscopy**” (like in chapter Camelpox).

LINES 234-257: 234-235: we consider that the first sentence is correct but other sentences (sentences 235-245) can be deleted – negative staining is a cheap and very simple technique which is ruinously used in electron microscopy; a special description is not needed for an electron microscopy specialist;

We suggest adding instead the sentences: “The characteristic poxvirus virion can be visualised using the negative staining preparation technique followed by examination with a transmission electron microscope (Biel and Gelderblom 1999). The following procedures will improve the sensitivity of negative staining technique: enrichment techniques (e.g. ultracentrifugation with an airfuge) used beside simple preparation enhances the sensitivity of the method, and grids should be placed on top as well as on the ground of the suspension droplets as the large poxviruses are often found at the bottom of the droplets. The capripox virion ….” Sentences 246-257 should follow.

LINE 374: “…is stopped by washing the “membrane (?)” in PBS”, we suggest rewording as follows: is stopped by washing the NCM in PBS

LINE 392: please add this sentence: “The use of a vaccine based on a homologous LSD strain during the outbreak of LSD in Europe showed that is more effective than a vaccine consisting of a heterologous strain (SPPV or GTPV).”

LINE 625: we suggest adding new references:

Biel, St.S., Gelderblom, H.R. (1999). Electron microscopy of viruses. Virus Culture – a Practical Approach (Cann, A.J. ed.). Oxford University Press, pp. 111-147.

Haegeman, A., De Vleeschauwer, A., De Leeuw, I., Vidanović, D., Šekler, M., Petrović, T., Demarez, C., Lefebvre, D., De Clercq, K. (2019): Overview of diagnostic tools for Capripox virus infections. Preventive Veterinary Medicine, corrected proof; pp. 104704 https://doi.org/10.1016/j.prevetmed.2019.104704

Menasherow, S., Rubinstein-Giuni, M., Kovtunenko, A., Eyngor, Y., Fridgut, O., Rotenberg, D., Khinich, Y., Stram, Y., 2014. Development of an assay to differentiate between virulent and vaccine strains of lumpy skin disease virus (LSDV). J. Virol. Methods 199, 95–101

Vidanović, D., Šekler, M., Petrović, T., Debeljak, Z., Vasković, N., Matović, K., Hoffmann,B., 2016. Real-time PCR assays for the specific detection of field balkan strains of lumpy skin disease virus. Acta Vet. 66 (4), 444–454.

3.4.16 Animal trypanosomoses of African origin (*excluding Trypanosoma evansi and T. equiperdum*)

General comment:

The EU can in general support this revised chapter, but we believe that this chapter should be read alongside Chapter 3.1.21. on Trypanosoma evansi to ensure a consistent approach between the two.

Specific comments:

LINE 52: typo, “and” appears two times.

LINE 53: editorial: the second “brucei” should be written not in italics

LINE 69: we suggest harmonising this text with 1.1. of Chapter 3.1.21. on Trypanosoma evansi

LINE197: This is a difference to the protocol described in Chapter 3.1.21. on Trypanosoma evansi. For consistency, we suggest reviewing the two chapters .

LINE 306: in line with previous comments, we suggest harmonising 1.2. in this chapter with 1.2. in Chapter 3.1.21. on Trypanosoma evansi

LINE 324: Chapter 3.1.21 mentions “14.000 g”, in this chapter “9000 g”. We suggest checking both chapters.

LINE 400: we suggest to harmonise 1.2.3. and a similar paragraph in Chapter 3.1.21. on Trypanosoma evansi. In 3.1.21. the description is much more detailed and we consider that it is better.

LINE 458: “..but some strains are cross-reacting …” we suggest mentioning this in Table 2.

LINE 476, Table 2, we suggest to coordinate Table 2 with that of Chapter 3.1.21. on Trypanosoma evansi. E.g. In contrast to Table 2 here, Table 2 in Chapter 3.1.21 says “…and some T. brucei & T. equiperdum” for both primers for T. evansi type A and T. evansi type B.

LINE 586: we think this paragraph should be adapted and should refer to Chapter 1.1.6 – Terrestrial Manual

3.6.2. Rabbit haemorrhagic disease

General comment:

The EU can support this chapter. We would like to mention that HA and HI are the most simple and less cost intense test systems that are available worldwide. We do not advise to delete both methods, which are still important for developed as well as for developing countries. Moreover, the manual refers frequently to these tests in the chapter.

Specific comments:

LINE 14: we suggest deleting ”more frequently”.

LINE 21: we believe that the period of time should be less than one week of age onwards as this is repeatedly observed.

LINE 24: editorial comments, ‘in-vitro’ in italics.

LINE 35: we suggest deleting ”a full”; especially in young or compromised animals failure of RHDV2 vaccines has been reported in a relevant number of cases; even a vaccination interval of 12 month is under discussion.

LINE 46: we suggest replacing ”the capsid RHDVs” by RHDV capsid proteins.

LINE 48: we suggest deleting ”solid”.

LINE 56: we suggest replacing ”rabbits” by leporids.

LINE 59:we suggest that deleting ”very similaer to RHD”.

LINE 109-121: we suggest including bibliographical references.

LINE 125: comment of grammar, we believe the verb should be ” became”.

LINE 132: we suggest reviewing the mortality range. We believe a narrower range is more appropriate, i.e. mortality 60-95%.

LINE 134: we believe the age range should be 7-14 days.

LINE 135: we suggest replacing ”a fever” by “high fever”.

LINE 153: Table 1, we suggest restoring antigen detection (ELISA/HA). For the Confirmation of clinical cases: we believe that antigen detection and EM in comparison to RT-PCR are only ++. We also suggest restore HI

LINE 165: we suggest replacing ”useful” by suitable.

LINE 166: we sugget deleting ”animals that die from”.

LINE 167: grammatical comment: use the plural in the sentence ”So that RHD-virus-like particles (VLPs) are detected”.

LINE 185-186: we suggest deleting ”Therefore....acute RHD” as it is questionable if ELISA is the best method.

LINE 250: we suggest replacing ”reveal” by “detect” or “visualise”.

LINE 262: typo, Oligonucle**o**tides

LINE 290-292: we suggest restoring the deleted text.

LINE 298: we suggest replacing “could” by “can”.

LINE 302: we suggest including an explanation of Alsever’s solution. For HA: positive > 1:64; we suggest restoring deleted text; we also consider that HA is an easy, reliable, quick and cheap procedure and we strongly suggest that it is not deleted.

LINE 321: we suggest being more clear stating that the test ”should be replaced”.

LINE 347: we suggest adding at the end of the sentence “...however modern genome detection methods (qRT-PCR) can be preferably used for confirmation”.

LINE 382: we suggest replacing ”grounds” by “reasons”; and deleting ”when a case can be made for this procedure”.

LINE 389: we suggest saying that the percentage should be “more than” 80%.

LINE 390: we suggest replacing ”survive until” by “die not before”.

LINE 399: we suggest deleting ”or in convalescent animals”

LINE 403: we suggest rewording to say “...should be comparatively performed…”.

LINE 432: as per our generic comment above, we suggest restoring HI.

LINE 433: the Antigen preparation section in line 460 refers to 2.1. that has been deleted (see comment above about not deleting 2.1.).

LINE 456: we suggest rewording to mean that the replacement of the HI by antibody detection ELISA is recommended.

LINE 460: see comment in LINE 43, Antigen preparation refers to the deleted HI section 2.1.

LINE 467: we believe that due to animal welfare reasons, the focus should clearly lie on immunisation with inactivated antigens. Based on this we suggest that a more

detailed description of the immunisation procedure/ immunisation schedule should be given.

LINE 503-506: we suggest deleting ”add 7µl.....first column” and delete the sentence that follows and replace it by ”If qualitative data (positive/negative) are requested, 7µl of each serum sample are tested in duplicates.”

LINE 517, 521: we suggest replacing ”decided” by “calculated”.

LINE 543: we believe there is typo, and the word ”row” is meant to be “raw”.

LINE 576: typo, we suggest deleting the semicolon ”;”

LINE 603: we suggest deleting ”Normally...” and replace it by ”Usually the vaccine dose is inoculated subcutaneously or intramuscularly.

LINE 611: we suggest deleting the following sentence: ”and their natural resistance......but not by RHDV2,”

LINE 618: we suggest including (≥3) to read “Only after several (≥3) production cycles is…”.

LINE 621: we suggest reviewing the rage of days as normally immunity is operational approximately by day 7.

LINE 623: we suggest delete ”regularly”.

LINE 657-665: we suggest that due to animal welfare reasons replaced by determination of the HA titer; purity: qRT-PCR analyses.

LINE 672: we believe the title of this section does not correspond to the text.

LINE 720: we suggest that EM should be replaced by qRT-PCR analyses.

LINE 726: we believe that animal numbers could be reduced by highly sensitive PCR analyses of the livers.

LINE 750: we consider that in bullet ii~~i~~), safety has only to be confirmed for the prototype vaccine, not to release each batch.

LINE 754: we consider that an overdose is at least 10 doses.

LINE 771: we suggest that animal numbers are reconsidered in bullet iii) Batch potency

LINE 847: we suggest that animal numbers are reassessed.

LINE 851: we suggest revision the % for RHDV2

LINE 859: we suggest deleting ”or the presence.....1/10”.

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LINE 894: we suggest that in the case of homologous vaccines 3 and 6 months are deleted.

LINE 911: we suggest including the word ‘capsid’ to read: “Expressing the main RHDV capsid protein”

3.7.4 Contagious caprine pleuropneumonia

General comment:

The EU can support the revised chapter. WE wish to flag up that this chapter clearly states that the cELISA is the preferred method although it is only available from one supplier, but refers to the CFT at various points in the chapter. Details of the CFT methodology have been deleted. As yet not all laboratories have validated the cELISA for use in their laboratories (possibly due to limited availability of positive samples), so for that reason the methodology for the CFT should remain.

Specific comments:

LINE 573: the Western Blot is mentioned, we suggest provide details or references for this method.

LINE 185: typo, we believe the word ‘stain’ should read ‘strain’.

LINE 264: we suggest revising the sentences starting from “bovine group 7” to “M.leachii”.

3.7.9 *Peste des petits ruminants* (*infection with peste des petits ruminants virus*

General comment:

In general the EU can support the revised text. We would like the OIE to consider changing the title of the chapter based on the official virus taxonomy published by the ICTV. We would like to propose the following title:

PESTE DES PETITS RUMINANTS (INFECTION WITH SMALL RUMINANT MORBILLIVIRUS, formerly PESTE DES PETITS RUMINANTS VIRUS)

In addition, if the above is considered appropriate, the abbreviation PPRV in the text should be replaced by SRMV.

Specific comments:

LINE 539: we have an editorial comment, we believe when the verb "submitted" is used, it is meant to be "subjected"

LINE 636: another editorial comment, we consider that "protection to" reads better using the words "protection against".

3.9.2 Camelpox

General comment:

The EU can in general can support this revised chapter and has one specific comment.

Specific comments:

LINE 3: in the Summary, we recommend that the Summary is updated to recommend PCR as the method of choice for identification of the agent.

LINE 24: we suggest that the following sentence "The best method of laboratory confirmation of camelpox is by the demonstration of the characteristic, brick-shaped orthopoxvirions in skin lesions…." is replaced by "The fastest method of laboratory confirmation of camelpox is by polymerase chain reaction".

This comments is related to the intimal one when we highlight that PCR is not mentioned in the summary as a method of diagnosis for camelpox, however it is the method of choice. Electron microscopy is slower, more expensive, requires greater technical expertise, and available to fewer labs worldwide than PCR. Scientific references can be found in the body of chapter sections 1.5 and 1.6.

LINE 65: we suggest using the correction nomenclature for Camelpox virus. Where it says "camelpox is caused by Orthopoxvirus cameli" we suggest using the name "Camelpox virus" as recommended by the International Committee on Taxonomy of Viruses (ICTV). Scientific reference: www.talk.ictvonline.org.

LINES 151-155: we suggest rewording the following phrase as follows: "TEM is the best and fastest method for distinguishing clinical cases of camelpox and parapox ~~orf caused by camelpox and parapox viruses~~, respectively, although ~~the viruses~~ the poxvirus infections can be differentiated by serological techniques and by PCR (Mayer & Czerny, 1990)".

LINES 156-173: we believe that this method is too difficult and virus particles are lost by preparing the sample in this way. Please, see chapter on LSD, where native material and simple negative staining technique is recommended. We consider that 1.1.1. and most of 1.1.2 can be deleted.

We suggest the following text: "The characteristic poxvirus virions can be visualised using a negative staining preparation technique followed by examination with a transmission electron microscope (Biel and Gelderblom 1999). The following procedures will improve the sensitivity of negative staining: enrichment techniques (e.g. ultracentrifugation with an airfuge) used beside simple preparation enhances the sensitivity of the method, and grids should be placed on top as well as on the

ground of the suspension droplets as the large poxviruses are often found at the bottom of the droplets."

LINE 553: based on the suggested text above a citation will have to be added: Biel, St.S., Gelderblom, H.R. (1999). Electron microscopy of viruses. Virus Culture – a Practical Approach (Cann, A.J. ed.). Oxford University Press, pp. 111-147.