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REPORT OF THE MEETING OF THE OIE BIOLOGICAL STANDARDS COMMISSION

Paris, 20–22 February 2013

The OIE Biological Standards Commission (the Commission) met at the OIE Headquarters from 20 to 22 February 2013. Dr Kazuaki Miyagishima, Deputy Director General and Head of the OIE Scientific and Technical Department, welcomed the Members of the Commission on behalf of Dr Bernard Vallat, Director General of the OIE: Prof. Vincenzo Caporale, President, Dr Hualan Chen and Dr Rodolfo Rivero, Vice-Presidents, Dr Beverly Schmitt, Dr Paul Townsend and Dr Peter Daniels, Members of the Commission.

Dr Miyagishima informed the Commission of the process going on between the OIE and the FAO[1] towards a possible greater convergence of each organisation's Reference Centres. In the near future, an indication would be added to the list of OIE Reference Centres available on the OIE web site identifying those Reference Centres that had both OIE and FAO status. Harmonisation of annual reporting procedures for OIE and FAO Reference Centres was also being pursued.

In follow-up to a question raised by the Commission at its previous meeting, Dr Miyagishima reported that the OIE Council would present a Resolution in May this year that would formalise the role of the OIE Delegate in the procedure to replace experts at OIE Reference Laboratories. Under the proposed procedure, nominations would receive final approval by the Council at one of its three annual meetings. This would streamline the process in that the new experts could be approved and the on-line list of OIE Reference Laboratories updated after each Council meeting rather than waiting for approval at the General Session in May of each year. The Council would also present Resolutions for both the designation and the withdrawal of Reference Centres.

At present, a new edition of the *Aquatic Manual* was being published in hardcopy every 3 years. To avoid coinciding with publication of new editions of the *Terrestrial Manual* (every leap year), it had been decided to extend the publication cycle of the *Aquatic Manual* by 1 year (i.e. publish a new edition every 4 years) beginning in 2014. For both publications, a number of updated chapters would continue to be proposed for adoption at the annual General Session, and if adopted, added to the on-line version; the online versions therefore would remain the most up-to-date versions. Dr Miyagishima added that French translations of the *Manuals* had been abandoned due to lack of funding; Spanish translations would continue for the present thanks to donations from the Government of Spain.

The OIE publication: OIE *Quality Standard and Guidelines for Veterinary Laboratories: Infectious Diseases*, 2008, had been withdrawn from sale as two of the four guidelines it contained were obsolete, one of the remaining two would shortly be obsolete, and the last one needed to be updated. The Director General of the OIE had proposed that the OIE continue to publish such a booklet without the obsolete guidelines based on the OIE PVS[2] for laboratories and relevant annexes to be submitted for endorsement by the Commission.

[1] FAO: Food and Agriculture Organization of the United Nations
[2] PVS: Performance of Veterinary Services

Finally, Dr Miyagishima updated the Commission on the work of the FAO-OIE Rinderpest Joint Advisory Committee and reminded the Commission of Resolution No. 33: *The OIE Role in Maintaining World Freedom from Rinderpest*, which had been adopted in May 2012 and in which it was recommended that a limited number of OIE Reference Laboratories be appointed with an even geographical distribution. It was clarified that it was the prerogative of the OIE, and the Commission, not the Joint Advisory Committee, to assess applications for reference laboratory status and recommend adoption to the World Assembly so that the world would better be served by a network of OIE Reference Laboratories. This would facilitate transfer of field rinderpest virus samples and isolates scattered over the world to a limited number of high containment facilities. The Joint Advisory Committee would meet the week following the meeting of the Biological Standards Commission and would finalise procedures for approval of facilities authorised to hold the rinderpest virus containing material including vaccines and the criteria for approval of research projects involving rinderpest virus. The work of the OIE and the Joint Advisory Committee would thus be complementary.

1. Adoption of Agenda

The proposed agenda was presented and adopted.

The Agenda and List of Participants are given at Annexes 1 and 2, respectively.

2. OIE Reference Centres

2.1. Applications for the status of OIE Reference Centre

The Commission recommended acceptance of the following eight applications for OIE Collaborating Centre and Reference Laboratory status:

OIE *Collaborating Centre for Quality Control of Veterinary Vaccines*
African Union Pan African Veterinary Vaccine Centre (AU-PANVAC), P.O. BOX 1746, Debre-Zeit, ETHIOPIA
Tel: (+251) 11.433.80.01 / 11.437.12.86; Fax: (+251) 11.433.88.44;
E-mail: panvac@africa-union.org; panvac@ethionet.et
Contact point: Dr Karim Tounkara.

OIE *Reference Laboratory for Swine streptococcosis*
Nanjing Agricultural University (NAU), Branch of Swine Streptococcosis Diagnostic Laboratory (BSSDL), Weigang No.1, Nanjing, Jiangsu Province, CHINA (PEOPLE'S REP. OF)
Tel./Fax: (+86) 258.439.5328 / 258.439.6517; E-mail: lucp@njau.edu.cn; yaohch@njau.edu.cn
Web site: ssuis.njau.edu.cn, www.chinasscontrol.com
Designated Reference Expert: Prof. Chengping Lu.

OIE *Reference Laboratory for Ovine theileriosis*
Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS), Vector and Vector-borne Diseases Control Laboratory (VVBDC), Xujiaping 1, Chengguan District, Lanzhou, Gansu Province, CHINA (PEOPLE'S REP. OF)
Tel.: (+86) 931.834.0977 / 931.834.2515; Fax: (+86) 931.834.2681 / 931.834.2515;
E-mail: yinhong@caas.net.cn
Web site: http://www.chvst.com
Designated Reference Expert: Prof. Hong Yin.

It was noted that neither Swine streptococcosis nor Ovine theileriosis were listed by the OIE but may be of importance in the region. The Commission agreed that for such applications, the designated OIE Reference Expert would be requested to draft a proposal for a chapter for the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*).

OIE *Reference Laboratory for Q fever*
Anses (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail), Laboratoire de Sophia-Antipolis, Unité de Pathologie des Ruminants, 105, route des Chappes, BP 111, 06902 Sophia-Antipolis, FRANCE
Tel.: (+33 [0]4) 92.94.37.00; Fax: (+33 [0]4) 92.94.37.01; E-mail: elodie.rousset@anses.fr
Designated Reference Expert: Dr Elodie Rousset.

OIE *Collaborating Centre for Cell Cultures*
Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Via Bianchi No. 9, 25124 Brescia, ITALY
Tel.: (+390-30) 229.01; Fax: (+390-30) 242.5251; E-mail: info@izsler.it
Website: http://www.izsler.it
Contact point: Dr Maura Ferrari.

OIE *Reference Laboratory for Foot and mouth disease*
Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Via Bianchi No. 9, 25124 Brescia, ITALY
Tel.: (+390-30) 229.03.10; Fax: (+390-30) 229.03.69; E-mail: emiliana.brocchi@izsler.it
Website: http://www.izsler.it
Designated Reference Expert: Dr Emiliana Brocchi.

OIE *Reference Laboratory for Japanese encephalitis*
Rabies Research Laboratory, Division of Viral Disease, Animal, Plant and Fisheries Quarantine and Inspection Agency (QIA), Ministry of Food, Agriculture, Forestry, and Fisheries (MIFAFF), 175 Anyang-ro, Manan-gu, Anyang-si, Gyeonggi-do, 430-757, KOREA (REP. OF)
Tel.: (+82) 31.467.1783; Fax: (+82) 31.467.1797; E-mail: yangdk@korea.kr; ydk40@hanmail.net
Web site: http://www.qia.go.kr/eng/index.asp
Designated Reference Expert: Dr Dong-Kin Yang.

OIE *Reference Laboratory for Swine influenza*
Animal Health and Veterinary Laboratories Agency (AHVLA), New Haw, Addlestone, Surrey KT15 3NB, Weybridge, UNITED KINGDOM
Tel.: (+44-1932) 357.339; /Fax: (+44-1932) 357.239; E-mail: ian.brown@ahvla.gsi.gov.uk
Designated Reference Expert: Prof. Ian Brown.

Two applications had been received from a country in the Americas for OIE Collaborating Centre status: one for Research, Diagnosis and Training on Bovine Viral Diseases, and the second for Training and Diagnosis of Emerging Diseases of Swine. The Commission reiterated that the purpose of Collaborating Centres was to act as centres of expertise in a specific designated sphere of competence relating to the management of general questions on animal health issues ("specialty"). Reference Laboratories act as centres of expertise on designated pathogens or diseases. The proposed specialties of the two applications were not in line with the Terms of Reference of a Collaborating Centre. The Commission proposed that the applicants choose one or two named diseases and submit one application for Reference Laboratory status for each individual named disease.

A country in Europe had sent an application for recognition of a Collaborating Centre for Food-Borne Parasites. The Commission believed that the activities detailed in the application fit more suitably in the remit of a Reference Laboratory. Again, it proposed that the applicant choose one or two named diseases and re-apply for Reference Laboratory status for an individual named disease.

An application had been received from an African country for a Reference Laboratory for African horse sickness and Bluetongue. The Commission proposed that the applicants submit two separate applications, one for each disease. A similar situation arose with an application from a European country for a Reference Laboratory for *Chlamydiaceae* (avian, ovine and caprine chlamydiosis). Again the Commission proposed the applicants submit two separate applications, one for avian chlamydiosis and the second for ruminant chlamydiosis).

A European country had submitted an application for a Reference Laboratory for Q fever. The application was put on hold pending receipt of more information on research and international activities and a full list of its recent publications. Another European country had submitted an application for a Reference Laboratory for Foot and mouth disease (FMD). Although the institution is world renowned for its competence and expertise, the application did not demonstrate sufficient international activity or provision of expert consultations specifically on FMD but rather detailed several other important diseases. The Commission requested that the applicants provide additional information and focus the application on their activities in FMD.

Finally, an application from a country in Asia for a Reference Laboratory for Equine piroplasmosis was put on hold; the laboratory is currently completing an OIE Twinning project and the Commission deferred its decision until receipt of the final report of this project.

2.2 Changes of experts in the List of Reference Centres

The OIE Council would present a Resolution in May this year that would formalise the role of the OIE Delegate in the procedure to replace experts at OIE Reference Laboratories and also streamline the process (see opening address by Dr Miyagishima).

The Delegates of the countries concerned had submitted to the OIE the following nominations for changes of experts at OIE Reference Laboratories. The Commission recommended their acceptance:

African horse sickness
Dr Montserrat Agüero to replace Dr Concepción Gómez-Tejedor at the Laboratorio Central de Veterinaria, Algete (Madrid), SPAIN.

Avian influenza
Dr Frank Wong to replace Dr Paul Selleck at the Australian Animal Health Laboratory, Geelong, AUSTRALIA.

Avian influenza and Newcastle disease
Dr Mia Torchetti to replace Dr Janice Pedersen at the National Veterinary Services Laboratories, Ames, UNITED STATES OF AMERICA.

Classical swine fever
Dr Paul Becher to replace Prof. Volker Moennig at the University of Veterinary Medicine of Hannover, GERMANY.

Equine influenza and Equine rhinopneumonitis
Dr Armando Daminai to replace Dr Kerstin Borchers at the Free University of Berlin, GERMANY.

Foot and mouth disease
Dr Somjai Kamolsiripichaiporn to replace Dr Wilai Linchongsubongkoch at the National Institute of Animal Health, Pakchong, THAILAND.

Newcastle disease
Dr Sam McCullough to replace Dr Paul Selleck at the Australian Animal Health Laboratory, Geelong, AUSTRALIA.

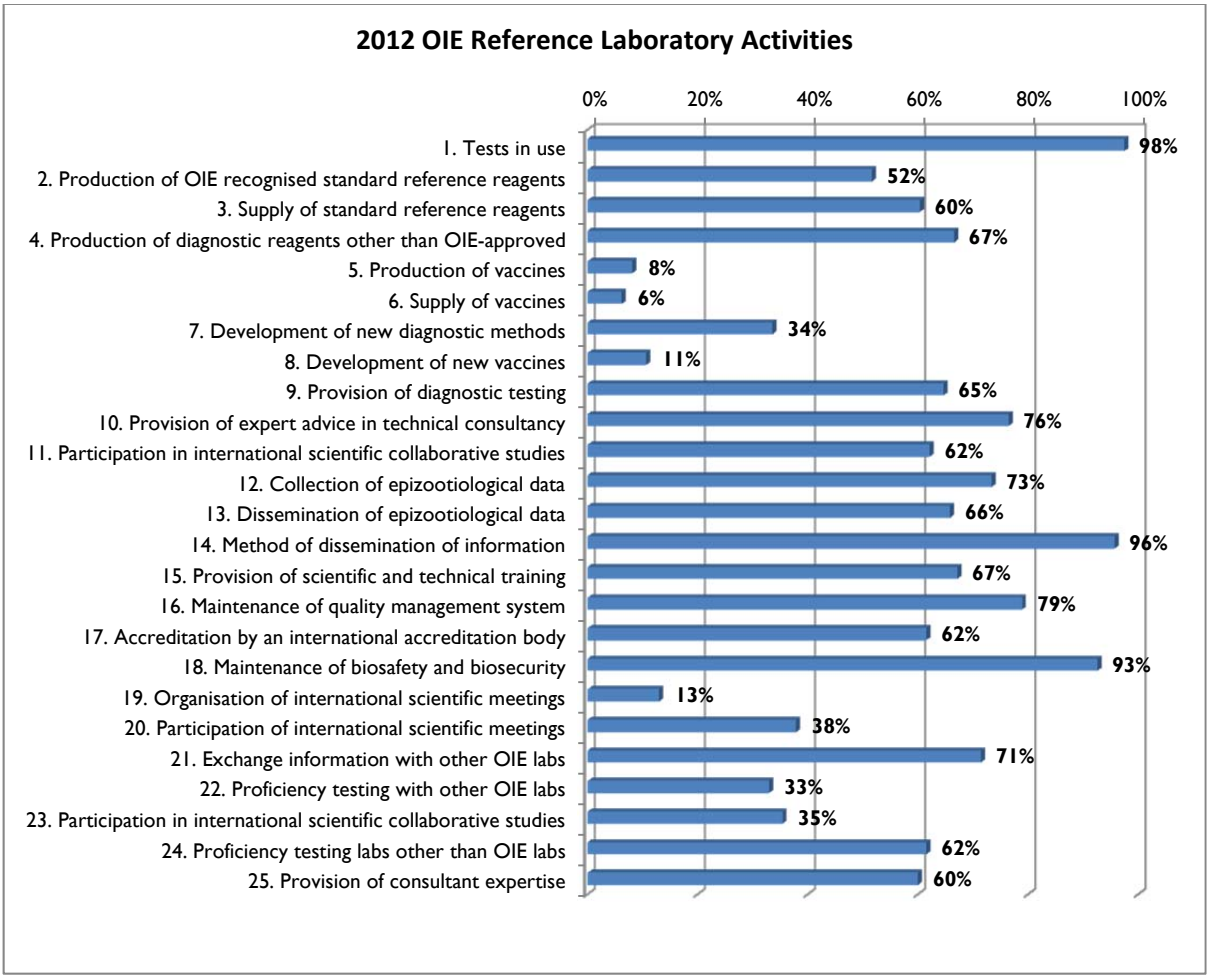
Rabies
Dr Richard Franka to replace Dr Charles Rupprecht at the CDC (Centers for Disease Control and Prevention), Atlanta, Georgia, UNITED STATES OF AMERICA.

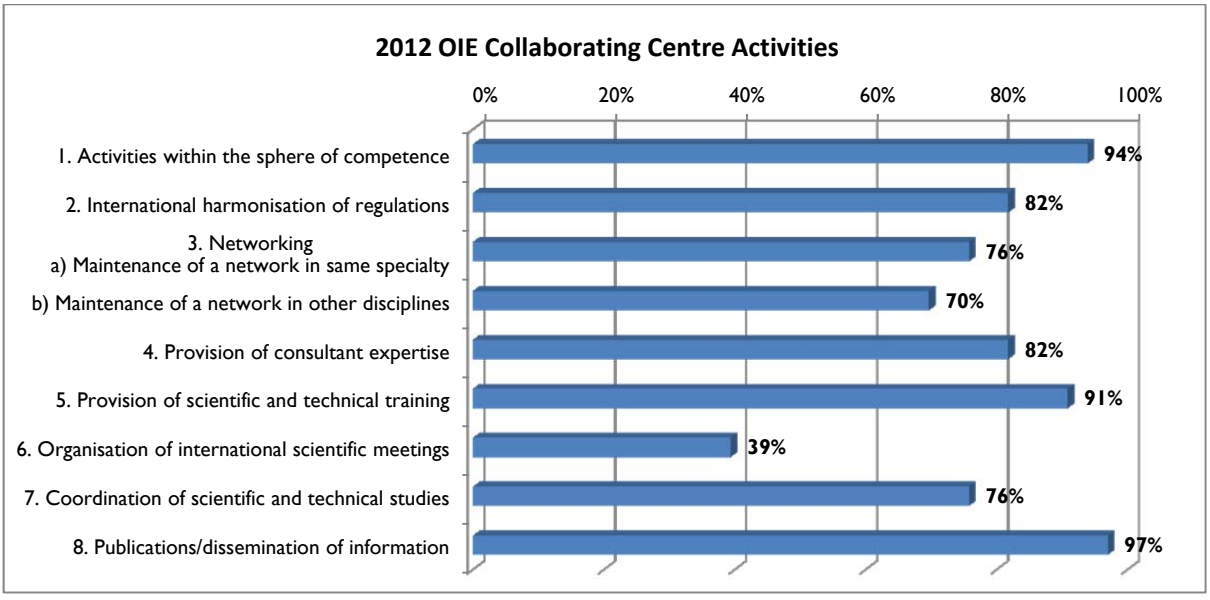
West Nile fever
Dr Federica Monaco to replace Dr Rossella Lelli at the IZS dell'Abruzzo e del Molise "G. Caporale", ITALY.

2.3. Annual reports of Reference Centre activities for 2012

Ms Min-Kyung Park, Intern, Scientific and Technical Department of the OIE, joined the meeting. The Commission was reminded that a new annual report template had been used by the Reference Laboratories for the first time. Though the majority of experts welcomed the new format, some constructive criticisms had also been received. Ms Park presented an analysis of the activities along with some proposed improvements to the template. The revised template would be sent to the Commission members for comment.

The Commission expressed its on-going appreciation for the enthusiastic support and expert advice given to the OIE by the Reference Centres. To further improve communication and effective collaboration between the Commission and the network, it was agreed that the Reference Laboratories should be given some feedback on their activities. This could be included in the letter of announcement of the next Conference for OIE Reference Centres (see item 7.2). The international activities relevant to the work of the OIE are summarised in the following graphics:





For the 2012 reports, the template was an Office document rather than a web-based tool. An electronic version of the template should be available for the 2013 reports.

2.4. Mutual recognition of OIE and FAO Reference Centres

An agreement had been signed between the OIE and the FAO for the mutual recognition of each organisation's Reference Centres. In the near future, an indication would be added to the list of OIE Reference Centres available on the OIE web site identifying those Reference Centres that had both OIE and FAO status (see opening address by Dr Miyagishima).

2.5. Review of new and pending applications for laboratory twinning

Dr Gounalan Pavade, Scientific and Technical Department of the OIE, provided an update on the OIE Laboratory Twinning programme. As of 20 February 2013, eight projects have been completed, 32 were underway and ten were approved and due to start.

He presented a model of a certificate for successful completion of Twinning projects. This certificate will be presented to representatives from parent and candidate institutes at the OIE General Session, acknowledging their efforts and achievements.

Four twinning proposals were presented for the Commission's technical input. The Commission supported the technical content of two of them; Canada–Peru for Rabies and Transmissible spongiform encephalopathies (TSE); and Canada–Cuba for Bovine spongiform encephalopathy (BSE).

Exceptionally, the Commission requested that letters of support be provided for the other two projects, UK–Tanzania for Peste des petits ruminants and Sweden–Uganda for African swine fever and Foot and mouth disease, before the members would consider providing their technical input. These two proposals therefore did not receive the Commission's technical input. OIE would clarify the procedures for review and approval of twinning projects with the Commission at a later date.

3. *Ad hoc* Groups

■ Past *ad hoc* Group meetings

3.1. Report of the Meeting of the *ad hoc* Group on Rift Valley fever, 9–11 October 2012

Dr François Diaz, Scientific and Technical Department of the OIE, presented the report of the meeting of this *ad hoc* Group. The Commission noted that the Group proposed changing the categories (i.e. the purposes themselves) in Table 1. *Test methods available and their purpose*. As it is planned to add such a table to all the disease-specific chapters, in lieu of prescribed and alternative tests (see item 4.2), the Commission did not accept this change and asked the Consultant Editor to amend the table such that it is consistent with the other chapters. The Commission adopted the report, which can be found at Annex 3 of this report; Member Country comments are invited on the updated *Terrestrial Manual* chapter included therein.

3.2. Report of the Meeting of the *ad hoc* Brainstorming Group on New Approaches to Diagnosis: Applied Genomics, 10–12 December 2012

Prof. Vincenzo Caporale presented the report of the meeting of this Brainstorming Group and the White Paper: *High-throughput Sequencing in Veterinary Infection Biology and Diagnostics*. One of the recommendations in the report was that the OIE convene an *ad hoc* Group on high throughput sequencing and bioinformatics and computational genomics (HTS-BCG). This item was added to the Commission's work plan (see item 10.1). The Commission adopted the report, which can be found at Annex 4 of this report.

3.3. Report of the Meeting of the *ad hoc* Group on Validation of Diagnostic Tests for Wildlife, 15–17 January 2013

Dr Diaz presented the report of the meeting of this *ad hoc* Group. The Commission adopted the report, which can be found at Annex 5 of this report; Member Country comments are invited on the appendix: *Principles and Methods for the Validation of Diagnostic Tests for Infectious Diseases Applicable to Wildlife*, included therein. Once adopted, this appendix would be integrated into the *Terrestrial Manual* chapter on the principles and methods of validation of diagnostic assays for infectious diseases (see item 5.1)

3.4. Report of the Meeting of the *ad hoc* Group on Classical Swine Fever, 4–6 September 2012

The Commission reviewed the report of the meeting of the *ad hoc* Group. The Group's recommendation that consideration be given to revising section A of the *Terrestrial Manual* chapter on classical swine fever to give a more comprehensive introduction to the different aspects of the disease and its control, was noted, but the Commission commented that the *Terrestrial Manual* is for the provision of standards for diagnostic tests and vaccines and these other aspects of the disease should be addressed elsewhere. The Commission adopted the report, which can be found at Annex 6 of this report; Member Country comments are invited on the updated *Terrestrial Manual* chapter included therein.

Future *ad hoc* Groups: scheduling and drafting ToRs

3.5. *Ad hoc* Group to update chapter 1.1.6 *Principles of veterinary vaccine production* and to draft two new chapters: 1.1.8 *Minimum requirements for vaccine production facilities* and 1.1.9 *Quality control of vaccines*

The OIE Collaborating Centre for Veterinary Medicinal Products in Fougères, France, had offered to update chapter 1.1.6 and to draft new chapters 1.1.8 and 1.1.9. The Commission accepted the offer and asked that the Centre liaise with other OIE Collaborating Centres having vaccine production and evaluation in their remit, to produce consensus documents. Once received, the texts would be reviewed to determine whether they could be sent directly to Member Countries for comment or whether they could be used by an *ad hoc* Group as base document for further elaboration.

3.6. Other *ad hoc* Groups identified during this meeting

The following *ad hoc* Groups were identified and were added to the Commission's work plan (item 10.1):

- High throughput sequencing and bioinformatics and computational genomics (HTS-BCG) (item 3.2)
- Biosafety and Biosecurity in Veterinary Laboratories (item 5.1)
- Shortening the vaccine registration process when simply updating and incorporating relevant strains in equine influenza vaccines (item 9.1).

4. International Standardisation/Harmonisation

■ Diagnostic tests

4.1. OIE Register of diagnostic kits: review of applications

Dr François Diaz, Scientific and Technical Department of the OIE, updated the Commission on the current status of the dossiers submitted according to the OIE Procedure for Registration of Diagnostic Kits.

According to the procedure, each kit included in the OIE Register must have its registration renewed every 5 years. Dr Diaz informed the Commission that one diagnostic kit (Bio-Rad TeSeE™ Western Blot), added to the OIE Register in 2009, was reaching the end of its 5-year term; the renewal would take place under the aegis of this Commission. In accordance with protocol, the kit manufacturer had been contacted to indicate whether they wished to maintain the same purposes for which their kit had been certified as validated or to add new purposes. The OIE experts for the diseases targeted by the kit had also been contacted and asked their opinion on the need for a new evaluation of the purposes for which the kit had been certified as validated. Based on this information, the Commission decided to ask the kit manufacturer for some additional data and to take a decision at its next meeting in September.

4.2. Prescribed and Alternative Tests

In September 2011, one of the proposals of the Brainstorming Meeting for Modernising the OIE *Terrestrial Manual*[3] was to include a table in the disease-specific chapters that lists the diagnostic methods available for the disease in question alongside the purpose for which the assay has been validated; such a table already existed in the *Manual of Diagnostic Tests for Aquatic Animals*. Each chapter that has been updated since this proposal was approved has included such a table; the first of these chapters will be proposed for adoption in May 2013. The purposes listed in the table are those listed in the chapter on the principles of validation and are:

- Purpose
  - Population freedom from infection
  - Individual animal freedom from infection
  - Efficiency of eradication policies
  - Confirmation of clinical cases
  - Prevalence of infection – surveillance
  - Immune status in individual animals or populations post-vaccination.

Given that the table better reflects testing purposes and practices, it could potentially replace the current list of prescribed and alternative tests that can be found in both the *Terrestrial Manual* and the *Terrestrial Code*. In a first step, the column of alternative tests would be removed. For those *Terrestrial Manual* disease chapters that have a table of test methods available and their purpose, a reference to the chapter would be added to the list of prescribed tests (in some cases this will replace the current prescribed test or tests). Eventually all the chapters will have such a table; at that time, the list of prescribed tests could be removed and individual *Terrestrial Manual* chapters consulted for those interested in knowing the tests available and the fitness of purpose for which they have been validated. Prof. Caporale would highlight this proposal in his presentation to the Assembly at the General Session in May.

The Commission provided feedback on a text explaining how a test can be approved for inclusion in the *Terrestrial Manual*. The amended text would eventually be made available on the web site.

4.3. The polymerase chain reaction test for contagious equine metritis

A query from a Member Country regarding the polymerase chain reaction (PCR) test for contagious equine metritis (CEM) had been referred to the OIE experts for advice. The experts felt that the PCR could be included, in due course, in the *Terrestrial Manual* chapter as a suitable test for animal movement but they were uncertain if there were currently sufficient validation data.

5. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*

For this agenda item, the Commission was joined by the Consultant Editor of the *Terrestrial Manual*, Prof. Steven Edwards.

5.1. Decision on proposals of the Enlarged Bureau Group

The Commission approved the proposals of the EBG Group, which met from 18 to 19 February 2013 (see Annex 7).

The Commission reviewed the outcome of the Enlarged Bureau Group (EBG) meeting. Twenty-one chapters were approved for circulation to Member Countries as the final versions that would be proposed for adoption in May this year. One chapter required further review, and the chapter and guideline on biorisk management had received a large volume of comments that need to be addressed by a specific *ad hoc* Group.

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[3] Brainstorming Meeting for Modernising the OIE the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, which was held at the OIE Headquarters from 12 to 13 September 2011; the report can be found as Appendix V of the Report of the Meeting of the OIE Biological Standards Commission September, 14 to 16 September 2011.

Those chapters that had been identified for proposal for adoption in May 2014, and those that had been identified for adoption in May 2013 but which had been submitted too late for first round circulation or had yet to be received, would be reviewed at the September meeting.

5.2. Decision on the booklet OIE *Quality Standard and Guidelines for Veterinary Laboratories: Infectious diseases*

The Commission reiterated its belief that it was timely to discontinue publication of the booklet in its current format. The quality standard had been drafted at a time when veterinary diagnostic laboratories needed guidance as many of them were not accredited, but this situation was no longer the case. Most of the information it contains can now be found in the *Terrestrial Manual*. The Commission asked for the rationale for continuing this work and guidance on updating this publication.

6. Resolutions for the General Session

6.1. Resolutions that will be presented in May 2013

From May 2013, the President of the Commission would continue to present a resolution on new or revised texts for the *Terrestrial Manual* and one on the register of diagnostic tests validated and certified by the OIE. However, as responsibility for reviewing applications for Collaborating Centre status is now shared among the four Specialist Commissions, the OIE Council would present the resolutions for both the designation and the withdrawal of Reference Centres. This year, a resolution would also be proposed to formalise the procedure to replace experts at OIE Reference Laboratories (see opening address by Dr Miyagishima).

7. Conferences, Workshops, Meetings

7.1. Update on 1-day OIE Seminar, 7 June 2013 (theme: New approaches to diagnosis: applied genomics) to be held during the WAVLD[4], 5–8 June 2013, Berlin, Germany

The Commission endorsed the programme and list of speakers for this 1-day OIE Seminar.

7.2. International Conference of the OIE Reference Centres, Seoul, Korea (Rep. of), 2014

The Commission noted that the third International Conference of the OIE Reference Centres would be held in Seoul, Korea (Rep. of) from 7 to 9 October 2014. The Members agreed to finalise the agenda for the Conference at its next meeting in September 2013.

8. Liaison with other Commissions

8.1. Scientific Commission for Animal Diseases (Scientific Commission)

Matters from the Scientific Commission to the Biological Standards Commission

The Scientific Commission asked if there was a suitable diagnostic test for glanders that would allow the OIE to add the disease to the list of diseases for which there is a procedure for official recognition of Member Country status. The Commission referred the Scientific Commission to the updated chapter that would be proposed for adoption in May 2013, and in particular to the table it includes of test methods available and the purpose for which they have been validated.

The Commission referred the request for an internationally agreed upon and validated PCR method for African horse sickness to the OIE Reference Laboratories.

The Commission also referred the request for new information on the gamma interferon test for bovine tuberculosis to the OIE Reference Laboratories. It noted that an *ad hoc* Group on Bovine Tuberculosis would meet in April 2013.

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[4] WAVLD: World Association of Veterinary Laboratory Diagnosticians

The Commission noted that a new generation of live classical swine fever marker vaccine would shortly be registered in the European Union.

Finally, the Commission noted that an *ad hoc* Group on Rift Valley fever would meet to review the *Terrestrial Code* chapter.

The Commission called for a formal procedure for "joint" *ad hoc* Group meetings: when a Group is convened to discuss a *Terrestrial Code* chapter but where the *Terrestrial Manual* chapter will also be affected, the Biological Standards Commission should be informed at the planning stage so that the agenda item can be prepared and scheduled in collaboration with the Commission.

8.2. Terrestrial Animal Health Standards Commission (Code Commission)

Matters from the Code Commission to the Biological Standards Commission

A Member Country had queried the need for details on diagnostic tests in the *Terrestrial Code* chapter on *Zoonoses transmissible from non-human primates*. The Code Commission was requested to remove the text concerned and provide it to the Biological Standards Commission, which in turn would request the Consultant Editor to incorporate it, if necessary, into the *Terrestrial Manual* chapter.

The Commission referred the request for an opinion on delisting paratuberculosis to the OIE Reference Laboratories.

9. Matters of Interest for Information

9.1. Expert Surveillance Panel on Equine Influenza Vaccine Composition – Conclusions and Recommendations (4 March 2013)

The Report of the Meeting of Expert Surveillance Panel (ESP) on Equine Influenza Vaccine Composition, which had been held 4 March 2013 and which is appended to this report at Annex 8, was sent to the OIE Headquarters after the Commission meeting. The main conclusions of the ESP meeting were:

Vaccines for the international market should contain both clade 1 and clade 2 viruses of the Florida sublineage.
Clade 1 is represented by A/eq/South Africa/04/2003-like or A/eq/Ohio/2003-like viruses.
Clade 2 is represented by A/eq/Richmond/1/2007-like viruses.

The Commission stressed the importance of the ESP report and the need for its widespread distribution. It would be published in the OIE *Bulletin*.

In follow up to a question raised at the General Session in May 2012 on the possibility of shortening the vaccine registration process when simply updating and incorporating relevant strains in equine influenza vaccines, the Commission clarified that it would add to its work plan an *ad hoc* Group on this issue composed of representatives from industry, from the regulatory agencies and from the ESP on Equine Influenza Vaccine Composition.

9.2. Update on OFFLU

Dr Peter Daniels updated the Commission on OFFLU – the joint OIE-FAO network of expertise on animal influenza. OFFLU held two Executive Committee meetings to review and coordinate the progress of ten technical activities. One of the technical activities run at Friedrich-Loeffler-Institute, Germany (OIE Reference Laboratory for Avian influenza) achieved its objective by developing a universally usable RNA for H5 avian influenza virus targeted PCR assays. In September 2012, WHO[5] Meeting on Vaccine Composition in the Northern Hemisphere was held in Beijing (China [People's Rep. of]); the OFFLU network contributed 118 H5 sequences and 17 H9 sequences to help WHO in pandemic preparedness. OFFLU swine influenza virus group paper on "Review of influenza A virus in swine worldwide" was accepted for publication in the journal *Zoonoses and Public Health*. An editorial

[5] WHO: World Health Organization

highlighting the OFFLU research agenda is due for publication in the journal *Influenza and Other Respiratory Viruses*. OFFLU developed a *modus operandi* document detailing the terms of reference for the various committees and different positions within its structure. The OFFLU Annual Newsletter for 2012 compiling the achievements for the year had been prepared.

9.3. United Nations Environment Programme use of mercury in vaccines

The Commission noted the outcome of the United Nations Environment Programme's Inter-governmental negotiating committee to prepare a global legally binding instrument on mercury. The Convention text, once adopted, will allow the continued use of thiomersal in vaccines, as "vaccines containing thiomersal as preservatives" are specifically excluded from the list of prohibited products. In addition, for further clarity, there is a footnote on the prohibited category of "biocides" that explains that "preservatives in pharmaceuticals or vaccines" are not included in this category. This was of importance as there were currently no alternatives to thiomersal for the preservation and production of veterinary vaccines, particularly those for livestock, which are mostly multi-dose preparations

The list of exclusions may be re-visited in the future for revision, but no earlier than five years after the entry into force of the Convention.

9.4. OIE PVS Laboratory Mission Manual

In follow-up to the last meeting, the Commission's opinion was now being sought on the PVS Laboratory Mission Manual, which was still a 'work in progress'. Members agreed to send their comments to Dr Beverly Schmitt, who agreed to collate them and then send them to the OIE Headquarters. The Commission stressed that it was logical that there should be interaction between the laboratory component of the PVS evaluation tool and it, the Laboratories Commission, and reiterated their willingness to support this initiative (see item 10.1).

9.5. 3rd Next Generation Sequencing Conference

The Commission noted this conference announcement.

10. Any Other Business

10.1. Work plan and activities (as of February 2013)

At its last meeting, Prof. Caporale had invited all the Commission members to bring to this meeting their proposals for the Commission's programme of activities for the years 2013 to 2015. The work plan could then be established.

The Commission felt that the service it provides to the OIE could be extended beyond reviewing Reference Centre applications and updating the *Terrestrial Manual*. Following long discussions, the Commission identified three areas on which it proposed to focus:

- Evaluating the mandated activities of the OIE Reference Laboratories: the OIE would like to have a worldwide network of laboratories providing coherent test results; to achieve this goal it would need to more closely monitor Reference Laboratory activities through, for example, the development of performance criteria followed by auditing the laboratories to ascertain their scientific vigour. A procedure such as that applied by the World Health Organization to monitor its influenza laboratories could be adapted.

- Laboratory PVS: the Commission strongly believes that there should be a laboratory component to the PVS missions and that it should be involved, in particular in assessing the quality management of laboratory systems under evaluation. Apart from developing a system to harmonise laboratory activities with the ultimate aim of achieving standardised diagnostic results worldwide, the experts who conduct the PVS missions must be provided with training materials. The Commission could also be involved in such a developmental process.

- Coordinating the interactions between the Focal Points for Laboratories and the OIE Reference Laboratories: the OIE has organised pilot training seminars for Focal Points for laboratory issues in two regions. To date, Focal Point activities are not driven by any OIE Specialist Commission or Working Group, but 'make use of' experts from OIE Reference Laboratories and Collaborating Centres. The Focal Points for Laboratories, if extended to all regions, should interact with the experts at the OIE Reference Laboratories. The Commission would like to be involved in future activities and help to coordinate this relationship.

The Commission emphasised its willingness to support the work of the OIE in these three areas, and in any other area identified by the OIE Headquarters.

The current work plan can be found at Annex 9.

10.2. Dates of the next Biological Standards Commission meeting

The Commission noted the dates for its next meeting: 10–12 September 2013.

11. Adoption of the Report

The report was adopted by the Commission.

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.../Annexes

Annex 1

MEETING OF THE OIE BIOLOGICAL STANDARDS COMMISSION

Paris, 20–22 February 2013

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Agenda

1. Adoption of the Agenda

2. OIE Reference Centres

2.1. Applications for the status of OIE Reference Centre
2.2. Changes of experts at OIE Reference Centres
2.3. Annual reports of Reference Centre activities for 2012
2.4. Mutual recognition of OIE and FAO Reference Centres
2.5. Review of new and pending applications for laboratory twinning

3. *Ad hoc* Groups

Past *ad hoc* Group meetings, reports for adoption:

3.1. Report of the Meeting of the *ad hoc* Group on Rift Valley fever, 9–11 October 2012
3.2. Report of the Meeting of the *ad hoc* Brainstorming Group on New Approaches to Diagnosis: Applied Genomics, 10–12 December 2012
3.3. Report of the Meeting of the *ad hoc* Group on Validation of Diagnostic Tests for Wildlife, 15–17 January 2013
3.4. Report of the Meeting of the *ad hoc* Group on Classical Swine Fever, 4–6 September 2012

Future *ad hoc* Groups:

3.5. *Ad hoc* Group to update chapter 1.1.6 *Principles of veterinary vaccine production* and to draft two new chapters: 1.1.8 *Minimum requirements for vaccine production facilities* and 1.1.9 *Quality control of vaccines*
3.6. Other *ad hoc* Groups identified during this meeting

4 International Standardisation/Harmonisation

- **Diagnostic tests**

4.1. OIE Register of diagnostic tests: review of applications
4.2. Prescribed and Alternative Tests
4.3. The polymerase chain reaction test for contagious equine metritis

5. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*

5.1. Decision on proposals of the Enlarged Bureau Group
5.2. Decision on the booklet OIE *Quality Standard and Guidelines for Veterinary Laboratories: Infectious diseases*

6. Resolutions for the General Session

6.1. Resolutions that will be presented in May 2013

7. Conferences, Workshops, Meetings

7.1. Update on 1-day OIE Seminar, 7 June 2013 (theme: New approaches to diagnosis: applied genomics) to be held during the WAVLD, 5–8 June 2013, Berlin, Germany
7.2. Meeting of the OIE Reference Centres, Seoul, Korea, 2014

8. Liaison with other Commissions

8.1. Scientific Commission for Animal Diseases

8.2. Terrestrial Animal Health Standards Commission

9. Matters of Interest for Information

9.1. Expert Surveillance Panel on Equine Influenza Vaccine Composition – Conclusions and Recommendations (4 March 2013)
9.2. Update on OFFLU
9.3. United Nations Environment Programme use of mercury in vaccines
9.4. OIE PVS Laboratory Mission Manual
9.5. 3rd Next Generation Sequencing Conference

10. Any Other Business

10.1. Work plan
10.2. Dates of the next Biological Standards Commission meeting: 10–12 September 2013

11. Adoption of Report

Annex 2

MEETING OF THE OIE BIOLOGICAL STANDARDS COMMISSION
Paris, 20–22 February 2013

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Annex 3

Original: English
October 2012

REPORT OF THE MEETING OF THE OIE *AD HOC* GROUP ON RIFT VALLEY FEVER

Paris, 9–11 October 2012

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1. Opening

The OIE *ad hoc* Group on Rift Valley Fever (RVF) met from 9 to 11 October 2012 at the OIE Headquarters in Paris, France. Dr Kazuaki Miyagishima, Deputy Director General of the OIE and Head of the Scientific and Technical Department, welcomed the participants on behalf of the OIE Director General, Dr Bernard Vallat.

2. Appointment of chairperson and rapporteur

Dr Pierre Rollin and Dr Jeroen Kortekaas were appointed as chairman and rapporteur, respectively.

3. Adoption of the agenda

The Agenda and the List of Participants are presented in Appendices I and II of this report, respectively.

4. Finalisation of the updating of Chapter 2.1.14 Rift Valley fever of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*

The Group reviewed the chapter in the light of recent scientific advances and the latest available technologies in diagnostic tests and vaccine development, taking into account the updated version of the instructions for authors adopted by the Biological Standards Commission in 2012.

a) Summary and Section A: Introduction

Only minor modification was made to the text: a sentence was added to the introduction to provide information on differential diagnosis.

b) Section B: Diagnostic techniques

In accordance with the instructions for authors, the Group added a table at the beginning of section B summarising the test methods available and their 'purposes'. This Group was the first *ad hoc* Group to implement this instruction. During the discussion on the development of the table, the Group felt that it was difficult to fit into table format the test methods for RVF and their proposed purposes. In addition, the Group was of the opinion that some of the proposed purposes overlapped with each other and were difficult to apply to a generic model. The Group therefore adapted the proposed purposes to make them more applicable to RVF, while ensuring their broader applicability (for other diseases) and avoiding overlapping among the purposes. The purposes included in the table were the following: Surveillance, Laboratory confirmation of clinical cases, Humoral immune status in individual animals or populations post-vaccination, and Population free from infection. Some footnotes were also added in the table to provide more information when relevant. The Group felt however that the table format tended to over-simplify actual situations and might not be so useful. As an alternative, the Group strongly suggested developing short paragraphs describing the advantages and disadvantages of each diagnostic test method and the advice on how to apply these methods in different epidemiological situations.

At the request of the Biological Standards Commission, the Group included detailed protocols for the different test methods, where relevant. The Group agreed that these protocols should preferably describe the use of diagnostic tests that are validated by the OIE Reference Laboratories for RVF.

The Group was informed that references to commercial diagnostic kits should be avoided in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual)*. However the Group stressed that the protocols used by OIE Reference Laboratories were validated using commercially obtained materials and that these materials must therefore be used when these protocols were followed.

The Group also pointed out that the diagnostic test methods recommended in the chapter should be validated in each laboratory implementing these techniques in collaboration with the OIE Reference Laboratories for RVF. The Group agreed to include a statement in the introduction of Section B to reflect this point: "*All the test methods described below have to be validated in each laboratory using them.*" In addition a reference was made to Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases* of the *Terrestrial Manual*.

c) Section C: Requirements for vaccines

1. Background

A table was added to briefly describe vaccines currently in use. Apart from the commercially available veterinary vaccines (Smithburn-based live-attenuated vaccines, inactivated vaccines and the Clone-13 vaccine), the inactivated human vaccine TSI-GSD-200, although no longer available, was added to this table. The Group was of the opinion that this information might be useful to the users of the *Terrestrial Manual*.

2. Outline of production and minimum requirements for conventional vaccines

Section 2a: Characteristics of the seed virus

Apart from minor modifications, subsection iii) *Validation of a vaccine strain* was added to this section. Subsection iv) *Emergency procedure for provisional acceptance of new master seed virus* (*MSV*) *in the case of an epizootic* was not added as this did not apply to RVF virus given that only one serotype of RVF virus exists.

Section 2b: Method of manufacture

Besides some minor modifications, in subsection iii) *In-process controls* a relevant sentence from the European Pharmacopoeia was added, briefly describing an *in vitro* test of the inactivation procedure.

In the subsection iv) *Final product batch tests* (*Safety*), a short protocol for batch safety testing was provided.

Section 2c: Requirements for authorisation/registration/licensing

The Group developed text for subsection i) *Manufacturing process*, and added more details to subsection ii) *Safety requirements* by providing protocols for the safety tests (in young animals and in pregnant animals) and information on the precautions to take for both live and inactivated vaccines. The Group also provided a protocol for the reversion-to-virulence test and information on environmental considerations for live vaccines.

Subsection 3: Efficacy requirements

The Group agreed that the demonstration of vaccine efficacy in lambs would be sufficient evidence of the quality of a vaccine to endorse its use in other species, while it might be more appropriate to test the efficacy of the vaccine in the species for which the vaccine would be applied. The rationale for this recommendation was, first, to reduce the number of experimental animals, and, second, to address the fact that, apart from cattle, no protocols to evaluate vaccine efficacy in other target species (goats, wildlife species) were available.

Protocols to evaluate immunogenicity in lambs and pregnant ewes were provided and it was specified in the text that appropriate modification should be made when dealing with other species.

The Group agreed that it was important to determine challenge virus load in the blood by both quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and virus isolation. By combining these methods, analysis of viraemia in control animals would demonstrate the successful challenge as well as sensitivity of virus isolation procedures and qRT-PCR.

Subsection 4: Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

The Group mentioned that no DIVA strategy was currently applicable for existing RVF vaccines.

Subsection 5: Duration of immunity

The Group developed a paragraph for this section.

Subsection 6: Stability

The Group developed a paragraph for this section.

5. Adoption of the report

The Group adopted the report at the end of the meeting.

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…/Appendices

Appendix I

OIE *AD HOC* GROUP ON RIFT VALLEY FEVER

Paris, 9–11 October 2012

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Agenda

1. Opening
2. Appointment of chairperson and rapporteur
3. Adoption of the agenda
4. Finalisation of the updating of Chapter 2.1.14 *Rift Valley fever* of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*
5. Adoption of the report

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Appendix II

OIE *AD HOC* GROUP ON RIFT VALLEY FEVER

Paris, 9–11 October 2012

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Appendix III

CHAPTER 2.1.14.

RIFT VALLEY FEVER

SUMMARY

Rift Valley fever (*RVF*) *is a peracute or acute zoonotic disease of domestic ruminants ~~in Africa~~. The virus is currently confined to the African continent and Arabian Peninsula. It is caused by a single serotype of a mosquito-borne ~~bunya~~virus of the* Bunyaviridae *family* (*genus* Phlebovirus). *The disease occurs in climatic conditions favouring the breeding of mosquito vectors and is characterised by abortion, neonatal mortality and liver damage. The disease is most severe in sheep, goats and cattle~~, in which it produces abortions in pregnant animals and a high mortality rate in the newborn~~. Older non-pregnant animals, although susceptible to infection, are more resistant to clinical disease. There is considerable variation in the susceptibility to RVF of animals of different ~~breeds~~ species. ~~Those breeds or strains that are exotic to Africa or are from areas where RVF is not endemic, tend to be more susceptible.~~ Camels usually suffer an inapparent infection with RVF virus (RVFV), but sudden mortality, neonatal mortality and abortion occurs and abortion rates can be as high as in cattle. ~~Among ruminant game, buffalo also abort during an inapparent RVF infection.~~*

Humans are susceptible to infection and get contaminated through contact with infected animal material (body fluids or tissues) or mosquito bites. ~~Infection of humans by vectors is a striking feature in countries with a relatively small population of animal hosts. In such areas, RVF may be recognised first in humans. It~~ RVFV has also caused serious infections ~~disease~~ in laboratory workers and must be handled with high level ~~biosecurity~~ containment. It is recommended that laboratory workers be vaccinated if possible.

Identification of the agent: *RVFV consists of a single serotype of ~~a bunyavirus of the genus~~* Phlebovirus *that has morphological and physicochemical properties typical of ~~bunyaviruses~~ this genus.*

Identification of RVFV can be achieved by virus isolation, antigen-detection enzyme-linked immunosorbent assay (ELISA) or immunopathology. Viral RNA can be detected by reverse transcription-polymerase chain reaction.

The virus can be isolated from blood, preferably collected with ~~in an~~ anticoagulant, during the febrile stage of the disease, or from organs (e.g. liver, spleen and brain tissues) of animals that have died and from the organs of aborted fetuses. Primary isolations are usually made on cell cultures of various types, such as African green monkey kidney (Vero) and baby hamster kidney (BHK) cells~~, chicken embryo reticulum, or primary cells of sheep or cattle origin~~. Alternatively, sucking~~, hamsters, adult or suckling~~ mice~~, embryonated chicken eggs or 2-day-old lambs~~ may be used for primary virus isolation.

~~A rapid diagnosis can be achieved by using the supernatant of homogenised samples as antigen in virus neutralisation (VN) tests; immunofluorescent staining of impression smears of liver, spleen, brain or infected cell cultures; or by the demonstration of virus in serum, taken during the febrile stage of the disease, by enzyme immunoassay or immunodiffusion.~~

~~The presence of characteristic histopathological lesions in the liver assists in the diagnosis.~~

Serological tests: *Identification of specific antibodies is mostly achieved by ELISA or virus neutralisation test. ~~Infected animals develop specific antibodies that may become demonstrable by VN as early as 3 days following infection and after 6–7 days by enzyme-linked immunosorbent assay, and by haemagglutination inhibition. Serological tests used less often include immunofluorescence, complement fixation and immunodiffusion.~~*

Requirements for vaccines ~~and diagnostic biologicals~~: *Live attenuated or inactivated ~~virus~~ vaccines ~~and antigens for~~ can be used in countries where RVF is endemic or at risk of introduction ~~during outbreaks~~. These vaccines should preferably be prepared from ~~nonpathogenic mouse- or mutagen-~~attenuated strains of RVFV grown in cell cultures. ~~The mutagen-attenuated strain of RVF is not yet at a stage where it can be recommended for use.~~*

In RVF-free countries, vaccines and diagnostic tests should preferably be limited to those using inactivated virus. Work with live virus should be performed by trained personnel in biocontainment facilities following appropriate biosafety procedures.

~~*Suitable virus strains can be obtained from the*~~ *There are two OIE Reference Laboratories for RVF* (*see Table given in Part 3 of this* Terrestrial Manual).

A. INTRODUCTION

~~Rift Valley fever (RVF) virus consists of a single serotype of a bunyavirus of the genus *Phlebovirus* and has morphological and physicochemical properties typical of bunyaviruses. The virus is enveloped, spherical and 80–120 nm in diameter. Short Glycoprotein spikes project through a bilayered lipid envelope. The virus is readily inactivated by lipid solvents and acid conditions below pH 6. RVF virus (RVFV) has a three-segmented, single-stranded, negative-sense RNA genome and consists of the three segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The S segment is ambisense RNA, i.e. has bi-directional coding (Gentsch & Bishop, 1979).~~

Rift Valley fever (RVF) is a peracute or acute, febrile, mosquito-borne, zoonotic disease caused by a virus of the family *Bunyaviridae*, genus *Phlebovirus*. It is usually present~~s~~ in epizootic form over large areas of a country following heavy rains and flooding, and is characterised by high rates of abortion and neonatal mortality, primarily in sheep, goats, cattle and camels. The susceptibility of different species and breeds to RVF may vary considerably. Some ~~indigenous African~~ animals may have ~~only~~ inapparent infections, while ~~exotic or~~ other~~s~~ ~~breeds~~ suffer severe clinical disease with mortality and abortion. Susceptible, older non-pregnant animals ~~and some other species usually~~ often do not show signs of disease. ~~Camels have been regularly involved in the RVF epidemics in East Africa and Egypt. Clinical disease is not seen in adult camels, but abortion occurs and some early post-natal deaths have been observed.~~

Signs of the disease tend to be nonspecific, rendering it difficult to recognise individual cases (Coackley *et al.*, 1967; Coetzer, 1982; Coetzer & Barnard, 1977; Easterday, 1965; Gerdes, 2004; Meegan & Bailey, 1989; Swanepoel & Coetzer, 1994; Weiss, 1957) during epidemics; however, the occurrence of numerous abortions and mortalities among young animals, together with disease in humans, is characteristic of RVF. RVF has a short incubation period~~:~~ of about 12–36 hours in lambs. A biphasic fever of up to 41°C may develop, and the fever remains high until shortly before death. Affected animals are listless, disinclined to move or feed, and may show enlarged superficial lymph nodes and evidence of abdominal pain. Lambs rarely survive longer than 36 hours after the onset of signs of illness. Animals older than 2 weeks may die peracutely, acutely or may recover or develop an inapparent infection. Some animals may regurgitate ingesta and may show melaena or bloody, foul-smelling diarrhoea and bloodstained mucopurulent nasal discharge. Icterus may sometimes be observed, particularly in cattle. In addition to these signs, adult cattle may show lachrymation, salivation and dysgalactia. In pregnant sheep, the mortality and abortion rates vary from 5% to almost 100% in different outbreaks and between different flocks. The death rate in cattle is usually less than 10%. Camels have been regularly involved in the RVF epidemics in East Africa, Egypt and more recently Mauritania. Clinical disease is usually not seen in adult camels, but sudden deaths, abortion and some early post-natal deaths have been observed. Differential diagnosis includes: bluetongue, Wesselsbron disease, enterotoxemia of sheep, ephemeral fever, brucellosis, vibriosis, trichomonosis, Nairobi sheep disease, heartwater, ovine enzootic abortion, toxic plants, bacterial septicaemias, peste des petits ruminants, anthrax and Schmallenberg disease.

The hepatic lesions of RVF are very similar in all species, varying mainly with the age of the infected individual (Coetzer, 1982). The most severe lesion, occurring in aborted fetuses and newborn lambs, is a moderately to greatly enlarged, soft, friable liver with a yellowish-brown to dark reddish-brown colour with irregular congested patches. Numerous greyish-white necrotic foci are invariably present in the parenchyma, but may not be clearly discernible. In adult sheep, the lesions are less severe and pinpoint reddish to greyish-white necrotic foci are distributed throughout the parenchyma. Haemorrhage and oedema of the wall of the gallbladder are common. Hepatic lesions in lambs are almost invariably accompanied by numerous small haemorrhages in the mucosa of the abomasum. The contents of the small intestine and abomasum can be ~~are~~ dark chocolate-brown as a result of the presence of partially digested blood. In all animals, the spleen and peripheral lymph nodes can be ~~are~~ enlarged, oedematous and may have petechiae.

Microscopically, hepatic necrosis is the most obvious lesion of RVF in both animals and humans. In fetuses and neonates of cattle and sheep, foci of necrosis consist of dense aggregates of cellular and nuclear debris, some fibrin and a few inflammatory cells. There is a severe lytic necrosis of most hepatocytes and the normal architecture of the

liver is lost. In about 50% of affected livers, intranuclear inclusion bodies that are eosinophilic and oval or rod-shaped are found. Mineralisation of necrotic hepatocytes is also seen. In adult animals, hepatic necrosis is less diffuse and in sheep, icterus is more common than in lambs (Coetzer, 1982; Swanepoel & Coetzer, 1994).

In humans, RVF infections are usually inapparent or associated with a moderate to severe, nonfatal, influenza-like illness (Madani *et al.*, 2003; McIntosh *et al.*, 1980; Meegan, 1981). A minority of patients may develop ~~ocular~~ retinal lesions, encephalitis, or severe hepatic disease with haemorrhagic manifestations, which is generally fatal.

RVF virus (RVFV) has caused serious human infections in laboratory workers. Staff should ~~either~~ be vaccinated when a vaccine is available and work should be performed under high containment level with respiratory protection ~~3, work under containment level 4 conditions, or wear respiratory protection~~. Particular care needs to be exercised when working with infected animals or when performing *post-mortem* examinations (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

RVFV consists of a single serotype of the *Bunyaviridae* family (genus *Phlebovirus*) and has morphological and physicochemical properties typical of bunyaviruses. The virus is enveloped, spherical and 80–120 nm in diameter. Glycoprotein spikes project through a bilayered lipid envelope. The virus is readily inactivated by lipid solvents and acid conditions below pH 6. RVFV has a three-segmented, single-stranded, negative-sense RNA genome and consists of the following segments: L (large), M (medium) and S (small), each of which is contained in a separate nucleocapsid within the virion. The S segment is an ambisense RNA, i.e. has bi-directional coding (Giorgi, 1991).

No significant antigenic differences have been demonstrated between RVF isolates and laboratory-passaged strains from many countries, but differences in pathogenicity between genotypes have been shown (Bird *et al.*, 2007; Swanepoel *et al.*, 1986).

~~Infection of humans by mosquito vectors is a striking feature in countries, such as Egypt, with a relatively small population of animal hosts and a large population of mosquitoes.~~

RVFV is endemic in many African countries and may involve several countries in the region at the same time or progressively expand geographically over the course of a few years. In addition to Africa, large outbreaks have been observed in the Arabian Peninsula and some Indian Ocean Islands ~~usually occurs in epizootics in Africa, which may involve several countries in a region at one and the same time~~. These generally, but not exclusively, ~~,~~ follow the periodic cycles of unusually heavy rainfall, which may occur at intervals of several years ~~very rarely in semi-arid zones (25–35-year cycles), or more frequently (5–15-year cycles) in higher rainfall savannah grasslands~~, or the flooding of wide areas favouring the proliferation of mosquitoes.

Rainfall facilitates mosquito eggs to hatch. *Aedes mosquitoes* acquire the virus from feeding on infected animals, and may potentially vertically transmit the virus, so that new generations of infected mosquitoes may hatch from their eggs (Linthicum, 1985). This provides a potential mechanism for maintaining the virus in nature, as the eggs of these mosquitoes may survive for periods of up to several years in dry conditions. Once livestock is infected, a wide variety of mosquito species may act as the vector for transmission of RVFV and can spread the disease.

Low level ~~undetectable~~ RVF activity may take place during inter-epizootic periods. RVF should be suspected when exceptional ~~unusually~~ flooding and subsequent abundant mosquitoes populations ~~heavy rains~~ are followed by the occurrence of abortions together with fatal disease marked by necrosis and haemorrhages in the liver that particularly affect newborn lambs, kids and calves, potentially concurrent with the occurrence of an influenza-like illness in farm workers and people handling raw meat.

During an outbreak, preventive measures to protect workers from infection should be employed when there are suspicions that RVF-virus-infected animals or animal products ~~meat and tissue samples~~ are to be handled.

B. DIAGNOSTIC TECHNIQUES

The collection of specimens and their transport should comply with the Chapter 1.1.1 *Collection and storage of diagnostic specimens* and Chapter 1.1.2 *Transport of specimens of animal origin* of this *Terrestrial Manual*.

Proper diagnosis should always use a combination of techniques based on history, the purpose of the testing and the stage of the suspected infection. For a definitive interpretation, combined epidemiological, clinical and laboratory information should be evaluated carefully.

All the test methods described below should be validated in each laboratory using them (see Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases* of this *Terrestrial Manual*). The OIE Reference Laboratories for RVF should be contacted for technical support. Table 1 provides a general guidance

summary on the use of the diagnostic tests methods. More detailed aspects are addressed in the test descriptions that follow.

Table 1. *Test methods available and their purposes*

Method	**Purpose**					
	Population freedom from infection (non-vaccinated animals)	Individual animal freedom from infection	Efficiency of eradication policies[1]	Confirmation of clinical cases[2]	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Virus isolation in cell culture	=	=	=	+++	+	n/a
Virus isolation in sucking mice	=	=	=	+	+	n/a
Reverse transcriptase polymerase chain reaction	=	=	=	+++	+	n/a
Antigen detection	=	=	=	++	+	n/a
Histopathology with immuno-histochemistry	=	=	=	++	=	n/a
Enzyme-linked immunosorbent assay	+++	++	+++	++	+++	+++
Virus neutralisation	+++	+++	+++	++	++	+++

Key: +++ = recommended, validated method; ++ = suitable, but requires further validation;
+ = may be used, but should be interpreted with caution; = – not appropriate for this purpose.

1. Identification of the agent

RVFV may be isolated from serum but preferentially from plasma or blood collected with anticoagulant during the febrile stage of the disease in live animals, or from liver, spleen and brain of animals that have died, or from aborted fetuses. Primary isolation is usually performed in ~~hamsters, infant or adult mice, or on~~ cell cultures of various types or by intracerebral inoculation of sucking mice.

a) Specimen collection

Using appropriate protective equipment to ensure biosafety of the staff, approximately 5 ml of blood with anticoagulant (preferably ethylene diamine tetra-acetic acid [EDTA]) collected during the febrile stage of the disease or approximately 1 cm^3 of liver, spleen, brain and/or abortion products collected post-mortem should be submitted for virus isolation. The samples should be kept at 0–4°C during transit. If transport to the laboratory is likely to take more than 24 hours, the samples should be frozen and sent on dry ice or frozen cold pack. In case of blood sample, plasma should be collected and frozen for transport.

~~a~~b) Isolation in cell cultures ~~Culture~~

~~Approximately 5 ml of blood collected during the febrile stage of the disease or approximately 5 g of liver, spleen and brain collected after death should be presented for virus isolation. The samples should be kept at 0–4°C during transit. If transport to the laboratory is likely to take more than 24 hours, the samples should be frozen and sent on dry ice.~~

1 If vaccination by DIVA vaccines are used in these policies then DIVA discriminatory assays should be useful (ELISA assay because the neutralisation assay do not allow the distinction between antibody following naturally infection or following vaccination)". The sentence between

2 Laboratory confirmation of clinical cases should require a combination of at least two positive results from two different diagnostic test methods: either positive for virus/viral RNA and antibodies or positive for IgM and IgG with demonstration of rising titres between paired sera samples collected 2-4 weeks apart. Depending of the stage of the disease, virus and/or antibodies will be detected.

~~Approximately 1 g of homogenised tissue is suspended 1/10 in cell culture medium or buffered saline, pH 7.5, containing sodium penicillin (1000 International Units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml), or fungizone (2.5 µg/ml). The suspension is centrifuged at 1000 ***g*** for 10 minutes and the supernatant fluid is injected intracerebrally into 1–5 day-old mice or intraperitoneally into hamsters or adult mice. Infant mice will either die or be obviously ill by day 2. Adult mice are affected 1–3 days later. Although mice or hamsters are the laboratory animal of choice, lambs and embryonated chicken eggs may also be used.~~

A variety of cell lines ~~monolayers~~ including African green monkey kidney (Vero), baby hamster kidney (BHK) ~~chicken embryo reticulum (CER: cells developed by Tsunemasa Motohashi at the Nippon Institute for Biological Science, Tokyo, Japan; recharacterised as a hamster line) (4)~~ and AP61 mosquito cells (Digoutte *et al.*, 1989) ~~primary kidney or testis cells of calves and lambs~~ may be used. They are inoculated with 1/10 ~~1 ml of clarified~~ dilution of the sample ~~supernates~~ and incubated at 37°C for 1 hour (with mosquito cell lines, the incubation should be done at 27°C for 1 hour). It is advisable to also inoculate some cultures with a further 1/100 dilution of the inoculum. This is to avoid the production of defective particles, which follows the use of very high virus inocula~~u~~m. ~~Some tubes containing flying cover slips should also be prepared. The cultures are washed with phosphate buffered saline at room temperature and covered with medium containing 2% serum free from antibodies against RVF.~~ The inoculum is removed and the monolayer is washed with phosphate buffered saline or culture medium. The wash solution is removed, replaced by fresh culture medium and incubated at appropriate temperature. The cultures are observed ~~microscopically~~ for 5–6 days. Mammalian cell lines are preferably used since RVFV induces a consistent cytopathic effect (CPE) characterised by slight rounding of cells followed by destruction of the whole cell sheet within 12–24 hours. Confirmation of virus isolation should be performed preferably by immunostaining or reverse transcription-polymerase chain reaction (RT-PCR).~~Specific identification of RVF virus antigen may be made 18–24 hours after infection by immunofluorescent staining of the cover-slip preparations.~~

~~The virus may also be detected by immunofluorescence carried out on impression smears of liver, spleen and brain. A rapid diagnosis can sometimes be made by demonstrating viral antigen in tissues or in serum of febrile animals by a complement fixation or agar gel immunodiffusion (AGID) test. A rapid diagnosis can also be made by detection of viral RNA using a reverse transcription polymerase chain reaction (RT-PCR).~~

~~b~~c) Isolation in sucking mice ~~Agar gel immunodiffusion~~

This method should be avoided if possible, for reasons of animal welfare and biosafety. Approximately 1 g of homogenised tissue is suspended 1/10 in cell culture medium or buffered saline, pH 7.5, containing sodium penicillin (1000 International Units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml), or fungizone (2.5 µg/ml). The suspension is centrifuged at 1000 ***g*** for 10 minutes and the supernatant fluid is injected intracerebrally into 1- to 5-day-old mice. Sucking mice will either die or be obviously ill by day 2 post-inoculation.

Confirmation of virus isolation should be performed preferably by immunostaining or polymerase chain reaction (PCR).

~~The AGID test is useful in laboratories without tissue culture facilities. Approximately 1 gram of tissue, preferably liver, is homogenised and made up to a 10–20% suspension in borate saline buffer, pH 9.0. The material is centrifuged at 1000 ***g*** and the supernatant is used in the test. Micro-AGIDs are performed on standard microscope slides covered with 3 ml of 1% agarose in borate saline. Patterns of six peripheral wells and a central well are prepared and filled with reagents as follows: a positive, preferably hyperimmune serum in the central well, positive control antigen in wells 1 and 4, test tissues in wells 2 and 5 and negative tissues in wells 3 and 6. A precipitin line of continuity should be formed between control antigen and positive serum that extends to include a line between test tissue and serum for a case to be considered positive.~~

~~c~~d) Reverse transcription polymerase chain reaction

A rapid diagnosis can also be made by detection of viral RNA (Sall *et al.*, 2001) using validated conventional or real-time RT-PCR (Bird *et al.*, 2007; Drosten *et al.*, 2002; Garcia *et al.*, 2001~~; Le Roux *et al.*, 2009~~; Sall *et al.*, 2001). These techniques were very useful during RVF outbreaks in Africa. ~~The PCR was used, among other techniques, for antigen detection in two recent RVF virus outbreaks in Africa – one in Kenya in 1998 and a limited outbreak in South Africa in 1999~~. It may also be used to detect RVFV in mosquito pools (Jupp *et al.*, 2000). ~~RT-PCR followed by sequencing of the NS(S) protein coding region has been used in phylogenetic analysis to characterise two distinct lineages of RVF virus – one Egyptian and the other sub-Saharan – making this technique a powerful molecular epidemiological tool (29).~~

This technique should be followed by sequencing on selected samples. Below are proposed protocols for conventional and real time RT-PCR. For information on specific procedures consult the OIE Reference Laboratories.

Agarose gel-based RT-PCR assay

This procedure is used at the OIE Reference Laboratories. The RT-PCR assay consists of the three successive procedures of (a) extraction of template RNA from the test or control sample followed by (b) RT of the extracted RNA, (c) PCR amplification of the RT product and (d) detection of the PCR products by agarose gel electrophoresis.

- **Test procedure**

RNA is extracted by an appropriate chemical method according to the procedure recommended by the commercial kit's manufacturers. When the procedure is finished, keep the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at –20°C or –70°C. For RT-PCR, the protocol from Sall *et al*. (2001) are used. For the first RT-PCR step, NSca (5-'CCT-TAA-CCT-CTA-ATC-AAC-3') and NSng (5'-TA-TCA-TGG-ATT-ACT-TTC-C-3') primers are used.

i) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (15.5 µl); RT-PCR reaction buffer, 5× conc (10 µl); $MgCl_2$, 25 mM (1 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer NSca, 10 µM (2.5 µl); primer NSng 10 µM (2.5 µl): *Enzyme Mix*, 5 units/µl (0.25 µl).

ii) Add 40 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample to be assayed followed by 10 µl of the RNA (prepared in step ix) to give a final reaction volume of 50 µl.

iii) Spin the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

iv) Place the plate in a thermal cycler for PCR amplification and run the following programme:

45°C for 30 minutes: 1 cycle;

95°C for 2 minutes: 1 cycle;

94°C for 30 seconds, 44°C for 30 seconds, 72°C for 1 minute: 40 cycles;

72°C for 5 minutes: 1 cycle.

v) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.2% agarose gel. After electrophoresis a positive result is indicated by the presence of a 810 bp (242 bp for Clone 13) band corresponding to RVFV sequence in the NSs coding region of the S segment of the genome.

For the nested RT-PCR step, NS3a (5'-ATG-CTG-GGA-AGT-GAT-GAG-CG-3') and NS2g (5'-GAT-TTG-CAG-AGT-GGT-CGT-C-3') are used.

vi) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (35.5 µl); RT-PCR reaction buffer, 10× conc (5 µl); $MgCl_2$, 25 mM (1.25 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); primer NS3a (5'-ATG-CTG-GGA-AGT-GAT-GAG-CG-3'), 10 µM (2.5 µl); primer NS2g (5'-GAT-TTG-CAG-AGT-GGT-CGT-C-3'), 10 µM (2.5 µl): *Enzyme Mix*, 5 units/µl (0.25 µl).

vii) Add 49 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample to be assayed followed by 1 µl of the amplicon obtained from RT-PCR reaction with NSca and NSng to give a final reaction volume of 50 µl.

viii) Spin the plate or tubes for 1 min in a suitable centrifuge to mix the contents of each well.

ix) Place the plate in a thermal cycler for PCR amplification and run the following programme:

95°C for 2 minutes: 1 cycle;

94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute: 25 cycles;

72°C for 5 minutes: 1 cycle.

x) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.2% agarose gel. After electrophoresis a positive result is indicated by the presence of a 668 bp (129 bp for Clone 13) band corresponding to RVFV sequence in the NSs coding region of the S segment of the genome.

Real-time RT-PCR assay

The real-time RT-PCR assay can use the same procedures of extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional procedure. The protocol is adapted from Drosten *et al*. (2002). If commercial kits are used the manufacturer's method should be followed.

- **Test procedure**

i) Prepare the PCR mix described below for each sample. Again it is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample: nuclease-free water (1.4 µl); RT-PCR reaction master mix, 2× conc. (10 µl); real-time PCR forward primer **RVS**: 5'-AAA-GGA-ACA-ATG-GAC-TCT-GGT-CA-3', 10 µM (2 µl); real-time PCR reverse primer **RVAs**: 5'-CAC-TTC-TTA-CTA-CCA-TGT-CCT-CCA-AT-3', 10 µM (2 µl); **RVP**: **FAM** 5'-AAA-GCT-TTG-ATA-TCT-CTC-AGT-GCC-CCA-A-3' **TAMRA** 20 µM (0.2 µl).

ii) Add 17 µl PCR reaction mix to a well of a real-time PCR plate for each sample to be assayed followed by 3 µl of the prepared RNA to give a final reaction volume of 20 µl.

iii) Spin the plate for 1 minute in a suitable centrifuge to mix the contents of each well.

iv) Place the plate in a real-time PCR machine for PCR amplification and run the following programme:

45°C for 30 minutes: 1 cycle;

95°C for 5 minutes: 1 cycle;

95°C for 5 seconds, 57°C for 35 seconds: 45 cycles.

v) *Reading the results:* Assign a threshold cycle (CT) value to each PCR reaction from the amplification plots (a plot of the fluorescence signal versus cycle number; different cut-off values may be appropriate for different sample types; Parida *et al.* (2007). The CT values used to assign samples as either RVFV positive or negative should be defined by individual laboratories using appropriate reference material.

e) Antigen detection

The antigen detection enzyme-linked immunosorbent assay (ELISA) is an immunocapture test. Samples are tested at different dilutions with appropriate positive and negative controls. This test has been used for human and animal samples during outbreaks in Saudi Arabia and Kenya (Madani *et al.*, 2003; Munyua *et al.*, 2010).

- **Test procedure**

i) The controls and antisera used in the performance of this assay have been treated to kill any RVFV that may have been in them at the time of production. Within the limits of our ability to detect viable virus these products are safe. The material to be tested for the presence of RVF viral antigen is potentially contaminated with viable RVFV or other agents for which a differential determination is being sought. Good laboratory practices should be used at minimum. Samples could be inactivated using appropriate detergent and heat inactivation.

ii) The basic approach is that of a double antibody sandwich capture assay in which the antigen is captured by antibody on a solid phase and then detected by a second antibody. A detection system using the horseradish peroxidase (HRPO)–ABTS is then applied to determine how much of the detection antibody has been retained on the solid phase of the system.

iii) *Capture* (*coating*) *antibody* (*diluted 1/2000 in PBS* [*no Tween*] *pH 7.4 and coated overnight at 4°C; control wells are coated with a similar dilution of normal fluid*): Plates are coated with a specific anti-viral antibody (available in OIE or WHO Reference Laboratories) capable of capturing viral antigen from the test sample. Normal serum is added to rows to serve as controls used to determine the non-specific background or noise of the system. In this instance it is a hyperimmune mouse ascitic fluid (HMAF) (it could also be monoclonal antibodies) specific for RVF viruses.

iv) *Suspect samples and control antigen* (*diluted 1/4 and then diluted four-fold, down the plate*): These are added in serum diluent to allow specific viral antigens to bind to the capture antibody. Serum diluent (phosphate buffered saline, 0.01 M, pH 7.4, with or without thiomersal) contains 5% skim milk and 0.1% Tween 20 to reduce nonspecific binding.

v) *Detection antibody:* An antibody, high titred for specific viral antigen, is added to allow detection of the bound viral antigen. In this experiment, it is an anti-RVF hyperimmune rabbit serum (available in OIE or WHO Reference Laboratories) that has a high titre against RVF viruses.

vi) *Anti-rabbit conjugated to HRPO* (*commercial product*): Used to detect the rabbit anti-RVF that binds to the antigen.

vii) Criteria for determining positives. A standard control antigen has been provided and will be run in a standard dilution series. This, in effect, provides a standard curve which will determine the limits of

detection of the assay. A group of normal tissues or samples, uncontaminated with antigen, are tested to determine the background of the assay and the limit at which the standard was positive. The values of these normal controls are used to generate the mean and standard deviation of the random background to be expected with negative samples. A sample is considered positive if its optical density (OD) value exceeds the mean plus 3 standard deviations of these normal controls.

~~d~~f) Histopathology

Histopathological examination of the liver of affected animals will reveal characteristic cytopathology, and immunostaining will allow the specific identification of RVF viral antigen in ~~infected cells~~ tissue (Coetzer, 1982; Swanepoel *et al.*, 1986). This is an important diagnostic tool because liver or other tissue placed in ~~formol saline~~ neutral buffered formaldehyde in the field is inactivated ~~for diagnostic purposes~~ and does not require a cold chain, which facilitates handling and transport from remote areas ~~remote from the laboratory~~.

2. Serological tests

Samples collected from animals for antibody testing may contain live virus and appropriate inactivation steps should be put in place. A combination of heat and chemical inactivation has been described (Van Vuren & Paweska, 2010). Immunofluorescence assays are still used, although cross-reactions may occur between RVFV and other phleboviruses. Techniques such as agar gel immunodiffusion (AGID), radioimmunoassays, haemagglutination inhibition (HI), and complement fixation are no longer used.

Several assays are available for detection of anti-RVFV antibodies in a variety of animal species. Currently the most widely used technique is the ELISA for the detection of IgM and IgG. Virus neutralisation tests (VNT) ~~including microneutralisation, plaque reduction neutralisation (PRN) and neutralisation in mice~~ have been used to detect antibodies against RVFV in the serum of a variety of species. Neutralisation tests are the most specific diagnostic serological tests ~~and will record the earliest response~~, but these tests can only be performed with live virus and are not recommended for use outside endemic areas and/or in laboratories without appropriate biosecurity facilities and vaccinated personnel. However, alternative neutralisation assays not requiring handling of highly virulent RVFV and not requiring high containment, are being developed and validated.

~~Other available tests include enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HI), AGID, immunofluorescence, radioimmunoassay and complement fixation. In these tests, however, cross-reactions may occur between RVF virus and other phleboviruses. An advantage of these tests is the fact that they can be performed with inactivated antigen and can therefore be used in RVF-free countries.~~

a) Enzyme-linked immunosorbent assay

The ELISA is a reliable and sensitive test ~~that may be employed with several species~~ to detect antibodies against RVFV. Both IgG and IgM ELISAs are available for most species. IgM-capture ELISA allows diagnosis of recent infections ~~to be made on a single serum sample~~.

A number of ELISAs using different formats are commercially available and others are under development (Afetine *et al.*, 2007; Cettre-Sossah *et al.*, 2009; Jansen Van Vuren *et al.*, 2007; Madani *et al.*, 2003; Munyua *et al.*, 2010; Paweska *et al.*, 2003; Paweska *et al.*, 2005; Van Vuren & Paweska, 2010). They are used routinely in many countries for single case diagnosis, outbreak management, and surveillance.

~~The HI test can be employed with great confidence in nonendemic areas. However, sera from individuals that have had previous infections with phleboviruses other than RVF may react with RVF antigen to titres as high as 40 and, rarely, to titres of 320 (33). In suspected cases, the OIE Reference Laboratory for RVF (see Table given in Part 3 of this *Terrestrial Manual*) can be of assistance in carrying out neutralisation tests for specificity. The HI antibody titre after vaccination with RVF virus vaccine may be as high as 640 or, rarely, 1280, whereas titres following natural infections with RVF virus are usually significantly higher.~~

Below are two such tests used at the OIE Reference Laboratory for RVF at ARC-Onderstepoort Veterinary Institute. Both assays use 10% non-fat milk/Tris salt Tween (NFM/TST) as blocking and dilution buffer and TST buffer: 50 mM Tris/150 mM NaCl/0.1% Tween 20 as washing buffer (pH 8.0). The reactions are stopped with 2N H_2SO_4.

The recombinant nucleoprotein (rN) of RVFV is produced and purified as described by Williams *et al*. (2011). Conjugation of the protein to HRPO is performed as per the Nakane & Akira Kawaoi (1974) protocol. The rN antigen is stable for up to 1 year at 4°C.

To prepare plates for immediate use, make a checkerboard titration of the capture antibody or antigen against the conjugate in a 96-well ELISA plate to determine the minimum reagent concentration that would give an OD

value of 0.5–0.6 when read at 650 nm after an incubation period of 20 minutes. This will inform how the antibody/antigen and conjugate must be diluted for coating of the plates and detection of the antigen/antibody binding in the test.

IgM capture ELISA

- **Test procedure**

i) Coat each well of the 96-well ELISA plates with 100 µl of the capture antibody (affinity purified Rabbit anti-sheep IgM1) diluted to 1 µg/ml in PBS (that is a 1/1000 dilution if so determined by the titration), and incubate overnight at room temperature in a humid chamber.

ii) Wash the plates three times with wash buffer.

iii) Block the plates with 300 µl blocking buffer and incubate for 1 hour at 37°C.

iv) Wash the plates again three times with wash buffer.

v) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer and add each serum in a designated well at volumes of 100 µl /well.

vi) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.

vii) Following the incubation step, wash the ELISA plates with wash buffer three times.

viii) Dilute the rN-HRP conjugate 1/6000 and add 100 µl of this in each well. Use blocking buffer as the conjugate control (cc).

ix) The plates are then incubated for 60 minutes at 37°C.

x) The plates are washed, as in step ii above. Ready to use tetra methyl benzidine (TMB) substrate at 100 µl quantities is then transferred to each well, and the plates allowed to stand at room temperature for a few minutes, until development of a colour change or OD values of 0.5 when the plates are read at 650 nm. Exposure to direct light should be avoided.

xi) Stop the reaction with 100 µl stop solution, and read the OD values using an ELISA plate reader at 450 nm.

xii) *Interpretation of results*: results are expressed as percentage of the positive serum control (PP) using the formula: [(mean OD of duplicate test serum/mean OD of positive control)]×100, where a positive and negative cut-off values are determined by receiver operating characteristic (ROC) curve analysis.

It should be noted that the cut-off value for an ELISA can be adjusted for different target populations as well as for different diagnostic purposes (Jacobson, 1998). The cut-off values determined by the recent validation exercise at ARC-OVI are the following: PP (%) values: negative <4; suspicious 4–5; positive>6.

Indirect IgG ELISA

- **Test procedure**

i) Coat each well of the 96 well ELISA plate with 100 µl of rN diluted in 50mM of carbonate buffer (pH 9.6) using the dilution ratio determined by prior titration as explained above; and incubate overnight at room temperature in a humid chamber.

ii) Wash the plates three times with approximately 300 µl wash buffer per well.

iii) Block the plates with approximately 300 µl blocking buffer and incubate for 1 hour at 37°C.

iv) Wash the plates again three times with nearly 300 µl of wash buffer per well.

v) Dilute both control (positive and negative) and test sera 1/100 in blocking buffer.

vi) Add 100 µl of the diluted sera in designated wells in duplicate.

vii) Incubate the plates for 1 hour at 37°C. Avoid drying by putting plates in a humid chamber.

viii) Following the incubation step, wash the ELISA plates with wash buffer three times.

ix) Dilute Protein G-HRP conjugate 1/32000 in blocking buffer and add 100 µl of the conjugate in each well.

x) Incubate for 60 minutes at 37°C.

xi) The plates are washed, as in step ii above. Add 100 µl of ready to use TMB substrate to each well and allow the plates to stand at room temperature for a few minutes, while avoiding exposure to direct light. The plates are read at 650 nm to determine if OD of 0.4–0.6 has been reached.

xii) Stop the reaction with 100 µl stop solution, and read the plates using ELISA plate reader at 450 nm.

xiii) *Interpretation of results*: results are expressed as percentage of the positive serum (PP) using the formula: [(mean OD of duplicate test serum/mean OD of positive control)]×100, where a positive–negative cut-off is determined by receiver operating characteristic (ROC) curve analysis.

It should be noted that the cut-off value for an ELISA can be adjusted for different target populations as well as for different diagnostic purposes (Jacobson, 1998). The cut-off values determined by the recent validation exercise at ARC-OVI are the following: PP values (%): negative <4; suspicious 4–6; positive>7.

b) Virus neutralisation (the prescribed test for international trade)

The VN test may be employed to determine the presence of antibodies in naturally infected animals and in vaccinated animals ~~vaccinated with RVF vaccine~~. The test is highly specific and can be used to test serum of any species. It is generally used to measure vaccine efficacy. ~~Factors other than neutralising antibodies may play a part in resistance to RVF.~~ The Smithburn neurotropic mouse brain strain of highly attenuated RVFV (Smithburn, 1949) or any other, preferably attenuated, RVFV, ~~also referred to as modified live virus and adapted to cell culture~~ is used as challenge virus. The virus ~~antigen~~ is stored at –80°C, or 4°C in freeze-dried form. The stock is titrated to determine the dilution that will give 100 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl under the conditions of the test.

- **Test procedure**

i) Inactivate the test sera for 30 min in a water bath at 56°C.

ii) Add 25 µl of cell culture medium with 5% RVF-negative serum and antibiotics to each well of a 96-well cell culture plate.

iii) Add 25 µl of test serum to the first well of each row and make twofold dilutions. Titrate each serum in duplicate from 1/10 to 1/80 for screening purposes or in quadruplet and to higher dilutions for determination of end-point titres. Include known positive and negative control sera.

iv) Add 25 µl per well of RVFV ~~antigen~~ (diluted in cell culture medium and calculated to provide 100 $TCID_{50}$ per well) to each well that contains diluted test serum and to wells in rows containing negative and positive control serum. In addition, make twofold dilutions of challenge virus ~~antigen~~ in at least two rows each containing cell culture medium only.

v) Incubate for 30 min at 37°C.

vi) Add 50 µl per well of Vero, BHK ~~CER~~ or any other suitable cell suspension at 3×10^5 cells/ml or at a dilution known to produce a confluent monolayer within 12 hours.

vii) Incubate the plates in an atmosphere of 3–5% CO_2 for 3–5 days.

viii) Using an inverted microscope, the monolayers are examined daily for evidence of CPE. There should be no CPE in rows containing positive control serum and clear evidence of CPE in rows containing negative control serum indicating the presence of virus. Determine the results by the Spearman–Kärber method.

~~**b) Enzyme-linked immunosorbent assay**~~

~~For the serodiagnosis of RVFV a number of ELISAs using different formats have been published and are commercially available (1, 28). The use of inactivated whole virus or mouse liver antigens has recently been replaced by recombinant nucleocapsid (N) protein as antigen.~~

~~These ELISAs are at present in an indirect format and apart from the very important safety consideration also have the advantage of antigen stability and the ability to test 40 sera in duplicate per plate instead of only 20.~~

~~An indirect ELISA with pre-coated plates using a nucleocapsid protein (NC) recombinant antigen and Protein G peroxidase conjugate is described below (17).~~

- ~~**Test procedure**~~

~~Unless otherwise stated, all dilutions are made with 10% (w/v) dried milk buffer and all washes performed three times with volumes of 250–300 µl/well.~~

~~i) Using pre-coated plates add 50 µl of diluted (1/100) serum in duplicate wells~~

~~ii) Add control sera at predetermined dilutions in duplicate wells. Incubate for 60 minutes at 37°C. Wash the plate.~~

~~iii) Add Protein G/horseradish peroxidase conjugate at a working dilution to all wells of the plate. Incubate for 60 minutes at 37°C. Wash the plate~~

iv) Add 50 µl of ready-to-use TMB Substrate to all wells of the plate. Cover the plate and incubate at room temperature in the darkness for 20–30 minutes.

v) Add 50 µl of ready-to-use Stop solution to all wells of the plate. Tap plate gently to allow contents to mix. Wait 5 minutes and read plate using a spectrophotometer equipped with a 450 nm filter.

vi) Suggested plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	CC	CC	1	2	3	4	5	6	7	8	9	10
B	CC	CC	1	2	3	4	5	6	7	8	9	10
C	C++	C++	11	12	13	14	15	16	17	18	19	20
D	C++	C++	11	12	13	14	15	16	17	18	19	20
E	C+	C+	21	22	23	24	25	26	27	28	29	30
F	C+	C+	21	22	23	24	25	26	27	28	29	30
G	C–	C–	31	32	33	34	35	36	37	38	39	40
H	C–	C–	31	32	33	34	35	36	37	38	39	40

CC: Conjugate control; C++: High positive control serum; C+: low positive control serum; C–: negative control serum; 1–40: test samples.

Newer ELISA formats are being introduced, including formats that are more specific for IgG and IgM (27).

c) Haemagglutination inhibition

The HI test adapted to a microtechnique is based on Clarke & Casals (7). A sucrose/acetone extracted hamster liver antigen is used in a 96-well U-bottomed plate test and antigen is diluted so that 4 haemagglutinating units are used in the test. Nonspecific inhibitors of haemagglutinin are removed by kaolin extraction of sera followed by adsorption with packed goose erythrocytes (RBC) prior to testing. Doubling dilutions of sera made in borate saline buffer, pH 9, are tested against equal volumes of antigen. Plates are held overnight at 4°C before the addition of 50 µl of 0.5% RBC to each of the wells. Plates are read after 30 minutes at room temperature and end-points are recorded as the reciprocal of the highest serum dilution producing complete inhibition of agglutination.

Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Sera with titres below 1/40 are considered to be negative.

HI is an appropriate screening test for surveys although it is not specific. Marked cross-reactions do occur between the phleboviruses, but homologous titres exceed heterologous titres. Experimentally, African phleboviruses other than RVF have been shown to be nonpathogenic for ruminants, and antibodies that they might induce are unlikely to cause confusion in RVF diagnosis (33).

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

This Section has been extensively revised and updated. Although some portions of the existing text have been incorporated, new text and deleted text have not been marked, in the interests of clarity.

1. Background

Currently available RVF vaccines are either live attenuated or inactivated vaccines.

Table 2. *Summary of the current RVF vaccine strains*

	Smithburn live attenuated virus vaccines	**Clone-13 live attenuated virus vaccine**	**Inactivated virus vaccines**	**TSI–GSD–200 inactivated human vaccine (presently not available)**
Origin of the isolate	Mosquito isolate, Uganda, 1948	Human isolate, 1974	Field strains (South Africa and Egypt) used	Mosquito isolate, Uganda, 1944
Attenuation	More than 200 passages in murine brain	Natural deletion in NSs gene	Not applicable	Not applicable
Production substrate	BHK cell line	Vero cell line	BHK cell line	Diploid fetal rhesus lung cell line
Target	livestock	livestock	livestock	human
DIVA policy	No	No	No	Not applicable

- *The live attenuated Smithburn RVF vaccine:* the vaccine virus is derived from Smithburn's original neurotropic strain. This strain is not lethal to adult mice inoculated intraperitoneally and is safe for use in all breeds of cattle, sheep and goats (Barnard, 1979; Smithburn, 1949). However, it may cause fetal abnormalities or abortion in pregnant animals. The Smithburn RVF vaccine has been used for decades in the control of RVF in Eastern and Southern Africa and in the Middle East, and is still used to date in different endemic region.

- *The Clone 13 RVF vaccine:* Clone 13 is a naturally attenuated strain characterised by a large deletion of the gene encoding for the main virulent factor, the NSs (Muller *et al.*, 1995). The risk of reversion is considered unlikely. No abortion or side effects have been seen in experimental vaccine trials (Dungu *et al.*, 2010; Hunter & Bouloy, 2001). It was recently introduced in South Africa for use in sheep and cattle using a single injection regimen.

- *The inactivated RVF vaccine:* the currently produced formalin-inactivated vaccines derived from a field strain of RVFV adapted to growth in cell culture (Barnard, 1979; Barnard & Botha, 1977). These vaccines are currently adjuvanted in aluminium hydroxide. However inactivated RVF vaccines need a booster 3–6 months following initial vaccination, followed by yearly boosters. Inactivated RVF vaccine is also used in outbreak situations, and in pregnant animals as the attenuated Smithburn vaccine is not suitable for this group.

- *Inactivated experimental human vaccine* formerly produced by the Salk Institute (USA) is no longer available (Meadors, 1986).

Many other candidate vaccines are either being developed and evaluated in target animals or are in early stage of development (Food and Agriculture Organization of the United Nations [FAO], 2011).

There are a number of product characteristics that it would be preferable to have in an effective and safe RVF vaccine, and that should be used to define a target product profile. Elements of a target product profile for a RVF vaccine should preferably meet recommendation 2 of the report of the FAO meeting, 2011 (FAO, 2011) and as indicated below.

The main purpose of a RVF vaccine is to prevent epizootics and epidemics in species of economic interest (susceptible livestock species [ruminants] and, potentially, camelids), and limit the impact on animal and public health. In addition to the potential economic impact, it could also have some implications in international movements of animals. It is relevant to distinguish specific requirements for endemic regions and regions free of the disease.

Endemic region: the objective is the prevention and control of epizootics and epidemics in endemic areas and to contribute to the improvement of livestock production in endemic areas. In order of priorities, characteristics of the vaccines are:

- preferably one dose, resulting in a long-lasting immunity;

- preferably a life-long immunity after a limited number of doses.

Free or non-endemic region: vaccines would be used either for the prevention of, or the response to an introduction of the virus. The expected characteristics of the vaccines are: safe with a quick onset of protective immunity and protection in animals of all ages and physiological status. Although DIVA (detection of infection in vaccinated animals) is an important property of any future vaccine, a requirement for DIVA should not hinder or block the development or licensing of an effective RVF vaccine.

In all the cases, the vaccines should be:

- Safe for the staff involved in the production of the vaccines and for the users, safe to all physiological stages of animals, and with minimal risk of introduction into the environment (potential vectors);
- Protective in multi species and if possible in all susceptible species of economic importance, to prevent infection and transmission;
- Cost effective for producers and users preferentially with a single-dose vaccination;
- Easy to use (e.g. preferably needle-free delivery), suitable for stockpiling (vaccine bank) and quick availability.

Staff handling virulent RVFV should preferably work in high containment facilities and be vaccinated, if vaccines are available, to minimise the risk of infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given below and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

In the following description of vaccine production, information is given on live vaccine production adjacent to information on inactivated vaccine production.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed virus

i) *Biological characteristics of the master seed virus*

The exact source of the isolate should be recorded and should include the type of material from which the virus was derived. The *in-vitro* passage history of the virus and details of the ingredients should be recorded in accordance with Chapter 1.1.6 of this *Terrestrial Manual.*The master seed virus (MSV) should be tested for identity, purity (freedom from adventitious agents) and safety. Characterisation of the MSV should be done using biological or genetic parameters as relevant.

Assuming adequate immunogenicity and for obvious safety reasons, it is highly recommended that attenuated virus strains be used for the production of inactivated vaccines.

The number of virus passages from the MSV stock to the final product should not exceed five (European Pharmacopoeia, 2012).

ii) *Quality criteria*

The purity of the MSV and cells to be used for vaccine production must be maintained during the process. The seed virus should be free from adventitious agents, bacteria and *Mycoplasma*, using tests known to be sensitive for detection of these microorganisms (see Chapter 1.1.7 *Tests for sterility and freedom from contamination of biological materials*). The aliquot to be tested should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against a RVFV different from the seed virus and the virus/antibody mixture is cultured on several types of cell line monolayers. Neutralised cultures should be passaged and tested for adventitious viruses that may have infected the cells or virus seed during previous passages. As an example, Bovine viral diarrhoea virus (BVDV) is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems. A cell line highly permissive for BVDV types 1 and 2 is recommended as one of the cell lines chosen for evaluation of the MSV. Products of bovine origin should be obtained from countries with negligible bovine spongiform encephalopathy (BSE) risk.

iii) *Validation as a vaccine strain*

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy for the species for which it is intended.

b) Method of manufacture

i) *Procedure*

Live vaccines

Virus seed is produced in cell culture. Selection of a cell type for culture is dependent on the degree of virus adaptation, growth in medium, and viral yield in the specific culture system. Vaccine products should be limited to the number of passages from the MSV and should be restricted to five. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. Dose of virus used to inoculate cell culture should be kept to a minimum to reduce the potential for viral defective interfering particles. When the virus has reached its appropriate titre, as determined by CPE or other approved technique, the harvest can be clarified. Generally, the vaccine is freeze-dried, preferably in the presence of a suitable stabiliser.

Inactivated vaccines

Antigens used in inactivated vaccines are generally prepared in a similar way to live vaccines. The virus present in the virus maintenance medium is inactivated using a validated inactivation method then can be eventually concentrated/purified and formulated with a suitable adjuvant.

In the case where a virulent RVFV is used for inactivated vaccine production, staff handling the live virus should be vaccinated if vaccines are available and the facilities and practices should conform with high containment level minimising the risk of infection of the staff and release into the environment.

ii) *Requirements for ingredients*

Cell lines used for cell culture should be demonstrated free of extraneous agents. All animal origin products used in the production and maintenance of cells (i.e. trypsin, fetal bovine sera) and growth of virus should be free of extraneous agents, with special attention paid to the presence of BVDV.

iii) *In-process controls*

Yield can be assessed using antigenic mass or infectivity assays. Sterility of antigens should be checked throughout the process.

A validated inactivation control method is used to assure complete inactivation of the bulk material of each batch. For inactivated vaccines, samples taken at regular timed intervals during inactivation, then inoculated into a susceptible cell line (as used for production), should indicate a complete loss of titre by 2/3 of the total duration of the inactivation process.

For tests in cell cultures, not less than 150 cm^2 of cell culture monolayer is inoculated with 1.0 ml of inactivated harvest. The product complies with the test if no evidence of the presence of any live virus or other micro-organism is observed.

At the end of the production, antigen content is measured to establish that minimum bulk titres or antigenic mass have been achieved.

iv) *Final product batch tests*

Sterility

The final products should be tested for absence of bacteria, *Mycoplasma* and fungal contamination (see chapter 1.1.7).

Identity

The bulk live attenuated virus or the inactivated antigen as well as the final formulated product (freeze-dried or liquid) should undergo identity testing before release to demonstrate that the relevant RVF strain is present.

Safety

Unless consistent safety of the product is demonstrated and approved in a registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in Chapter 1.1.6 of this *Terrestrial Manual*, batch safety testing is to be performed. The final product batch safety test is designed to detect any abnormal local or systemic adverse reactions.

If batch safety testing needs to be performed, each of at least two healthy sero-negative target animals have to be inoculated by the recommended route of administration with the recommended dose of vaccine. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. If the potency test is performed in the target species, observation of the safety during this test can also be considered as an alternative to the batch safety test described here.

Batch potency

For live vaccines, potency is usually based on live virus titre. For batch release of inactivated vaccines, indirect tests can be used for practicability and animal welfare considerations, as long as correlation has been validated to the percentage of protection in the target animal. Frequently indirect potency tests include antibody titration after vaccination of suitable species. Alternative methods (antigen mass) could be used if suitably validated.

Moisture content

The moisture content of the lyophilised attenuated vaccine should not exceed 5%.

c) Requirements for authorisation/registration/licensing

i) *Manufacturing process*

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.b.i to iv of this chapter) should be submitted to the Regulatory Authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

The *in-process* controls are part of the manufacturing process.

ii) *Safety requirements*

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily. In addition of these tests, the vaccines should be tested for safety in the field (see Chapter 1.1.6 on field tests [safety and efficacy]).

Live vaccines

Vaccines should be tested for any pathogenic effects in each of the target species claimed on the label.

- *Safety test* (*overdose*) *in young animals*

  Carry out the test for each recommended route of application using in each young target animal not older than the minimum age recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

  Use not fewer than 8 healthy young target animals without antibodies against RVFV. Administer to each animal a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the animals daily for at least 14 days. The body temperature of each vaccinated animal is measured on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 hours after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all animals does not exceed 1.5°C, no animal shows a temperature rise greater than 1.5°C for a period exceeding 3 consecutive days, and no animal shows notable signs of disease or dies from causes attributable to the vaccine.

- *Safety test in pregnant animals*

  Safety at different stages of gestation should be demonstrated if the product is to be used in pregnant animals.

  Carry out the test with vaccination by a recommended route using not fewer than 16 healthy animals of the same age and origin and that do not have antibodies against RVFV: 8 in the first third of gestation and 8 in the second third (periods of time where the teratogenic risk of RVF is the highest [Botros, 2006; Hunter, 2002]). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

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

The test is invalid if the vaccinated animals do not seroconvert before parturition. The vaccine virus complies with the test if no abnormalities in the gestation or in the animals are noted. No animal shows notable signs of disease or dies from causes attributable to the vaccine.

Vaccine virus must not be present in blood samples from newborn animals.

- *Non-transmissibility*

This test should be performed in the most susceptible species which is sheep for RVF.

Keep together not fewer than 12 healthy lambs, at the minimum age recommended for vaccination and of the same origin, and that do not have antibodies against RVFV. Use vaccine virus at the lowest passage level that will be present between the MSV and a batch of the vaccine. Administer by a recommended route to not fewer than 6 lambs a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine.

Maintain not fewer than 6 lambs as contact controls. The mixing of vaccinated lambs and contact lambs is done 24 hours after vaccination.

After 45 days, euthanise all lambs. Carry out appropriate tests on the lambs to detect antibodies against RVF virus and on the control lambs to detect RVFV in the spleen and liver. The vaccine complies with the test if antibodies are found in all vaccinated lambs and if no antibodies and no virus are found in the control lambs.

- *Reversion-to-virulence*

This test is carried out using the master seed lot. If the quantity of the master seed lot sufficient for performing the test is not available, the lowest passage material used for the production that is available in sufficient quantity may be used. At the time of inoculation, the animals in all groups are of an age suitable for recovery of the strain. Serial passages are carried out in target animals using five groups of animals, unless there is justification to carry out more passages or unless the strain disappears from the test animal sooner. In vitro propagation may not be used to expand the passage inoculum.

The passages are carried out using animals most appropriate to the potential risk being assessed.

The initial administration is carried out using the recommended route of administration most likely to lead to reversion to virulence, using an initial inoculum containing the maximum release titre. After this, not fewer than four further serial passages through animals of the target species are undertaken. The passages are undertaken by the route of administration most likely to lead to reversion to virulence. If the properties of the strain allow sequential passage via natural spreading, this method may be used, otherwise passage of the virus is carried out and the virus that have been recovered at the final passage are tested for increase in virulence. For the first four groups, a minimum of two animals is used. The last group consists of a minimum of eight animals. At each passage, the presence of living vaccine-derived virus in the material used for passage is demonstrated. Care must be taken to avoid contamination by virus from previous passages. When the virus is not recovered from any intermediate in vivo passage, repeat the passage in ten animals using in vivo passaged material from the last passage in which the virus was recovered. The virus recovered is used as the inoculum for the next passage. If the vaccine virus is not recovered, the experiment is considered to be completed with the conclusion that the vaccine virus does not show an increase in virulence.

General clinical observations are made during the study. Animals in the last group are observed for 21 days unless otherwise justified. These observations include all relevant parameters typical for the disease that could indicate increase in virulence. Compare the clinical signs and other relevant parameters with those observed in the animals used in the test for safety of the administration of 1 dose. If the last group of animals shows no evidence of an increase in virulence, further testing is not required. Otherwise, material used for the first passage and the virus recovered at the final passage level are used in a separate experiment using at least eight animals per group, to compare directly the clinical signs and other relevant parameters. This study is carried out using the route of administration that was used for previous passages. An alternative route of administration may be used if justified.

Unless otherwise justified and authorised, the product complies with the test if no animal dies or shows signs attributable to the vaccine strain and no indication of increased virulence is observed in the animals of the last group.

- *Environmental considerations*

A risk assessment should be prepared where potential spread or risk of live vaccines to non-target species or spread by vector is considered.

- *Precautions* (*hazards*)

Modified live virus vaccines may pose a hazard to the vaccinator depending on the strain and level of attenuation of the virus. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of vaccine.

Inactivated vaccines

- *Safety test* (*of one dose and a repeated dose*)

For the purposes of gaining regulatory approval, a trial batch of inactivated vaccine should be tested for local and systemic safety by each recommended route of administration in an *in-vivo* test in eight animals of each target species. Single dose and repeat dose tests using vaccines formulated to contain the maximum permitted payload should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine.

- *Safety test in pregnant animals*

Safety at different stages of gestation should be demonstrated if the product is to be used in pregnant animals.

Carry out the test with vaccination by a recommended route using not fewer than 16 healthy animals of the same age and origin and without antibodies against RVFV: 8 in the first third of gestation and 8 in the second third.

Administer to each group a quantity of the vaccine equivalent to not less than the maximum antigen mass likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until parturition.

The test is invalid if the vaccinated animals do not seroconvert before parturition. The vaccine complies with the test if no abnormalities in the gestation or in the animals are noted. No animal shows notable signs of disease or dies from causes attributable to the vaccine.

- *Precautions* (*hazards*)

Inactivated RVFV vaccines present no danger to vaccinators, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of vaccine.

iii) *Efficacy requirements*

Vaccine efficacy is estimated in vaccinated animals directly by evaluating their resistance to live virus challenge. In general, a successful test in lamb is considered to be sufficient evidence of the quality of a vaccine to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in a species other than lamb, it may be more appropriate to test the efficacy of the vaccine in that same species. However, except for cattle, efficacy tests in other target species, such as goats or camelids have not been developed yet.

- *Immunogenicity test in young animals*

The following test is applicable to sheep. For other species, appropriate modifications could be made.

A test is carried out for each route and method of administration recommended for vaccination using in each case lambs of the minimum age to be recommended. The quantity of vaccine to be administered to each lamb for a live vaccine is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine. For inactivated vaccines, a minimum antigenic dose should be used according to the recommended vaccination schedule.

Use for the test not fewer than 16 lambs without antibodies against RVF.

For live vaccine, collect sera from the lambs before vaccination, 7 days and 14 days after vaccination and just before challenge. For inactivated vaccine, collect sera from the lambs before the first and second injection of the primo vaccination and at the time of the challenge.

Vaccinate not fewer than 8 lambs, according to the recommended schedule. Maintain not fewer than eight lambs as controls. For live vaccines, challenge each lamb after 20–22 days by an appropriate route with a virulent RVFV. In case of inactivated vaccines, challenge each lamb 14 days after completion of primo vaccination. Observe the lambs at least daily for 14 days after challenge and monitor for clinical signs and viral load by virus isolation and qRT-PCR in blood.

The test is invalid if antibodies against RVFV in the sera of the control animals indicate that there was intercurrent infection with the virus during the test.

The vaccine complies with the test if, during the observation period after challenge, in vaccinated lambs compared to controls there is a significant reduction in duration and titre of viraemia, and a notable reduction in clinical signs (if the challenge virus used produces such signs).

- *Immunogenicity test in pregnant animals*

The following test is applicable to sheep. For other species, appropriate modifications should be made, e.g. the most sensitive gestation period for challenge.

Use 16 ewes without antibodies against RVFV, randomly allocated to either the vaccine group (n = 8) or the control group (n = 8).

A test should be carried out for each of the recommended routes and methods of administration. The vaccine administered to each ewe is of minimum potency.

Vaccinate ewes free from RVFV and without antibodies against RVFV, before pregnancy, according to the recommended schedule. Use for the test not fewer than 16 pregnant ewes (eight vaccinated and eight controls). Keep all the animals as one group. Take a blood sample from non-vaccinated animals shortly before challenge. Challenge each animal between the 40^{th} and 60^{th} days of gestation. Two test models for the challenge may be performed with a sufficient quantity of virulent RVFV (observation until lambing or harvest of fetuses at 28 days). Observe the animals clinically at least daily from challenge, and either until the end of gestation or until harvest of fetuses after 28 days. If abortion occurs, examine the aborted fetus for the presence of the RVFV. If animals are observed until lambing, immediately after birth and prior to ingestion of colostrum, examine all lambs for viraemia and antibodies against RVFV. If fetuses are harvested 28 days after challenge, examine the fetuses for RVFV by suitable methods. Transplacental infection is considered to have occurred if virus is detected in fetal organs or in the blood of newborn lambs or if antibodies are detected in precolostral sera of lambs.

The test is invalid if any of the control animals have neutralising antibody before challenge. The test should show significant difference in protection and transplacental transmission between the vaccinated and control groups of animals.

iv) *Vaccines permitting a DIVA strategy* (*detection of infection in vaccinated animals*)

There is currently no DIVA strategy available for the existing RVF vaccines.

v) *Duration of immunity*

As part of the authorisation procedure the manufacturer should demonstrate the duration of immunity of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection.

vi) *Stability*

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for authorisation.

The period of validity of a batch of lyophilised RVF vaccine or a batch of liquid inactivated vaccine should not be less than 1 year.

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NB: There are OIE Reference Laboratories for Rift Valley fever
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Rift Valley fever

Annex 4

Original English
December 2012

AD HOC BRAINSTORMING GROUP ON NEW APPROACHES TO DIAGNOSIS: APPLIED GENOMICS

OIE Headquarters, Paris, 10–12 December 2012

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An *ad hoc* Brainstorming Group on New Approaches to Diagnosis: Applied Genomics was convened at the OIE Headquarters from 10 to 12 December 2012. Dr Elisabeth Erlacher-Vindel, Deputy Head of the OIE Scientific and Technical Department, welcomed the participants on behalf of Dr Bernard Vallat, Director General of the OIE. The specific task of the brainstorming Group was to advise the OIE on how it could identify the opportunities and challenges offered by high throughput sequencing (HTS) and computational genomics and harness their full potential in the context of animal health.

The Agenda and List of Participants are given at Appendices I and II, respectively.

1. Introduction

In recent years, diagnostic approaches using novel HTS, bioinformatics and computational genomics have resulted in a major transformation in the biomedical research area. Furthermore, these new technologies have set the stage for powerful new methods in the diagnosis, surveillance, control and reporting of infectious diseases.

For the scope of this report, HTS encompasses a variety of DNA sequencing methods that includes the "next generation sequencing" platforms, the newest single DNA molecule sequencers ("third generation sequencing") and nanotechnologies currently in the developmental phase. A detailed explanation of the principles and potentials of these sequencing platforms was beyond the scope of this report but would be described in detail in a White Paper prepared by Belák *et al.* (High-Throughput Sequencing in Veterinary Infection Biology and Diagnostics) for a future issue of the OIE *Scientific and Technical Review*. HTS enables the detection, identification and detailed analyses of both pathogen and host genomes on an unprecedented scale, speed and depth. Initially, the instrumentation necessary for HTS was extremely expensive and affordable only to large sequencing and research centres. However, in recent years, several new sequencing methodologies and platforms have been developed that make HTS affordable in small diagnostic laboratories and even in the field. Thus, there are a variety of potential applications that HTS offers to infectious disease diagnostics, surveillance and control.

The amount of data generated by HTS has been on an unprecedented scale. In this report the terms "bioinformatics and computational genomics" (BCG) have been used in a broad general context that includes both the general handling, retrieving and initial analyses of large data-sets, in addition to specific downstream computational analyses performed in order to investigate structure, characteristics and relationship of whole genomic sequences (either RNA or DNA). Thus, the full potential of HTS cannot be realised without bioinformatics and computational genomics and this report has used the abbreviation "HTS-BCG" to define the whole area ranging from sample collection and preparation for sequencing until downstream computational analyses.

The *ad hoc* Group stressed that in the context of animal health, several skill sets need to be available in any research/diagnostic centre wishing to exploit the full potential of HTS-BCG. Classic competencies in veterinary case management and diagnostics, laboratory information management systems, molecular biology and infection biology must be complemented with expertise in bioinformatics and computational genomics.

2. Opportunities

The Group noted that new developments in HTS-BCG offered a variety of opportunities in the context of animal health and, more specifically, in the diagnosis, surveillance and control of animal infectious diseases. Some of the key opportunities are summarised below:

- Detection, identification and characterisation of new microorganisms;
- Improved diagnosis of known diseases;
- Improved diagnosis of emerging or re-emerging diseases with known or unknown aetiology;
- Single "universal" diagnostic assays, able to identify any potential pathogen, can be developed in concert with established diagnostic approaches;
- Simultaneous and quick detection of multiple disease agents with multifactorial aetiologies;
- Increased capability to study the evolutionary dynamics of pathogens at the farm, local, national and global level;
- Deeper understanding of the epidemiology of infectious diseases and the phylogeography of infectious agents;
- Enhanced traceability of infectious diseases and modes of pathogen transmission including applications in forensic epidemiology;
- More extensive characterisation of "populations" of known pathogens (e.g. relevant minority strains, escape mutants) that in turn facilitate the design of better vaccines, antivirals etc.;
- Better links between pathogen genotype and phenotypes enabled through full genome sequence of multiple strains (including reference strains) of a single agent.

3. Challenges

The Group agreed that as with any revolutionary scientific development, HTS-BCG had also created some interesting challenges such as:

- Input parameters and software used to analyse different HTS technologies require optimisation;
- Approaches to sample preparation from the specimens biologically appropriate for veterinary investigations need to be improved and optimised;
- Systems and infrastructures for HTS data capturing, handling, archiving and access need to be optimised and standardised;
- Interpretation of HTS data in the context of animal health purposes requires careful harmonisation;
- Bioinformatic skills, not always available globally, are necessary to harness the full potential of HTS (e.g. by developing custom bioinformatics pipelines for different applications).

Other challenges that the OIE would face as HTS-BCG technologies become more widely used, perhaps even by users not traditionally or formally engaged in animal health investigations, are as follows:

- The continual detection and rapid identification of purported new infectious agents, without the rigorous evidence of their biological significance to disease, represents a challenge to OIE and animal health organisations;

- The diagnostic, surveillance and disease control issues that can be better addressed by HTS-BCG need to be identified and the opportunities understood;

- Standards for HTS platforms, including specimen collection, sample preparation, data management and analysis need to be developed and incorporated into the OIE *Manuals*;

- HTS-BCG needs to be fully validated and quality assured in order to be used as a diagnostic tool;

- Sources of OIE guidance and support (both established and new) regarding the implementation of HTS-BCG methods in the laboratory need to be identified and communicated to Member Countries;

- The technical feasibility of HTS data (including raw and metadata) being incorporated into the existing OIE World Animal Health Information System (WAHIS) must be evaluated;

- The processes to report and confirm HTS results (including the identification of putative new pathogens) need to be developed;

- Legal issues, including intellectual property rights, need to be addressed;

- The OIE must be careful not to recommend specific commercial HTS platforms in an environment where most operating systems are commonly known by their commercial names.

4. Recommendations to the OIE

HTS-BCG has been revolutionising biomedical research and is being applied in a variety of scientific contexts. Animal Health can benefit enormously from harnessing the power of these new technologies. The Group agreed that therefore, the OIE should provide the necessary guidance to successfully embrace these technologies wherever appropriate, and recommended the following:

a) OIE take a global leadership role to ensure that the full potential of HTS-BCG is applied to animal health and food safety.

b) OIE develop and adopt standards in the *Manuals* for the use of HTS-BCG.

c) OIE convene an *ad hoc* Group with experts in HTS-BCG, laboratory diagnosis, epidemiology and infectious disease investigation to:

   i) Identify the diagnostic, surveillance and disease control issues that can be addressed by HTS-BCG;

   ii) Recommend standard approaches on the harmonisation and interpretation of HTS data;

   iii) Advise OIE on best practices to follow in regard to HTS technologies; including data and metadata capture, handling and reporting and validation of the whole HTS-BCG based diagnostic method; and

   iv) Propose scientifically rigorous criteria for the identification of agents involved in pathological processes from complex metagenomic data (new or refined criteria for causality of disease).

d) OIE identify a network of HTS-BCG experts by collecting information existing within the Collaborating centres and Reference Laboratories that collectively can offer appropriate HTS-BCG expertise and training.

e) OIE consider extending the scope of the ad hoc group on “Validation” to include HTS-BCG.

f) OIE add the necessary functionality to the current WAHIS in order to include the pathogen genome sequence when available.

g) OIE assess the feasibility (utilising internal and external consultants) of extending the current WAHIS with HTS data and building standards for data archiving, management and access. OIE should take into account similar databases being developed by other international organisations.

h) OIE take legal advice on intellectual property rights issues concerning HTS data.

i) OIE Collaborating Centres, on request, provide advice and guidance on performance criteria useful to identify specific HTS commercial platforms that match the needs of individual laboratories.

5. Conclusions

In summary the Group concluded that HTS-BCG was having a major impact on the bio-medical science community and this impact would grow exponentially in the future. The cost of these technologies would continue to fall and at the same time there would be a colossal increase in the generation of scientific data.

Major challenges for the future would include data storage, structuring, management, analysis, retrieval and interpretation. There would also be a pressing need for international standards in relation to data management and validation of HTS BCG approaches to animal disease diagnosis. However, animal health could greatly benefit from harnessing the full power of these technologies.

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…/Appendices

Appendix I

AD HOC BRAINSTORMING GROUP ON NEW APPROACHES TO DIAGNOSIS: APPLIED GENOMICS

OIE Headquarters, Paris, 10–12 December 2012

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Agenda

1. Introduction
2. Opportunities
3. Challenges
4 Recommendations to the OIE
5. Conclusions

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Appendix II

AD HOC BRAINSTORMING GROUP ON NEW APPROACHES TO DIAGNOSIS: APPLIED GENOMICS

OIE Headquarters, Paris, 10–12 December 2012

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Annex 5

Original: English
January 2013

REPORT OF THE MEETING OF THE OIE *AD HOC* GROUP ON VALIDATION OF DIAGNOSTIC TESTS FOR WILDLIFE

Paris, 15 – 17 January 2013

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The second meeting of the OIE *ad hoc* Group on Validation of Diagnostic Tests for Wildlife (hereinafter the Group) was held at OIE Headquarters, Paris, France, from 15 to 17 January 2013.

1. Opening

Dr Elisabeth Erlacher-Vindel, Deputy Head of the Scientific and Technical Department, welcomed the participants of the meeting on behalf of Dr Bernard Vallat, Director General of the OIE. She provided a brief report of OIE's recent activities on diagnostic tests and wildlife and also reminded the Group of the procedure for the development of OIE Standards and Guidelines.

2. Appointment of chairperson and rapporteur

The meeting was chaired by Prof. John Fischer, and Prof. Anita L. Michel was designated as rapporteur.

3. Adoption of the agenda

Dr Fischer introduced the provisional agenda, which was subsequently adopted by the Group as agenda for the meeting. The agenda and list of participants are attached as Appendices I and II, respectively.

4. Finalisation of the Draft Standard "Principles and Methods for the Validation of Diagnostic Tests for Infectious Diseases Applicable to Wildlife"

Before starting the discussion on the draft standard, the Group was informed of the meeting, held in August 2012, of the *ad hoc* Group on Validation of Diagnostic Assays. The objective of the latter *ad hoc* Group had been to reply to the comments received from the Member Countries on the proposed updated version of the Chapter 1.1.5.of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*). Prof. Ian Gardner, who participated in this meeting, presented to the Group the outcomes of the meeting and the main changes to the Chapter that were suggested by that *ad hoc* Group.

The Group discussed the status of the document "Principles and Methods for the Validation of Diagnostic Tests for Infectious Diseases applicable to Wildlife" under development and concluded that it should preferably be adopted as an Appendix to Chapter 1.1.5. of the *Terrestrial Manual*, with the status of an OIE Standard and be approved by Member Countries.

The Group started its work on the document by addressing the following outstanding issues that were documented in the report of its first meeting:

- *Relatedness of species in relation to type of test (direct versus indirect tests)*: This was addressed by proposing new sentences in Section 3 of the standard;
- *Influence of type of test on the flow chart (parallel scenarios with modifications necessary?)*: This was addressed by proposing new sentences in Section 3 of the standard;

- *Guidance on how to take into account autolysed samples in the validation process*: the Group addressed this issue in Section 2.2.c. by recommending evaluation of effects of autolysis after provisional recognition of the assay;
- *Template for documenting the source and characteristics of reference samples*: The Group indicated that a template would not cover all situations and therefore was not appropriate. Rather than developing a template, the Group elected to amend the list in the second paragraph of the introduction of Section 2.2 by describing minimum requirements for the source and characteristics of reference samples;
- *Lack of a standard test for comparison for some diseases*: the Group considered that this issue was already addressed in Section 3 through the reference to latent class analysis;
- *"Grandfathering" tests that have been historically used successfully by reference laboratories:* The Group agreed that all tests should be validated without exceptions and that there should be no Grandfathering of tests that had been used by Reference Laboratories.

The Group added explanations and refinements in all sections of the Standard to provide the user with as much support as possible in addressing and overcoming common constraints in wildlife testing.

The Group recommended that Validation Stages 1 and 2a for field tests (rapid animal side tests) be completed by the manufacturer, prior to the commercial distribution of the kit, because this would enhance collaboration with diagnostic laboratories and potentially improve interpretation of results by end-users of the test.

The Group provided guidance on the selection of the most suitable validation pathway in the introduction of Section 3 of the Standard.

In an effort to harmonise the terminology used in this Standard with Chapter 1.1.5. of the *Terrestrial Manual*, it was agreed to re-word "provisional acceptance" at the end of Stage 2a back to "provisional recognition".

The Group added sections on Validation Stages 1, 3 and 4, which had not been discussed in detail at the last meeting owing to time constraints. Special care was taken to address aspects of reproducibility which might pose specific problems for the validation of diagnostic tests in wildlife.

The Group made recommendations regarding re-evaluation following modifications of validated tests.

The draft Standard as finalised by the Group is attached as Appendix III.

5. Other matters

After the draft Standard had been finalised, the Group made the following proposals on the draft updated version of the Chapter 1.1.5. to maintain consistency between the two documents:

Regarding the Introduction:

- to mention that all diagnostic assays (laboratory and field assays) need to be validated for the species in which they will be used;
- to add a reference to the "Principles and Methods for the Validation of Diagnostic Tests for Infectious Diseases Applicable to Wildlife";
- to highlight that the information provided in the wildlife appendix applied specifically to wildlife species but might also be useful for domestic animal test validation, for example, where the number or availability of samples was limited.

Regarding Section A. Assay Development Pathway:

- to move Paragraph 1.b) on the fitness for use into "Section *B. Assay Validation Pathway*, *4. Stage 4 – Programme implementation*" because it was of greater relevance to that section. In addition the paragraph should include considerations of other tests available for potential use in combination with the validated test.

Regarding Section B. Assay Validation Pathway:

- to move the provisional recognition currently at the end of Stage 1 of the Chapter to the beginning of Stage 2.

Additional comments:

The Group recommended that the Validation Guidelines 6 "Methods Comparability of Assays after Minor Changes in a Validated Test Method", which were under development, should also cover antibody detection tests because they were extensively used in wildlife and in domestic animals. The Group suggested a new document rather than addition to the current Validation Guidelines for ease of use.

The Group did not propose to meet further, unless required by the Biological Standards Commission.

6. Adoption of the report

The Group adopted the report prepared by the rapporteur assisted by the OIE secretariat.

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…/Appendices

Appendix I

OIE *AD HOC* GROUP ON VALIDATION OF DIAGNOSTIC TESTS FOR WILDLIFE

Paris, 15 – 17 January 2013

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Agenda

1. Opening
2. Appointment of chairperson and rapporteur
3. Adoption of the agenda
4. Finalisation of the Draft Standard on "Principles and Methods for the Validation of Diagnostic Tests for Infectious Diseases Applicable to Wildlife"
5. Other matters
6. Adoption of the report.

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Appendix II

OIE *AD HOC* GROUP ON VALIDATION OF DIAGNOSTIC TESTS FOR WILDLIFE

Paris, 15 – 17 January 2013

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Appendix III

Principles and Methods for the Validation of Diagnostic Tests for Infectious Diseases Applicable to Wildlife

1. Introduction

Diagnostic testing of wildlife for infectious diseases is becoming increasingly important as interest grows in diseases that occur in wildlife that may have an impact on wildlife populations and biodiversity, as well as on the health of humans and domestic animals. For the purposes of this standard, "wildlife" will be defined as animals belonging to one or more of the following groups:

- *Wild animals*: Those animals that do not live under human supervision or control and do not have their phenotype selected by humans.
- *Captive wild animals*: Those animals that live under human supervision or control but do not have their phenotype selected by humans.
- *Feral animals*: Those animals that do not live under human supervision or control but do have their phenotype selected by humans.

Wild animals generally are susceptible to infection with the same disease agents as domestic animals and in some cases, the tests developed and validated in other species may have utility for wildlife species. However, diagnostic testing of wildlife can be more challenging than in domestic animals for a variety of reasons, including difficulties in animal and sample accessibility, poor sample quality, poor knowledge of pathogenesis/epidemiology of the disease in wildlife, and local or international regulations limiting or prohibiting possession and/or international shipment of samples. Affordability of tests is a key consideration because wild and feral animals do not have owners who pay for testing. Hence, low cost may be a critical factor in test selection for use for a designated purpose.

Many routine diagnostic tests that have been developed and are currently used for detecting or confirming diseases in domestic animals have not been validated for wildlife. The question remains as to whether there are any essential differences in diagnostic sensitivity or specificity of these tests when they are applied to wildlife samples.

Diagnostic tests can arbitrarily be divided into two categories: direct and indirect identification techniques. Direct diagnostic test methods to identify agents include microscopic examinations, culture – commonly used to isolate bacteria, viruses, fungi and some protozoa; and molecular techniques – including polymerase chain reaction (PCR) amplification of the agent's genetic material and sequences coding for immunoproteins. Importantly, these direct agent identification diagnostic techniques should theoretically not be affected by the species of the host, i.e. domestic animal or wildlife. However, there may be some species variation in the proliferation rate or amplification of the agent, which may affect the amount and distribution of pathogens and their products in the hosts. Indirect test methods are based on the detection of an animal's cellular or antibody immune response against a pathogen. In contrast to direct methods, detection of the immune response often requires species-specific reagents, which complicates this diagnostic approach in wildlife in species that do not benefit from existing validated tests in a closely related species. Determination of the actual infection status of animals identified as infected or exposed in a serological test requires confirmation by a validated direct detection assay.

Validation of diagnostic tests for individual wildlife species presents challenges, including the accessibility of adequate sample numbers and volumes to be used in the validation process. The underlying principles and stepwise approach to the validation of a diagnostic test are outlined in the Chapter 1.1.5. of the OIE *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. The purpose of the standard described in the sections 2 and 3 of this document is to present information specifically for validation of diagnostic tests for wildlife species that will be recognised by the OIE (completion of Stages 1, 2 and 3 of the Validation Pathway). However, recognizing that completion of this process may not be necessary, or even possible, in all circumstances, guidance is provided for following the Validation Pathway to a point where the test can be provisionally recognised to provide confidence in results and for use in specific applications in a regional or national context (see section B.1.g of the Chapter 1.1.5. for more details).

Provisional recognition of an assay for wildlife species requires completion of Stage 1 (analytical characteristics) and Stage 2a (preliminary estimates of Diagnostic Sensitivity [DSe] and Diagnostic Specificity [DSp]) of the Validation Pathway. Stage 2a evaluation using a panel of positive and negative reference samples to evaluate diagnostic performance is considered essential because of the diversity of species within taxonomic families, varying host factors that may influence pathogenesis of infection, and different disease ecologies. Details about Stage 2a evaluation are provided in section 3 of this standard. Stages 2b and 3 of the pathway also need to be completed for a provisionally recognized assay to achieve full validation, through the OIE pathway, for its originally intended purpose.

2. Test validation principles

Validation is a process that determines the fitness of an assay, which has been properly developed, optimized and standardised, for an intended purpose. Ideally, the validation process of tests for wildlife should be conducted in the same way as for tests for domestic animals (presented in Chapter 1.1.5.). However, as explained above, wildlife diagnostic testing often meets difficulties, which also place limitations on the prospects for full validation. Therefore, in cases in which full validation is not feasible, the best possible alternative may be to evaluate the wildlife assay's fitness in a reduced number of reference samples. The preliminary estimates of a test's performance may provide sufficient information for government authorities to agree that a test can be provisionally recognised for testing of animals being moved or translocated or for pathogen surveillance within a country.

In many cases, pre-existing diagnostic tests validated for one species may be adapted to and evaluated in other species with minimal or no modifications. In other cases, new tests for wildlife may need to be developed. In all cases, the intended purpose(s) and application(s) of the test should be established and defined before it is developed and validated since this may impact selection of appropriate reference samples and ultimately, generalisability of the validation results.

The development of rapid and easy to perform field tests (animal-side or pen-side tests) for disease diagnosis in domestic animals has been well received by end users and these tests are becoming increasingly popular for use in wildlife. The use and interpretation of field tests is often the sole responsibility of the veterinary personnel attending to cases in the field without laboratory support. Therefore validation of these tests through Stage 2a by the manufacturer is essential to facilitate correct interpretation of the test results. Test kits used in the field rather than under laboratory conditions should be evaluated for reproducibility of results under different environmental conditions (temperature, humidity, etc.).

2.1. Fitness for purpose

A list of purposes for diagnostic testing is provided in the Chapter 1.1.5. More specifically for wildlife testing, the main purposes to develop and apply a diagnostic assay are:

1) Screening wildlife populations for the presence of infectious agents, for example:

    a) for surveillance (e.g. early detection, evaluation of trends in prevalence or incidence)
    b) to estimate prevalence of infection or exposure

2) Screening or testing vectors or environmental samples for the presence of infectious agents

3) Confirming a diagnosis of suspect or clinical cases (includes confirmation of positive results from a screening test)

4) Certifying freedom from infection or presence of the agent in individual animals or products, for

    a) movement or translocation
    b) human consumption

5) Monitoring of the geographical distribution and prevalence changes due to management interventions (including determining immune status of individual animals or populations)

6) Studying agent, host and environment factors associated with disease occurrence

2.2. Reference samples and sample quality

Reference samples should represent the target condition of interest e.g. clinically diseased, subclinically infected. Experience indicates that selection of inappropriate positive reference samples from clinically affected animals when the test will be used to detect subclinical infection results in overly optimistic estimates of sensitivity and specificity. Experimentally infected animals may be the only source of reference samples in some cases but their use should be supplemented with samples from naturally infected animals, wherever possible.

By definition, all reference samples should be well characterised in terms of the host and its source population, and the infectious agent involved. Although the same description details would be desirable for reference samples from wildlife as compared with domestic animals, the relevant information is often not available. In such cases as many details as possible should be recorded. Minimum requirements to adequately characterise a reference sample are: a) the precise host species and subspecies when possible, b) tests used for confirmation of the presence or absence of the pathogen/antibody, c) geographical location with reference to known disease free or infected areas/regions, d) the date of sample collection and e) specimen type. Wherever possible, information on sex, age category (juvenile, sub-adult, adult), absence or presence of clinical signs, and a description of the signs will add value.

a) ***Pooling of reference samples***

Ideally, reference samples should be obtained from individual animals and aliquoted into smaller volumes (weights) for subsequent testing. However, when animals of small body mass are the source of the reference samples or when very few animals are infected with the particular agent of interest, pooling of samples is acceptable to obtain a reference sample. Preferably the stage of infection of the individual animals should be known. A strongly positive sample of good quality can be diluted with the same sample matrix, for example faeces or serum, from the same host species to generate a series of samples with decreasing concentrations of the agent or products of the immune response. If certain stages of infection are not available, this should be documented.

In cases where only a limited volume of a suitable sample of good quality is available, it can be used as a reference sample in support of a well-planned set of test runs (e.g. for a repeatability study).

b) ***Negative reference samples and samples of unknown infection status***

If negative reference samples are not available to determine diagnostic specificity in terms of certain agents known to cause cross-reactivity, this should be documented.

Latent-class statistical models for estimating diagnostic sensitivity and specificity in the absence of a perfect reference standard (sometimes termed a gold standard) are appealing for validating diagnostic assays for wildlife. This approach may be particularly useful for evaluating the sensitivity of nucleic-acid detection assays compared with viral, bacterial, and parasite isolation. Latent class analysis models have inherent assumptions and require a thorough description of the source population(s), which may be difficult or impossible to obtain for free-ranging wildlife (see the Chapter 1.1.5. and Validation Guideline 5 on statistical evaluation for details).

c) ***Sample quality***

The sampling environment for wildlife is often sub-optimal and may lend itself to cross-contamination. In addition, opportunistic sampling constitutes an important aspect in screening and monitoring wildlife populations for infectious agents. This often results in the collection of samples with compromised integrity (e.g. contamination, advanced autolysis). Therefore, investigators are responsible to determine the suitability of such samples for test validation, but given the overall scarcity of samples for certain conditions or from certain host species (e.g. endangered species), great care should be taken to ensure maximum utilisation of samples of sub-optimal quality. A qualitative assessment of sample quality (e.g. good, poor, autolysed) should be recorded in databases documenting the characteristics of reference samples.

Therefore it is deemed useful and necessary to validate appropriate tests for a range of sample condition criteria such as changes in detectability over time, under different storage temperatures, during autolysis, etc. However, this step in the validation process should be conducted after the test has been provisionally recognised.

3. Test validation pathways and stages for wildlife

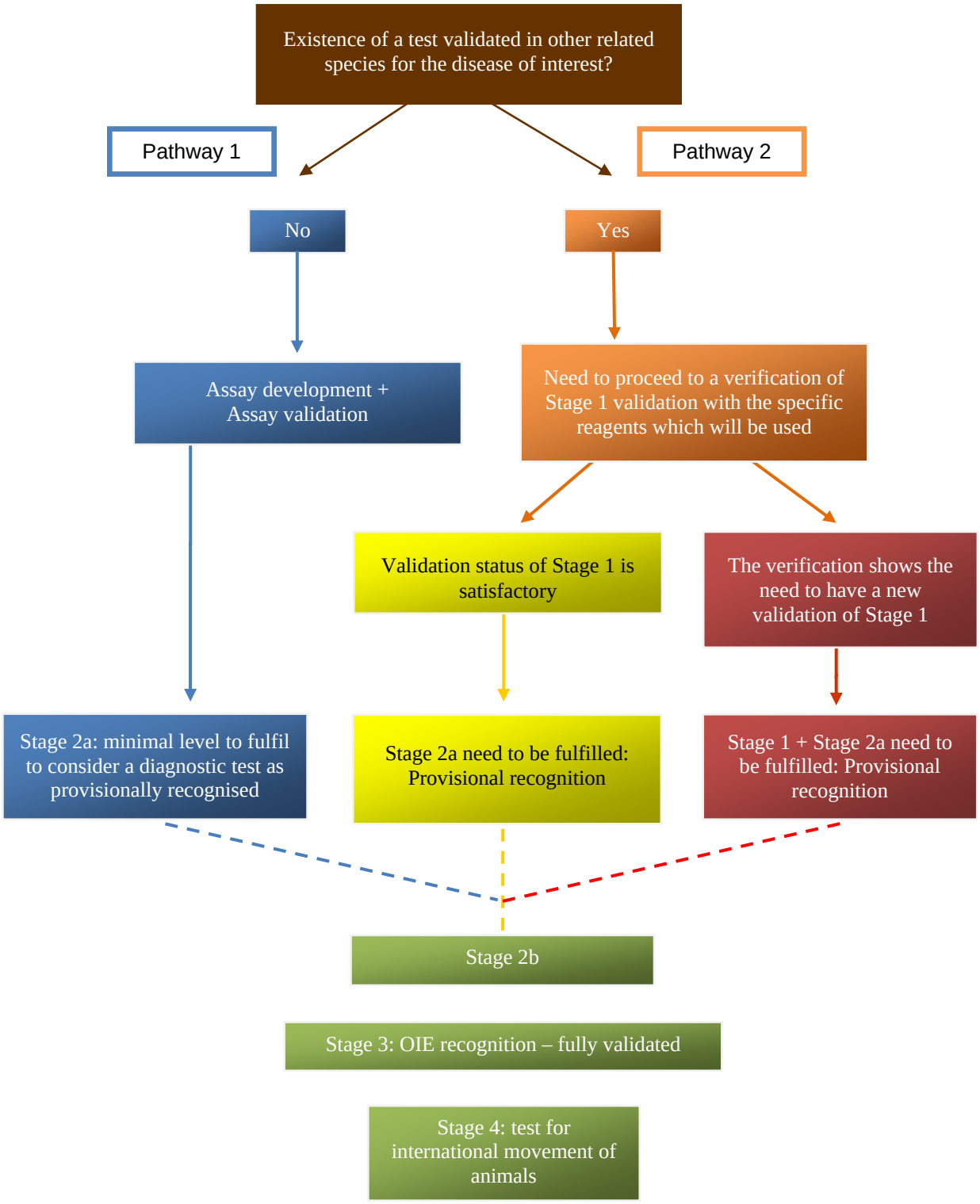
3.1. Introduction

The two scenarios, considered in this Standard, involve the lack of availability (Pathway 1) or the availability of a validated test in another related species (Pathway 2) for the same pathogen. The flowchart (Figure 1) and the Table 1 show the stages in the validation process. Corresponding requirements to meet validation criteria and estimate test performance characteristics are shown in Table 1. Taxonomic relatedness of species should be a primary consideration when choosing the applicable pathway (Pathway 1 or 2, see Table 1), particularly when indirect test methods are applied. Other criteria such as behaviour of the animals, variation in pathogen strains or ecology of the diseases should also be taken into consideration. In most cases involving wildlife, Pathway 1 is appropriate because of the lack of validated tests in closely related species. When Pathway 2 is chosen, justification for its use should be provided by documenting the existence of a validated test.

Table 1: Validation Pathway: Steps required to meet validation criteria described in the Chapter 1.1.5. and to estimate test characteristics. Requirements in the different Stages need to be fulfilled with an acceptable outcome

Validation pathway Chapter 1.1.5.	**Pathway 1:** ***No validated test in related species***	**Pathway 2:** ***Validated test in related species***
Stage 1	**Stage-1 verified in new target species**	**Stage-1 verified in new target species**
Analytical specificity	Yes	Yes
Analytical sensitivity	Yes	Yes
Repeatability	Yes	No
Reproducibility (preliminary)	Yes	No
Stage 2	**Stage 2a (Provisional recognition)**	**Stage 2a (Provisional recognition)**
Diagnostic sensitivity	Yes (minimum of 30 positive reference samples)	Yes (minimum of 10 positive reference samples)
Diagnostic specificity	Yes (minimum of 30 negative reference samples)	Yes (minimum of 10 negative reference samples)
Cut-off determination	Yes (total of 60 samples)	Yes (total of 20 samples)
Reference sample description	Yes	Yes
	Stage 2b	**Stage 2b**
Diagnostic sensitivity	Yes	Yes
Diagnostic specificity	Yes	Yes
Cut-off determination	Yes	Yes
Reference sample description	Yes	Yes
Stage 3	**Stage 3**	**Stage 3**
Reproducibility	Yes	Yes
Repeatability	Yes	Yes
Stage 4	**Stage 4**	**Stage 4**
Predictive values (populations)	Yes	Yes

Figure 1: Flowchart of pathways and stages of test validation in wildlife when a previously validated test exists or does not exist



3.2. Additional considerations with regards to the general validation pathway as described in the Chapter 1.1.5.

a) Stage 1 – Estimation of analytical characteristics

Estimation of analytical characteristics should follow recommendations set out in the Chapter 1.1.5.

Depending of the existence of diagnostic test methods validated according to the OIE pathway for another species, the characteristics requiring validation will differ. If there is no validated diagnostic test method, all the characteristics of the Stage 1 should be evaluated (Pathway 1). If there is already a validated diagnostic test method, repeatability and reproducibility (preliminary) will not need to be reassessed until Stage 3 (Pathway 2).

Because the diversity of cross reacting organisms is often unknown, evaluation of analytical specificity can be more difficult than in domestic animals.

b) Stage 2 – Estimation of diagnostic characteristics

Estimation of diagnostic characteristics should follow recommendations set out in the Chapter 1.1.5.

For purposes of wildlife testing, Stage 2 is proposed to be divided into Stages 2a and Stage 2b. Stage 2a needs to be completed for "provisional recognition", as previously described. In Stage 2a, the assumption is made that the pathway based on an existing validated test in a related domestic animal disease (compared with no validated test) was based on at least 10 positive reference samples and 10 negative reference samples and estimates of DSe and DSp are similar, if not identical, in the 2 species. These samples provide "credit" towards the reduced sample size (Pathway 1 vs. Pathway 2 in Table 1). Selection of the pathway with reduced sample size (Pathway 2) should be justified based on sample size and evidence of comparability (e.g. same test cut-off value and reagents) provided in peer-reviewed publications.

The selected sample size for completion of Stage 2 (Stage 2b) should be based on expected values for diagnostic sensitivity (DSe) and specificity (DSp), the desired confidence level and error margin as shown in Table 1 in Chapter 1.1.5. For example for an expected DSe or DSp of 90%, a sample size of 138 is required to yield an error margin of 5% with 95% confidence (see right panel of Table 1 in the Chapter 1.1.5.). However, it is acknowledged that this number of truly positive and negative reference samples may be difficult to obtain for some wildlife species and could potentially only be achieved when data from multiple testing laboratories using the same test in a standardized way are combined over time. Consequently, the initial number of samples tested may be lower than recommended numbers in Table 1of the Chapter 1.1.5.

If numbers of reference samples (positive and negative) are lower than numbers in Table 1 of the Chapter 1.1.5., the calculated error margins on estimates (typically represented as 95% confidence intervals) for DSe and DSp, respectively, will be wider than those on which the table was based. Consequently, small sample sizes increase the uncertainty in test performance characteristics. Use of reference samples that are representative of the target condition is critical to achieve an unbiased (and practically useful) estimate of DSe and DSp that will stand up to scrutiny over time. For example, samples should be obtained from subclinically infected animals if the test undergoing validation is to be used in apparently healthy animals. Obtaining and using representative samples of the target condition is therefore of greater importance than sample size.

The net effect of a lower sample size is greater uncertainty in estimates unless the prior information about the DSe and DSp in the related species is formally incorporated through a Bayesian analysis. Table 2 shows the effect of use of 140 or fewer known positive samples when the DSe estimate (90%) was calculated after field samples were collected and tested.

Table 2: Approximate error margins and 95% confidence intervals for diagnostic sensitivity (DSe) for decreasing numbers of positive reference samples

No. positive reference samples	No. positive	DSe (%)	Approximate error margin on estimate of DSe	95% exact binomial confidence interval for DSe (%)
140	126	90	± 0.05	83.8 – 94.4
100	90	90	± 0.06	82.4 – 95.1
60	54	90	± 0.08	79.5 – 96.2
30	27	90	± 0.10	73.5 – 97.9
10	9	90	± 0.18	55.5 – 99.7

Calculations for 95% confidence intervals for DSp are affected similarly by the number of negative reference samples that are used.

c) Stage 3 – Reproducibility

Generally the recommendations set out in Chapter 1.1.5 for the evaluation of the reproducibility are applicable, meaning that a minimum of 20 samples should be tested by 3 different laboratories in 3 distinct regions or countries. In cases where a particular test in wildlife is performed by very few laboratories or countries, or where the exchange of wildlife samples across international borders may be regulated by the CITES[1] agreement, the evaluation of the reproducibility may be postponed to a later stage when the test has been adopted by sufficient laboratories or the relevant CITES permit could be obtained.

d) Stage 4 Interpretation of test results

Interpretation of test results (predictive values) in all species is dependent on knowledge of prevalence in the targeted population. This is difficult to know a priori in most free-ranging wildlife populations and even in captive populations where the population size is known, there may be substantial variations in prevalence among populations. Hence, it may be unreasonable to expect that predictive value calculations can be made with certainty in most wildlife populations. In the limited situations in which true prevalence can be determined, the predictive values of test results in these populations should not be extrapolated to other populations.

e) Monitoring assay performance after initial validation: modifications and enhancements – considerations for changes in the assay

Modifications in the protocol of the validated test may have an important impact on the performance of the test. Examples include: the use of body fluids collected from live or dead animals (such as ascites fluid, lung extract or pleural fluid) for an antibody detection test validated for serum; a change in the nature or source of reagents, and a change in cycling parameters of a PCR protocol.

Any modification will therefore require a limited reevaluation of the analytical characteristics (Stage 1). If the characteristics are comparable with the initial protocol, with no significant change, the validation process can continue from the point where the change occurred. If the analytical characteristics change significantly, Stages 1 and 2a should be repeated in full.

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1 CITES: Convention on International Trade in Endangered Species of Wild Fauna and Flora

Annex 6

Original: English
September 2012

MEETING OF THE OIE *AD HOC* GROUP ON VACCINE QUALITY RELATED TO CLASSICAL SWINE FEVER (CSF)

Paris, 4–6 September 2012

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A meeting of the OIE *ad hoc* Group on Vaccine Quality related to Classical Swine Fever (CSF) was held at the OIE Headquarters, Paris from 4 to 6 September 2012. This meeting had been scheduled to revise Section C of Chapter 2.8.3 Classical Swine Fever of the *Terrestrial Manual*.

1. Opening and purpose of the meeting

Dr Kazuaki Miyagishima, Deputy Director General, welcomed the participants of the meeting on behalf of Dr Bernard Vallat, the Director General of the OIE. By way of introduction he explained that there was a new template to be used to re-write the section on vaccine requirements. He also informed that there was now an extended cycle for the member country comments on manual chapter revisions, which meant that it would be advisable to draft of the revision available for the Biological Standards Commission meeting in September so as to have a final draft before the end of year.

2. Adoption of agenda and appointment of chairman and a rapporteur

The meeting was chaired by Dr Michel Lombard and Dr Ralph Woodland was designated as rapporteur for this meeting. Dr Lombard introduced the provisional agenda, which was subsequently adopted by the Group. the adopted agenda and list of participants are attached as Appendices I and II, respectively.

3. Update and revision of Section C (Requirements for Vaccines and Diagnostic Biologicals) of Chapter 2.8.3 Classical Swine Fever of the *Terrestrial Manual*

The Group was requested to follow the newly adapted template for Section C, which is attached at Appendix III. The Group reviewed a draft text for a section on the background to vaccination against CSF that had been prepared by Dr Lombard based on the text previously adopted for the respective chapters on Foot and mouth misease (FMD) and Rabies. Further amendments proposed by Dr Sandra Blome, Dr Frank Koenen, Dr María T. Frías Lepoureau and Dr Catherine Charreyre were taken into account before the Group finally agreed on a text for the background section.

The Group noted that the general introduction to the chapter on classical swine fever (chapter 2.8.3, section A) was focussed mainly on virology and a brief description of the disease but contained no discussion on the impact of the disease, various epidemiological situations and the types of control measures that could be used.

Recommendation: The Group recommended that consideration is given to revising section A of the Chapter to give a more comprehensive introduction to the different aspects of the disease and its control.

The Group discussed how to present the information on production of the different types of vaccines (e.g. live attenuated, recombinant, oral, baculovirus derived subunit, marker vaccines) taking into account the standard template that had been developed. Although presentation of all types in section C would have some benefits by reducing replication of information it was considered that it might be clearer to present the information in separate sections. It was therefore agreed to go forward to outline the requirements for live, oral and subunit vaccines separately

With regard to the production of modified live virus (MLV) vaccines, it was agreed that tissue vaccines prepared in live animals were no longer respecting OIE animal welfare principles. For animal welfare reasons it was considered important to include a statement that CSF vaccines should no longer be manufactured using live animals for virus growth. Draft texts for the sections on biological characteristics of the master seed, quality criteria and validation as vaccine strain were agreed.

It was noted that some of the information included in the section on validation as a vaccine in the previous version of the chapter would be covered in the section on *Requirements for Authorisation/Registration/ Licensing* in the new template.

With regard to the *method of manufacture* it was agreed to modify the previous text to be less specific and prescriptive so as to leave the option of using alternative methods if justified. A statement that the cell cultures used should comply with the requirements of chapter 1.1.6 was included.

It was also agreed that all ingredients used for manufacture should comply with the requirements of chapter 1.1.6.

It was noted that the quality control tests carried out during production would depend on the manufacturing process but would include virus titration and sterility of the bulk antigen. An appropriate statement was agreed.

Requirements for finished product testing were also agreed. The preferred method for demonstrating *batch potency* was agreed to be an *in-vitro* virus titration, but there was concern that in some areas sufficient correlation between virus titre and vaccine potency may not be established. It was therefore decided to retain the option to carry out an efficacy test on each batch in such cases.

With regard to the *requirements for authorisation/registration/licensing* the Group agreed to include the same statement regarding the manufacturing process that had been used in the FMD chapter to avoid repeating information that was included in the previous section. It was however, decided to add additional comments that each batch should be manufactured from the same master seed virus (MSV).

The text of the European Pharmacopoeia (Ph. Eur.) was used as a basis for the *safety requirements* for registration. However the numbers of animals to be used were changed from 10 to 8 in line with VICH[1] GL44 (this change is also being made to the Ph. Eur. text). Also the period of observation of the animals was changed from 21 days to at least 14 days as proposed for the Ph. Eur. monograph.

With regard to the test for *safety in pregnant sows*, one particular point that was discussed was the stage of gestation to be used: 25–35 days as in the previous OIE text was considered to be too early and 80 days (as allowed by the Ph. Eur. text) was considered to be too late. The Group agreed that a range from 55 to 70 days of gestation would be most suitable. Furthermore, it was considered important to include a test for virus in the blood of piglets before ingestion of colostrum to ensure that potential vaccine strains do not persistently infect piglets.

With regard to a test for *non-transmissibility* of vaccine virus it was agreed to adopt the text of the Ph. Eur. without change, noting that this involved the use of fewer animals than the previous OIE text and that serological monitoring of in-contact controls should be used instead of challenge as in the previous OIE text.

The text in the Ph. Eur. monograph was also used as a basis for the *reversion-to-virulence* section, with amendments to bring in line with VICH GL41. It was not considered appropriate to allow for the possibility of any increase in virulence during passage in the case of CSF and therefore it was decided not to include the respective text from the VICH guideline.

[1] VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Products

The Group decided to base the section on vaccine efficacy on the text in the Ph. Eur. monograph with minor modifications. Advice on the dilutions of vaccine to be used was included as in the previous OIE text. There was also discussion about whether to include a requirement for induction of sterile immunity in addition to protection against clinical disease but it was decided not to include this.

The Group then proceeded to discuss the manufacture and *requirements for subunit marker* (DIVA: differentiating infection in vaccinated animals) vaccines. Text for the manufacturing procedure and quality control testing regime for the E2 subunit vaccine was modified from that previously agreed for live vaccines to take account of the different nature of this vaccine (inactivated, adjuvanted) and were based on the methods used for the only vaccine of this type that is currently authorised.

It was agreed that the safety testing required for authorisation of subunit vaccines should be those generally applicable for inactivated vaccines and the text finally agreed was adapted from that previously agreed for FMD vaccines.

The Group discussed at length the efficacy requirements for the subunit (DIVA) vaccines and concluded that in principle the same efficacy requirements as those adopted for live vaccines should apply, while recognising that in certain circumstances the use of an inactivated vaccine with DIVA capability might be considered more important than compliance with all of the efficacy standards, e.g. the ability to prevent viral shedding and transplacental infection.

The Group continued to discuss requirements for oral vaccines. Manufacturing and testing are similar to those for the injectable MLV vaccines except for the final formulation and consequential differences in final product testing. In addition, requirements for the bait formulation need to be presented for registration purposes. Safety and efficacy testing for registration purposes need to be carried out by oral administration of the liquid vaccine (not formulated into baits) and in addition efficacy of the vaccine in the baits needs to be confirmed. Demonstration of safety for the environment and to non-target species was also considered to be especially important for oral bait vaccines.

4. Other matters

There was no other business discussed under this item.

5. Finalisation and adoption of the draft report

The Group welcomed Dr Woodland's offer to edit the first draft of the revised Section C. Further comments from Group members would be invited and discussed by email exchange. This edited version would be attached to this report.

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…/Appendices

Appendix I

MEETING OF THE OIE *AD HOC* GROUP ON VACCINE QUALITY RELATED TO CLASSICAL SWINE FEVER (CSF)

Paris, 4–6 September 2012

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Agenda

1. Opening
2. Appointment of chairperson and rapporteur
3. Review and updating of Section C *Requirements for vaccines* of Chapter 2.8.3. Classical swine fever of the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*
4. Other matters
5. Adoption of the draft report.

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Appendix II

MEETING OF THE OIE *AD HOC* GROUP ON VACCINE QUALITY RELATED TO CLASSICAL SWINE FEVER (CSF)

Paris, 4–6 September 2012

List of participants

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Appendix III

CHAPTER 2.8.3.

CLASSICAL SWINE FEVER
(hog cholera)

SUMMARY

Classical swine fever (*CSF*), *also known as hog cholera, is a contagious viral disease of pigs. The causative virus is a member of the genus* Pestivirus *of the family* Flaviviridae*, and is closely related to the viruses of bovine viral diarrhoea and Border disease. There is only one serotype of CSF virus* (*CSFV*).

The disease may run an acute, subacute, chronic, late onset, or inapparent course, depending on a variety of viral and host factors of which the age of the animals, the virulence of the virus and the time of infection (*pre- or post-natal*) *are of greatest importance. Adult pigs usually display less severe signs of disease than young animals and stand a better chance of survival. In pregnant sows, the virus may cross the placental barrier and reach the fetuses.* In-utero *infection with strains of the virus of moderate or low virulence can result in what is referred to as the 'carrier sow' syndrome followed by prenatal or early post-natal death, the birth of diseased piglets or an apparently 'healthy' but persistently infected litter. An outbreak of CSF has serious consequences for trade in pigs and pig products.*

The highly variable clinical picture of CSF ~~often~~ precludes a diagnosis on clinical and pathological grounds alone. Laboratory methods are therefore essential for an unambiguous diagnosis. Detection of virus or viral nucleic acid in anticoagulated whole blood and of antibodies in serum are the methods of choice for diagnosing CSF in live pigs, whereas detection of virus, viral nucleic acid or antigen in organ samples is most suitable when the pig is dead.

Identification of the agent: *~~The direct fluorescent antibody test (FAT) on cryostat sections of organs from affected pigs can be used for the detection of CSF antigen. A panel of monoclonal antibodies (MAbs) is used to determine whether the fluorescence is due to CSF or non-CSF~~* Pestivirus *~~antigens. For the detection of CSF genome, polymerase chain reaction (PCR) is commonly used.~~ The isolation of CSFV should be attempted in pig kidney* (*PK-15, SK-6*) *cell lines, or other ~~suitable~~ CSFV permissive cell lines. The cultures, which are generated from stocks that are* Pestivirus *free (and preferably free of other contaminants, e.g. mycoplasmas, porcine circovirus), are examined for virus growth by immunofluorescence or immunoperoxidase staining; positive isolates are further characterised by ~~the use of MAbs and by~~ partial genetic sequencing or, if that method is not available, by the use of monoclonal antibodies (MAbs). Reverse transcription polymerase chain reaction protocols for the identification of CSFV nucleic acid have now gained international acceptance and are being used in ~~several~~ many laboratories, both for detection of the agent and differentiation from ~~ruminant~~ other pestiviruses. The direct fluorescent antibody test (FAT) on cryostat sections of organs from affected pigs can be used for the detection of CSF antigen. A panel of MAbs is used to determine whether the fluorescence is caused by CSF or non-CSF* Pestivirus *antigens.* Antigen-capture *enzyme-linked immunosorbent assays* (*ELISAs*) are also useful for herd screening, but must not be used on a single animal basis.

Serological tests: *Detection of virus-specific antibodies is particularly useful in herds suspected of having been infected at least 21 days previously with CSFV. Serological methods are also valuable for monitoring and for prevalence studies, and are essential if a country wishes to be internationally recognised as being free from the disease in the absence of vaccination.*

As CSFV cross-reactive antibodies against ~~ruminant~~ other pestiviruses are occasionally observed in ~~breeding~~ pigs, screening tests have to be followed by confirmatory tests that are CSFV-specific. Certain ELISAs are relatively CSFV-specific, but the definitive method of choice for differentiation is the comparative neutralisation test, which compares the ~~level~~ neutralising titre of antibodies to different Pestivirus *~~species~~ isolates.*

Requirements for vaccines ~~and diagnostic biologicals~~: *Vaccines against CSF are based on live virus that has been attenuated by passage through cell cultures or through a suitable host species that is not of the family* Suidae*. The production of these modified live virus* (*MLV*) *vaccines is based on a seed-lot system that has been validated with respect to virus identity, sterility, purity, safety, nontransmissibility, stability and immunogenicity.* If CSFV is used in the production of vaccine or in challenge studies, the facility should meet the OIE requirements for Containment Group 4 pathogens.

Effective inactivated, conventional whole virus vaccines are not available. In contrast to conventional MLV vaccines, new generation MLV ~~Subunit~~ 'marker vaccines' ~~are now available, which~~ capable of ~~in contrast to MLV vaccines,~~ inducing antibodies that can be distinguished from antibodies induced by field virus ~~using~~ when an ~~accompanying~~ appropriate companion discriminatory diagnostic test is used. may soon become available. The presently registered subunit 'marker vaccine' is based on the major envelope glycoprotein (*E2-subunit*) *of CSFV, and is produced in insect cells using recombinant DNA technology.*

A. INTRODUCTION

The viruses that cause classical swine fever (CSF), bovine viral diarrhoea (BVD) and Border disease (BD) are members of the family *Flaviviridae*, genus *Pestivirus*, and are closely related to each other, both antigenically and structurally. Clinical signs and lesions seen at post-mortem examination in pigs affected with CSF are highly variable due to both viral and host factors. Furthermore, (congenital) infections with ruminant pestiviruses in pigs ~~can~~ occasionally give rise to a clinical disease that is indistinguishable from CSF (Terpstra & Wensvoort, 1988; Vannier & Carnero, 1985; Wensvoort & Terpstra, 1988).

CSF affects the immune system, a main characteristic being generalised leukopenia, which can often be detected before the onset of fever. Immunosuppression may lead to concurrent infections, which can mask the clinical picture.

~~Spread of disease in all age groups, accompanied by~~ Pyrexia, huddling, inappetance, dullness, weakness, conjunctivitis and constipation followed by diarrhoea ~~and an unsteady gait~~ are the prevailing signs of disease in all age groups. In addition, animals may display a staggering gait, ataxia or convulsions. Several days after the onset of clinical signs, the ears, abdomen and inner thighs may especially show petechial haemorrhages or a purple discoloration. Animals with acute disease die within 1~~–3~~–4 weeks. Sudden death in the absence of clinical illness is not symptomatic of CSF.

Under certain circumstances related to the animals' age and condition, as well as to the virus strain involved, subacute or chronic clinical illness may develop, which can be protracted for ~~2–4~~ several weeks or even months. Chronic illness leads to a stunting of growth, anorexia, intermittent pyrexia and diarrhoea.

Congenital persistent infections may go undetected for months and may be confined to only a few piglets in the herd or may affect larger numbers. The clinical signs are nonspecific: wasting in the absence of pyrexia. Chronic and persistent infections always lead to the death of the animal. Herd mortality rates may be slightly above the expected level. ~~CSF affects the immune system, a main characteristic being generalised leukopenia, which can often be detected before the onset of fever. Immunosuppression may lead to concurrent infections.~~

In acute cases, gross pathological lesions might be inconspicuous or absent. In typical cases, the lymph nodes are swollen and marbled red, and haemorrhages occur on serosal and mucosal membranes of the intestinal organs. Splenic infarctions may occur. In subacute and chronic cases, necrotic or 'button' ulcers may be observed in the mucosa of the gastrointestinal tract, epiglottis and larynx, in addition to the above lesions.

Histopathological findings are not pathognomonic. Lesions may include parenchymatous degeneration of lymphatic tissue, cellular proliferation of vascular interstitial tissue, and a nonsuppurative meningo-encephalomyelitis, with or without vascular cuffing.

A useful critique of diagnostics and vaccination for CSF, from an authoritative source, has recently been published (Blome *et al.*, 2006), which, as well as general guidance, also provides sources of information on validation and scientific opinion on the applicability of certain commercial products in these areas.

B. DIAGNOSTIC TECHNIQUES

The variability of the clinical signs and post-mortem lesions does not provide firm evidence for unequivocal diagnosis. Other viral diseases, such as African swine fever, porcine dermatitis and nephropathy syndrome (PDNS), and post-weaning multisystemic wasting syndrome (PMWS), thrombocytopenic purpura and various septicaemic conditions including, amongst others, salmonellosis (especially caused by *Salmonella choleraesuis*), erysipelas, pasteurellosis, actinobacillosis (caused by *Actinobacillus suis*) and *Haemophilus parasuis* infections may be confused with acute CSF. In fact, these bacteria often cause concurrent infections, and isolating these pathogens may obscure the real cause of disease, the CSF virus (CSFV). Similarly concurrent PDNS can lead to oversight of an underlying CSF infection.

A tentative diagnosis based on clinical signs and post-mortem lesions must therefore be confirmed by laboratory investigations. As pyrexia is one of the first signs of CSF and is accompanied by viraemia (Depner *et al*., 1994), detection of virus or viral nucleic acid in whole blood, collected in heparin or ethylene diamine tetra-acetic acid (EDTA), or in tissues, collected from ~~a few~~ febrile animals, is the method of choice for detecting infected herds at an early stage. This is all the more necessary in view of the serious consequences of an outbreak of CSF for trade in pigs and pig products.

Laboratory methods for diagnosis of CSF are aimed at detection of the virus, viral nucleic acid or viral antigens, or detection of specific antibodies. Targeted and risk-based sampling should be performed, random sampling only being applied in cases where no clinical signs of disease are present. To increase the sensitivity of detection of virus, viral antigen or nucleic acid, clinically diseased animals and febrile animals should primarily be sampled. For the detection of antibodies, animals that have recovered from disease or animals that have been in contact with infected or diseased animals should be primarily targeted.

For a correct interpretation of the test results, the inspecting veterinarian should pay particular attention to the simultaneous and clustered occurrence of two or more of the prevailing signs of disease listed above. In suspected primary cases an initial positive test result needs to be confirmed using a second test method. ~~Random sampling is unsuitable for CSF diagnosis. Additionally, whole blood samples for virus detection and reverse-transcription polymerase chain reaction (RT-PCR) analyses can be collected from a larger group of pigs.~~

CSF is subject to official control and the virus has a high risk of spread from the laboratory, consequently, a risk analysis should be carried out to determine the level of biosecurity needed for the diagnosis and characterisation of the virus. The facility should meet the requirements for the appropriate Containment Group as determined by the risk assessment and as outlined in Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.* Countries lacking access to such a specialised national or regional laboratory should send specimens to an OIE Reference Laboratory.

Antibodies develop in the third week of illness and persist in the surviving animal for years or even life (except for chronic cases). Samples for antibody detection are collected in ordinary (non-heparinised) tubes from convalescent pigs and from contact herds ~~when ≥more than 3 weeks have elapsed since the suspected contact with a confirmed outbreak took place~~. All methods and protocols need to be validated in the respective laboratory and the laboratory has to prove that it is capable of performing the tests it uses for diagnostic purposes with satisfactory results. If feasible and possible, validation should be done according to OIE validation criteria.

1. Identification of the agent

a) Immunological methods

- **Fluorescent antibody test**

The fluorescent antibody test (FAT) is a rapid test that can be used to detect CSFV antigen in cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum. Tissues should be collected from several (febrile and/or diseased) animals (Bouma *et al*., 2001) and transported without preservatives under cool conditions, but not frozen. Cryostat sections are stained directly with anti-CSF immunoglobulin conjugated to a fluorescence marker such as fluorescein isothiocyanate (FITC) or indirectly using a secondary fluorescent conjugate and examined by fluorescence microscopy. During the first stage of the infection, tonsillar tissue is the most suitable, as this is the first to become affected by the virus irrespective of the route of infection (Ressang, 1973). In subacute and chronic cases, the ileum is frequently positive and occasionally may be the only tissue to display fluorescence.

A negative FAT result does not completely rule out CSF infection. When suspicion of CSF continues, further samples should be obtained or attempts made at reverse transcription polymerase chain reaction (RT-PCR) or virus isolation in cell culture. In some cases during the terminal stage of disease, neutralising antibodies can mask a positive reaction ~~(e.g. pig kidney [PK-15]) or another cell line of pig origin that is as sensitive and known to be free from *Pestivirus* contamination~~.

There is a relatively high risk of false (positive and negative) results when FAT is used by laboratories not thoroughly acquainted with the method. Thus FAT should only be used by laboratories that have experience of using the technique, perform the technique on a routine basis and have had training in interpreting the fluorescence.

- **Test procedure**

Include positive and negative control sections in each series of organ samples to be examined. In indirect labelling, an infected control section should also be included, which is treated without incubation of the first antibody. The control sections can be prepared in advance and stored after acetone fixation for 2–3 years at –70°C until use.

i) Cut out a piece of tonsil, spleen, kidney and ileum of approximately 1 × 1 × 0.5 cm, and mount it with a cryo-embedding compound or distilled water on a cryostat table.

ii) Freeze the piece of organ on to the cryostat table. The freezing temperature should be –15 to –20°C. Shock-freezing of the tissue in n-Heptan cooled with liquid N_2 is ideal.

iii) Cut sections not more than 4–8 µm thick and mount these on to ~~10 × 32 mm~~ grease-free cover-slips. It is helpful to mark these cover-slips by one cut-off corner~~. All sections~~ and to ~~are~~ mount them with this corner in the same position (e.g. top right).

iv) Prepare several cover slips for each tissue sample.

v) Dry for 20 minutes at room temperature.

vi) After drying, fix the mounted sections for 10 minutes ~~at room temperature~~ in acetone (analytical grade) at –20°C or air-dry for 20 minutes at 37°C.

vii) Immerse the sections briefly in phosphate buffered saline (PBS), remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in a humid incubation chamber. ~~humidified with a small volume of water placed in the bottom of the chamber.~~

viii) Dispense the anti-CSF immunoglobulin at working dilution (dilution in PBS) on to the entire section and incubate in a dark, closed chamber for 30 minutes at 37°C. Check afterwards that the conjugate solutions have not evaporated and that the tissues have not dried out.

If a secondary FITC conjugate is required, wash the section five times for 2 minutes each in PBS at room temperature, then add the FITC conjugate at working dilution and incubate as previously described.

ix) Wash the sections five times for 2 minutes (or three times for 5 minutes) each in PBS at room temperature.

x) Immerse the section briefly in A. bidest (solvent).

xi) If necessary, counterstain in Evans Blue for 30 seconds.

xii) Remove the remaining ~~PBS~~ fluid by touching the cover-slip against tissue paper and mount the cover-slip (with the section between cover-slip and slide) with mounting buffer on to a microscope slide.

xiii) Remove excess mounting fluid with tissue paper and examine the sections for fluorescence using a UV microscope.

A CSF-positive section shows brilliant ~~green~~ fluorescence in the cytoplasm of infected cells. In the tonsils, fluorescence in the epithelial lining of the crypts is particularly evident. In kidney sections, fluorescence is most abundant in the proximal and distal tubules of the renal cortex and the collecting ducts in the medulla. In the ileum, fluorescence is most prominent in the epithelial cells of the Lieberkühn glands, whereas in the spleen reactivity is more diffuse, with concentrations of lymphoid cells in the periarterial lymphoid sheath (PALS).

~~The FAT involves~~ It is recommended to ~~the~~ use ~~of an~~ anti-CSFV gamma~~-immuno~~globulins prepared from polyclonal ~~antibody to CSFV that will not distinguish between the antigens of different pestiviruses. Conjugates used for the FAT on cryostat sections or inoculated cell cultures should be prepared from anti-CSFV gamma-globulins raised in specific pathogen free pigs.~~ antibodies against CSFV raised in specific pathogen free pigs. This ensures that no minor variant viruses will be missed, but has the disadvantage that the test will not distinguish between the antigens of different pestiviruses. Thus, pigs infected with other pestiviruses can yield a positive result. To differentiate CSFV from other pestivirues, especially in CSFV-free areas, duplicate samples from FAT-positive samples should be examined using monoclonal antibodies (MAbs) that can distinguish between CSFV and other pestiviruses (especially BVDV and BDV). Alternatively, confirmatory diagnosis should await results of RT-PCR (followed by genetic typing) or virus isolation in cell culture with subsequent typing by MAbs.

Strains of modified live virus (MLV) vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Pigs vaccinated with MLV strains may yield a positive FAT for 2 weeks after vaccination (Ogawa *et al*., 1973; Terpstra, 1978). RT-PCR followed by nucleic acid sequencing of the RT-PCR amplicon allows differentiation between field isolates and vaccine strains of CSFV.

The working dilution of the conjugates (at least 1/30) should combine a maximum brilliance with a minimum of background. The test should only be performed on samples from freshly dead animals, as autolysis and bacterial contamination can often result in high background staining.

~~Strains of modified live virus (MLV) vaccine multiply mainly in the regional lymph nodes and in the crypt epithelium of the tonsils. Pigs vaccinated with MLV strains may yield a positive FAT for 2 weeks after vaccination (Ogawa *et al*., 1973; Terpstra, 1978). Rabbit inoculation is used to differentiate between lapinised and field strains of CSFV. In contrast to field strains, lapinised strains given intravenously cause a febrile reaction and induce an immune response in rabbits. As nucleic acid sequencing has become available and more reliable, animal inoculation is no longer necessary to differentiate between field strains and vaccine strains of CSFV.~~

~~Pigs infected with ruminant pestiviruses can give false-positive FAT reactions. Congenital infections with ruminant Pestiviruses can cause clinical signs and pathological lesions indistinguishable from those in chronic CSF (Terpstra & Wensvoort, 1988b; Vannier & Carnero, 1985; Wensvoort & Terpstra, 1988). Infections by CSFV or ruminant pestiviruses can be differentiated by testing sera from the dam and litter mates, or from other contacts of an FAT-positive piglet, for neutralising antibodies to each virus. If the virus was isolated, or viral nucleic acid can be detected, using RT-PCR, subsequent sequencing provides a rapid and accurate tool to distinguish ruminant pestiviruses from CSFV. Another method of differentiating these viruses is by the inoculation of seronegative piglets with a suspension of suspect material, followed at least 4 weeks by virus neutralisation (VN) tests on their sera for the respective antibodies. However, VN tests may take several days, and animal inoculation methods take several weeks.~~

- **Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies**

The use of a panel of three MAbs that are either horseradish peroxidase (HRPO) or ~~FITC~~ conjugated to a fluorescence marker, or used in conjunction with an anti-mouse conjugate and capable of specifically detecting all field strains of CSFV, vaccine strains of CSFV and ~~ruminant~~ other pestiviruses, respectively, would allow an unambiguous differentiation between field and vaccine strains of CSFV on the one hand, and between CSFV and other pestiviruses on the other (Edwards *et al*., 1991; Wensvoort *et al*., 1986; 1989b). A prerequisite is that the MAb against CSFV recognises all field strains and that the anti-vaccine MAb recognises all vaccine strains used in the country. *No single MAb selectively reacts with all other ~~ruminant~~ pestiviruses* (Edwards *et al*., 1991). The use of an MAb to differentiate a CSF vaccine strain can be omitted in nonvaccination areas. A polyclonal anti-CSF immunoglobulin conjugated to HRPO serves as a positive control. Caution should be exercised when using evidence of a single MAb as sole confirmation of an isolate as CSF.

Positive and negative control sections need to be included in each series of organ samples to be examined. In the case of indirect labelling, an infected control section, which is treated without incubation of the first antibody, should also be included. The control sections can be prepared in advance and stored after acetone fixation for 2–3 years at –70°C until use.

- **Test procedure**

i) Cut eight or more cryostat sections (4–8 µm) of the FAT-positive tonsil, or another positive organ if the tonsil is not available (as described above for the FAT method).

ii) Place ~~Fix~~ the sections on to ~~flying~~ cover-slips, allow to dry for 20 minutes at room temperature and fix for 10 minutes in acetone (analytical grade) at –20°C and allow to air dry.

iii) Prepare working dilutions of the respective MAb-peroxidase conjugates in PBS + 0.01% Tween 80 + 5% horse serum, pH 7.6. (FITC–MAb can be used as well as unconjugated MAb provided that a secondary conjugate is used.)

iv) ~~After rinsing with PBS,~~ Immerse the sections briefly in PBS, remove excess fluid with tissue paper and place them (cut off corner top right) on a frame in a humid incubation chamber.

v) Overlay two sections each with the working dilution of the respective monoclonal conjugates, and two sections with the working dilution of the polyclonal conjugate (controls).

vi) Incubate in a dark, closed chamber for 30 minutes at 37°C. Check afterwards that the solutions have not evaporated and that the tissues have not dried out. ~~Incubate for 1 hour at 37°C in a humid chamber.~~

vii) Wash the sections six times for 10 seconds each in PBS at room temperature.

viii) Stain the sections with freshly prepared chromogen–substrate solution* for 5–15 minutes at room temperature.

ix) Rinse the sections in 0.05 M sodium acetate, pH 5.0, in distilled water and mount them on microscope slides.

x) Examine sections with a light microscope. Dark red staining of the cytoplasm ~~of the epithelial cells lining the tonsillar crypts~~ indicates recognition of the virus isolate by the respective conjugate, and is considered to be positive.

xi) Interpretation of the test:

Polyclonal antibody	Monoclonal antibody specific for			Interpretation
	CSF strain	CSF vaccine strain	BVD/BD strain	
+	+	–	–	CSF field strain
+	+	+	–	CSF vaccine strain
+	–	–	+	BVD/BD strain
+	–	–	–	Other non-CSF *Pestivirus*[†]

[†]The existence of novel strains of CSF should always be considered and any isolate from cases where CSF is still suspected should be sent to an OIE Reference Laboratory.

- **Antigen-capture assay**

For rapid diagnosis of CSF in live pigs, antigen-capture enzyme-linked immunosorbent assays (ELISAs) have been developed for screening herds suspected of having been recently infected. The ELISAs are of the double-antibody sandwich type, using monoclonal and/or polyclonal antibodies against a variety of viral proteins in either serum, the blood leukocyte fraction or anticoagulated whole blood; in addition, some test kits can be used to test clarified tissue homogenates (Depner *et al*., 1995) or serum. The technique is relatively simple to perform, does not require tissue culture facilities, is suitable for automation and can provide results within half a day. The disadvantage of being less sensitive than virus isolation, especially in adult pigs and mild or subclinical cases, may be compensated by testing all pigs of the suspect herd showing pyrexia or clinical signs of disease. However, the lowered specificity of these tests should also be taken into consideration.

The test is not suitable for the diagnosis of CSF in a single animal, but should only be used on herd level!

In any primary case, positive results must be confirmed using another test (i.e. virus isolation, RT-PCR or FAT).

b) Isolation of virus

Isolation of virus in cell cultures is a more sensitive but slower method for diagnosis of CSF than immunofluorescence on frozen sections. Organ preparations, leukocyte preparations, or whole blood samples can be used. Isolation is best performed in rapidly dividing PK-15 cells seeded on to cover-slips simultaneously with a 2% suspension of the tonsil in growth medium. Other pig cell lines may be used, but should be demonstrably at least as sensitive as PK-15 cells for isolation of CSFV and must be free of pestiviruses and pestivirus antibodies. It is generally advantageous to use more than one porcine cell line for inoculation, to enhance the chances of a positive result. As growth of the virus does not cause a cytopathic effect, its presence must be demonstrated by an immunostaining method, which may be carried out after one or two virus passages. This can be done by examining the cultures ~~are examined~~ for fluorescent foci by FAT after 24–72 hours or by immunoperoxidase staining after ~~4–5~~ 3–4 days' incubation ~~are fixed for immunoperoxidase staining~~. NB: Positive and negative controls always need to be included.

The tonsil is the most suitable organ for virus isolation from pigs that died or were killed for diagnostic purposes. Alternatively or in addition, spleen, kidney, ileum or lymph nodes can also be used.

* **Chromogen–substrate solution**

A. Stock solution of chromogen: 0.4% 3-amino-9-ethyl carbazole; N,N-dimethyl-formamide (1 ml).
Caution **TOXIC compound**. Both chemicals are carcinogens and irritants to eyes, skin and respiratory tract.
B. 0.05 M sodium acetate, pH 5.0; 19 ml (sterile filtered through a membrane).
C. Stock solution of substrate (30% hydrogen peroxide).

Keep stock solutions A and C at 4°C in the dark and solution B at room temperature. Stock solution A can be kept at 4°C for at least 6 months and solution C for 1 year. Immediately before use, dilute 1 ml of solution A in 19 ml of solution B. Then add 10 µl of stock solution C. Mix well and stain the sections.

Fetal bovine serum (FBS) used in any diagnostic assay always needs to be free of pestiviruses and pestivirus antibodies. It might not be sufficient to rely on manufacturers' declarations and for this reason it is recommended that each lot of FBS be tested for the presence of pestiviruses and pestivirus antibodies prior to its use in diagnostic assays.

A detailed procedure for virus isolation is as follows:

i) Prepare a 100-fold strength glutamine–antibiotic stock solution: dissolve glutamine (2.92 g) in 50 ml distilled water (solution A) and sterilise by filtration. Dissolve each of the following antibiotics in 5–10 ml sterile distilled water: penicillin (10^6 International Units [IU]); streptomycin (1 g); mycostatin (5×10^5 U); polymixin B (15×10^4 U); and kanamycin (1 g). Pool these solutions (solution B). Mix aseptically solutions A and B, make up to 100 ml with sterile distilled water, and store in 5 ml aliquots at –20°C. Exact antibiotic constitution is not critical, provided sterility is achieved and cells are not affected.

ii) Cut 1–2 g of tissue (organ sample of approx. 1 cm^3) into small pieces and, using a mortar and pestle or other device, grind in a small amount of cell culture medium with sterile sand into a homogeneous paste. Alternatively, use an appropriate crushing machine or automatic homogenisator at 4°C. (Attention: high speeds can heat the sample and affect the virus!)

iii) Make a 20% (w/v) suspension by adding Hanks' balanced salts solution (BSS) or Hanks' minimal essential medium (MEM); 1 ml of the glutamine–antibiotic stock is added for each 10 ml of suspension. This mixture is held at room temperature for 1 hour.

iv) Centrifuge at 1000 or 2500 ***g*** for 15 minutes. The supernatant is used for inoculation of cell cultures. A 1/100 dilution can be processed in parallel in case of cytotoxic effects. Sterile filtration can be performed, if considered necessary using syringe filters (0.45 µm followed by 0.22 µm).

v) A PK-15 monolayer is trypsinised, the cell suspension is centrifuged at 160 ***g*** for 10 minutes. The supernatant is discarded and the pellet is resuspended to contain approximately 2×10^6 cells/ml in growth medium (Eagle's MEM with Earle's salts; 5% fetal bovine serum free of ruminant pestiviruses and pestivirus antibodies; and 0.2 ml of the glutamine–antibiotic stock solution per 10 ml cell suspension). As a guide, one 75 cm^2 flask will give approximately 50 ml of cell suspension at the appropriate concentration. It usually contains about 8.5×10^6 cells.

Alternatively a protocol without centrifugation can be performed:

Growth medium is removed from a PK-15 monolayer and cells are washed once or twice with 5 ml of adjusted trypsin/versen (ATV) solution (5 ml ATV for a 250 ml flask). ATV is removed and replaced with fresh ATV (2 ml ATV for a 250 ml flask). The flask is incubated at 37°C for 15 minutes or until cells are detached. It is then filled up with cell culture medium containing 5% fetal bovine serum (8 ml medium for a 250 ml flask) and the cells are resuspended.

vi) Either:

Suspension inoculation: mix nine parts of cell suspension (from step v) and one part of supernatant fluid (from step iv) and inoculate 1.0–1.5 ml into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks or plates. Three tubes are inoculated with 1.0–1.5 ml of cell suspension alone as controls. After completion of the sample inoculations, three tubes are inoculated with CSFV as positive controls. Careful precautions must be taken to avoid cross-contamination with this known positive virus suspension. Negative cultures should also be prepared. Incubate at 37°C.

Or:

Pre-formed monolayer inoculation: for each tissue, inoculate 1.0–1.5 ml of cell suspension (prepared as in step v) into 6–8 Leighton tubes with cover-slips or other appropriate cell culture flasks or plates. Incubate at 37°C for a minimum of 4 hours and a maximum of 36 hours (until 50–80% confluency is reached). Then drain the medium and inoculate 0.2 ml of supernatant fluid (from step iv), incubate for 1 to 2 hours at 37°C, rinse once with PBSM (PBS without Ca/Mg), overlay with 1 ml of growth medium and incubate at 37°C.

vii) At 1, 2 and 3 days after inoculation, two cultures, together with a positive and negative control culture are washed twice for 5 minutes each in Hanks' BSS, Hanks' MEM or PBS and fixed ~~with cold~~. Cell fixation is performed by 100% acetone (analytical grade) for 5 minutes for cell cultures grown on glass surfaces. ~~for 10 minutes, and stained with a direct anti-CSFV conjugate at its appropriate working dilution or indirectly, as described in Section B.1.a.~~

~~If the 2% tonsil suspension proves to be toxic for the cells, then the test should be repeated using a higher dilution or another organ. Use of the method employing pre-formed monolayers (above) will help to avoid such.~~

~~viii) After washing in PBS three times for 5 minutes each, the cover-slip cultures are mounted in 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examined for fluorescent foci~~

viii) After fixation staining with a direct or indirect anti-CSFV conjugate at its appropriate working dilution is performed as described in Section B.1.a. After washing in PBS three times for 5 minutes each, the cover-slip cultures are mounted in 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examined for fluorescent foci.

Instead of Leighton tubes, 6-well plates with cover-slips can be used. Alternatively, cultures growing on flat-bottomed microtitre plates or M24-plates can also be used for virus isolation. In such case, plates are fixed and stained as described later for the neutralising peroxidase-linked assay (NPLA; Section B.2.a).

ix) If the 2% tonsil suspension proves to be toxic for the cells, then the test should be repeated using a higher dilution or another organ. Use of the method employing pre-formed monolayers (above) will help to avoid such.

Whole blood (heparin or EDTA treated) from clinically diseased pigs is a suitable sample for early CSF diagnosis. The leukocyte fraction or other components may be used, but for reasons of ~~sensitivity~~ simplicity the use of whole blood is more practical and therefore preferred (De Smit *et al*., 1994). The procedure is as follows:

i) Freeze a sample of whole blood at –20°C and thaw in a waterbath at 37°C in order to lyse the cells.

ii) Inoculate 300 µl haemolysed blood on to a PK-15 monolayer grown to approximately ~~75~~ 50–80% confluence* in an M24-plate or Leighton tubes with cover slips, and allow adsorption for 1–2 hours at 37°C. Duplicate cultures of each sample should always be prepared.

ii) Remove inoculum, wash the monolayer once with Hanks' BSS or Hanks' MEM or PBSM, and add fresh growth medium.

iv) After a further incubation period of 3–4 days at 37°C in a CO_2 incubator, the plates are washed, fixed and stained, as described later for the NPLA, using in each step a volume of 300 µl to compensate for the larger cell surface.

Note: This method is less sensitive than conventional virus isolation for the detection of acute CSF.

To improve the sensitivity virus isolation can be performed over two passages:

i) 200–300 µl of organ preparation or blood lysate (see above) is inoculated on a cell culture tube. Always duplicate cultures should be prepared.

ii) Cell cultures are incubated for 37°C for 1–2 hours, and washed twice with PBSM.

iii) The cultures are incubated for 72 hours at 37°C in a CO_2 incubator. EMEM with 10% FBS is the ideal medium for virus growth. Simultaneous inoculation is possible if the sample is fresh and a cytotoxic effect is unlikely.

iv) The cell culture tubes or plates are frozen at –80°C for at least 1 hour and then thawed at room temperature.

v) When using cell culture tubes, the tubes are centrifuged for 10 minutes at 778 ***g***.

vi) 200–300 µl of the supernatant is incubated for 1–2 hours on a well of a multi-dish plate or Leighton tube as described above.

vii) The cell culture tubes or plates are washed with PBSM, refilled with cell culture medium and incubated for 72–96 hours in a CO_2 incubator.

viii) Cells are fixed and stained as described in Section B.2.1.a.

In case a slow-growing isolate is suspected, a second passage in a culture tube can be done, leading to a third passage in a culture dish.

Positive and negative controls must always be included and processed in the same way.

- **Reverse-transcription polymerase chain reaction**

Many methods for RT-PCR have been described and are still being developed (McGoldrick *et al*., 1998). ~~This internationally accepted method is rapid and more sensitive than antigen capture ELISAs or virus isolation making it particularly suited to preclinical diagnosis.~~ By using RT-PCR techniques, infected animals can be detected early during the incubation period and also for a longer period of time in cases where the pigs recover. RT-PCR detects viral nucleic acid only and positive results may be obtained in cases where virus isolation or

* Simultaneous inoculation, though slightly more sensitive, is less suitable as the anticoagulant may interfere with the adhesion of cells on to the surface.

other techniques yield negative results. It is therefore more sensitive than other techniques (such as antigen-capture ELISA, and FAT).

Owing to its speed and sensitivity, RT-PCR is a suitable approach for screening and confirmation of suspected cases of disease and is now accepted by a number of countries and the European Union (EU) (European Commission, 2002). It is however, important to bear in mind that false positive results due to laboratory contamination can occur as well as false negative results due to inhibitors contained in the sample. Therefore, any positive results from primary outbreaks must always be confirmed by other tests. It is mandatory to include an adequate number of positive and negative controls in each run; it is also strongly recommended that internal controls be included. See Chapter 1.1.5 *Principles and methods of validation of diagnostic assays for infectious diseases*, for further details on PCR techniques. ~~The test can be applied to individual or pooled blood samples as well as solid organs and has been used successfully to control outbreaks.~~

The test can be applied to blood and serum samples as well as solid organs and cell culture supernatants and has been used successfully in case of outbreaks.

Isolation of RNA is a critical step in RT-PCR analysis. RNA integrity is at the highest risk prior to and after extraction. Thus, treatment of samples prior to RNA extraction and storage of isolated RNA have to be carefully considered as they will influence the quality of the yielded RNA and the test result. Different methods for RNA isolation have been described and a wide variety of extraction kits is commercially available. It has to be ensured that RNA isolation is also validated in the laboratory.

Several conventional and real-time PCR protocols have been described (Hoffmann *et al*., 2005; McGoldrick *et al*., 1998; Paton *et al*., 2000b; Risatti *et al*., 2003; 2005) and a suitable protocol may be obtained from the literature or from the OIE Reference Laboratories for CSF (see Table given in Part 4 of this *Terrestrial Manual*). Evaluation of RT-PCR results can either be performed by agarose gel electrophoresis (standard RT-PCR) or by real-time techniques (RT-qPCR). Any RT-PCR protocol to be used must be thoroughly validated in each individual laboratory to show that the method is fit for purpose, before it can be used for diagnosis in that laboratory. Any RT-PCR protocol used must prove to be at least as sensitive as virus isolation. The RT-qPCR protocol described by Hoffmann *et al*. (2005) is widely used and the method yielded consistent results in international interlaboratory comparison testing.

In principle, pooling of samples is possible, but sensitivity must be shown to be at least as high as the sensitivity of virus isolation performed on single samples. Pooling must be properly validated prior to its use in each individual laboratory.

Quality control is an essential issue in PCR diagnosis and prevention of laboratory contamination is crucial.

- **Molecular epidemiology and genetic typing**

The molecular epidemiology of CSF is based on the comparison of genetic differences between virus isolates. RT-PCR amplification of CSFV RNA followed by nucleotide sequencing is the simplest way of obtaining the sequence data to make these comparisons. A number of different regions of the CSFV genome may be targeted for molecular epidemiological studies (Paton *et al*., 2000a). Two regions have been extensively studied and provide large sets of sequence data with which new isolates can be compared. One of these regions lies within the 5'-~~noncoding~~ nontranslated region (~~5'NCR~~ 5'NTR) of the genome (150 nucleotides) and the other lies within the E2 major glycoprotein gene (190 nucleotides). In brief, the method used involves extracting virus RNA from clinical samples or ~~infected PK-15~~ cell cultures infected with low passage CSFV, performing RT-PCR to amplify one or both targets within the ~~5'NCR~~ 5'NTR or the E2 gene, and then determining the nucleotide sequence of the products and comparing with stored sequence information held in the databases (Greiser-Wilke *et al.*, 1998; Lowings *et al*., 1996). A database of these sequences is available from the OIE Reference Laboratory for CSF (Hannover, Germany). Recent findings on analysing other ~~ruminant~~ pestivirus sequences highlight the need for analysis of multiple regions in order to accurately type strains by this method (Becher *et al.,* 2003; Hurtado *et al*., 2003; Liu *et al.,* 2009; Vilcek *et al.,* 2010). CSFV isolates from primary outbreaks should be sent to an OIE Reference Laboratory for investigation of molecular epidemiology. The receiving laboratory should be contacted first and an import permit should be obtained prior to dispatch.

2. Serological tests

~~Detection of virus-specific antibodies is particularly useful on premises suspected of having infections with CSF strains of low virulence.~~ Due to the immunosuppressive effect of CSFV, antibodies cannot be detected with certainty until at least 21 days post-infection. Serological investigations aimed at detecting residual foci of infection, especially in breeding herds, may also be useful in a terminal phase of CSF eradication. In addition, antibody titres provide valuable epidemiological information and may give hints to the entry route of the virus.

As the incidence of infection with ruminant pestiviruses may be high, particularly in breeding stock, only tests that will discriminate between CSF and BVD/BD antibodies are useful. Virus neutralisation (VN) and the ELISA using MAbs satisfy the requirements for sensitivity, but positive results should be confirmed by comparative VN testing.

Neutralisation tests are performed in cell cultures using a constant-virus/varying-serum method. As CSFV is noncytopathic, any non-neutralised virus must be detected, after multiplication, by an indicator system. The NPLA (Terpstra *et al*., 1984) and the fluorescent antibody virus neutralisation (FAVN) test (Liess & Prager, 1976) are the most commonly used techniques. Both tests can be carried out in microtitre plates. The NPLA system is now favoured, being easier to read and having the advantage that the results can be determined by use of an inverted light microscope, though a crude assessment of titre can be made with the naked eye.

a) Neutralising peroxidase-linked assay (a prescribed test for international trade)

The NPLA is carried out in flat-bottomed microtitre plates. Sera can first be inactivated for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution). For surveillance schemes within a country, a screening dilution of 1/10 (1/20 final dilution) may suffice. Appropriate controls to ensure specificity and sensitivity of reactions are incorporated into each test.

- **Test procedure**

i) Dispense dilutions of serum in growth medium (Eagle's MEM, 5% fetal bovine serum and antibiotics) in 50 µl volumes into duplicate wells of a microtitre plate. The fetal bovine serum must be free from both BVDV and antibodies to it. A third well ~~may~~ should be included for each sample. This well contains serum and not virus and is used as a serum control (for cytotoxicity and/or nonspecific staining).

ii) Add 50 µl of virus suspension to the wells, diluted in growth medium to contain approximately 100 $TCID_{50}$/50 µl, and mix the contents on a microplate shaker for 20 seconds. A commonly used virus is CSF Alfort 187 (genotype 1.1). Although there is only one CSFV serotype, it is recommended that recent genotypes or field virus isolates circulating in the country or relevant other countries should also be used as antibody titres can vary depending on the virus genotype used in the assay.

iii) Incubate the plates in a CO_2 incubator in a moist chamber for 1 hour at 37°C.

iv) Add to all wells 50 µl of growth medium containing 2×10^5 PK-15 cells/ml.

v) Titrate the virus dilution and incubate together with the NT plate.

vi) Allow the cells to grow at 37°C in 5% CO_2 to become confluent, usually within 3–4 days.

vii) Discard the growth medium and rinse the plates once in 0.15 M NaCl or PBS.

viii) Drain the plates by blotting on a towel.

ix) The cell monolayers may be fixed in one of several ways:

- The plates are incubated for 45 minutes at 37°C, and then for at least a further 45 minutes at –20°C. The plates are removed from the freezer, the wells are filled with 100 µl 4% paraformaldehyde in PBS and reincubated for 5–10 minutes at room temperature. The paraformaldehyde is discarded and the plates are rinsed with 0.15 M NaCl; or
- The plates are incubated at 70–80°C for ~~1–2~~ 2–3 hours; or
- The plates are fixed with 80% acetone and incubating at 70–80°C for 1 hour; or
- The plates are fixed in 20% acetone in PBS for 10 minutes followed by thorough drying at 25–30°C for 4 hours. (This can be done quickly with the aid of a hair-dryer – after 3–5 minutes complete dryness is obtained as observed by the whitish colour of the cell monolayer.) or
- Plates are washed with ice-cold 99.9% ethanol and fixed with 99.9% ethanol for 45 minutes at 4°C. (Staining should be done immediately.)

x) Add to each well (of a 96-well plate) 50 µl of a hyperimmune porcine CSF antiserum or monoclonal antibody, diluted in 0.5 M NaCl containing 1% Tween 80 + 0.1% sodium azide, pH 7.6. Incubate at 37°C for at least 15 minutes. The working dilution of the antiserum should be determined by prior titration: i.e. a serum with an NPLA titre of 1/30,000 could be used at 1/100.

xi) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6 or PBS containing Tween and once in distilled water.

xii) Add to each well 50 µl of an anti-porcine or anti-murine (as appropriate) IgG-HRPO conjugate, diluted to its working concentration in 0.5 M NaCl with 1% Tween 80, pH 7.6, and then incubate for at least ~~10~~ 15 minutes at 37°C.

xiii) Wash the plates five times in 0.15 M NaCl containing 1% Tween 80, pH 7.6.

xiv) Add 50 µl of chromogen–substrate solution to each well and stain for 15–30 minutes at room temperature. This solution is described in Section B.1.a Immunoperoxidase procedure for differentiation of pestiviruses by monoclonal antibodies.

xv) Discard the supernatant and wash once with 1/3 PBS/distilled water.

xvi) The test is read visually. Infected cell sheets are completely or partially stained reddish brown in the cytoplasm. The monolayer should be examined by low-power microscopy to determine the end-point of the titration. The cytoplasm of infected cells is stained dark red. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that prevents virus growth in 50% of two replicate wells. The titre can be calculated according to the equation of Karber (1931)

xvii) The following controls are included in the test: cell control, positive serum and back titration of test virus. Back titration of the virus dilution added to the NT plate is to be carried out. It should cover a range of 4 log dilutions and acts as an internal quality control. The back-titration should confirm that virus has been used at a concentration of between 30 and 300 $TCID_{50}$/50 µl. A CSF antibody positive reference serum with known titre needs to be included. If the reference serum does not give the expected result and the back titration is out of the limit, the test has to be repeated. Reference sera should be monitored over time using internal laboratory tracking charts.

xviii) The back-titration titre is calculated using the method described by Reed & Muench (1938). The number of virus infected wells at each dilution is recorded. The cumulative infected, cumulative not infected, ratio of infected to infected + not infected and percent infected at each dilution are then determined. An example calculation is illustrated below.

Log_{10} virus dilution	**No. infected wells**	**Cumulative infected wells (A)**	**Cumulative not infected wells (B)**	**Ratio of A/A+B**	**Per cent infected**
0	6/6	12	0	12/12	100%
–1	5/6	6	1	6/7	86%
–2	1/6	1	6	1/7	27%
–3	0/6	0	12	0/12	0%

Using the information from the above chart the 50% endpoint is calculated using the formula

Proportionate distance = (% Positive above 50%) – 50%/

(%Positive above 50%) – (% Positive below 50%)

= 86%–50%/86%–27%

= 0.61

Therefore the 50% endpoint for the above example = (–1) + [0.6 × (-1)] = –1.61 = $10^{1.61}$ = 40 $TCID_{50}$/50 µl.

The calculation is performed according to Kärber.

~~The back-titration should confirm that virus has been used at a concentration of between 30 and 300 $TCID_{50}$/50 µl.~~

NOTE: The incubation times given above are for guidance only. Longer incubation times, with reagent dilutions optimised to such times, may be used, in order to conserve reagents.

b) Fluorescent antibody virus neutralisation test (a prescribed test for international trade)

Leighton tube method:

i) Seed a suspension of PK-15 cells at a concentration of 2 × 10^5 cells/ml into Leighton tubes with a cover-slip.

ii) Incubate the cultures for 1–2 days at 37°C until they reach 70–80% confluency.

iii) Inactivate the sera for 30 minutes at 56°C. For international trade purposes, it is best to test with an initial serum dilution of 1/5 (1/10 final dilution).

iv) Incubate the ~~equal volumes of~~ diluted serum ~~and~~ with an equal volume of a virus suspension that contains 200 $TCID_{50}$ (50% tissue culture infective dose) ~~per 0.1 ml~~ of CSFV for 1–2 hours at 37°C. Thus a constant amount of CSFV of 100 $TCID_{50}$ is used for each reaction well.

v) Remove the cover-slips from the Leighton tubes, wash briefly in serum-free medium, overlay the cell sheet with the serum/virus mixture (from step iv) and incubate for 1 hour at 37°C in a humid atmosphere.

vi) Place the cover-slip in a clean Leighton tube and incubate the cultures in maintenance medium for 2 more days.

vii) Remove the cover-slips from the Leighton tubes, wash the monolayers twice for 5 minutes each in PBS, pH 7.2, fix in pure acetone for 10 minutes and stain with the working dilution of the conjugate for 30 minutes at 37°C before washing.

viii) Mount the cover-slips on grease-free microscope slides with 90% carbonate/bicarbonate buffered glycerol, pH>8.0, and examine for fluorescence.

When the FAVN test is performed in microtitre plates, the procedure for the NPLA (see below) can be followed up to step viii. The plates are then stained with the working dilution of the conjugate for 30 minutes at 37°C and examined for fluorescence. Note: When detecting fluorescence, microplates are best examined from above, using a long focal-length objective and an inverted microscope.

~~Occasionally~~Sera from pigs infected with BVDV or BDV may show cross neutralising antibody titres that react in the FAVN or NPLA ~~at low dilution~~ as if the pigs were infected with CSFV. The extent of cross-reactivity depends on the strain of ruminant pestivirus involved and the interval between infection and time of sampling (Wensvoort *et al*., 1989a). ~~The usually high antibody levels reached after exposure to CSF infection, including strains of low virulence, allow the use of comparatively high initial dilutions in NPLA tests for CSF antibody, thus avoiding most, but not all, cross-reactions (Terpstra *et al*., 1984; Terpstra & Wensvoort, 1988a).~~ In case of continued doubt, comparative tests using a strain of CSFV, a strain of BVDV and a strain of BDV, that are representative for the country or region, have proven useful. Comparative neutralisation tests are end-point titrations in which the same series of twofold dilutions of the suspected serum sample is tested in duplicate against 100 $TCID_{50}$ of each selected virus strain. The comparative tests are performed according to the protocols described for the FAVN or NPLA; the cell lines used must be suitable for BVDV and BDV. Neutralisation titres are expressed as the reciprocal of the highest serum dilution that prevents virus growth in 50% of two replicate wells. A three-fold difference or more between end-points of two titrations should be considered decisive for an infection by the virus species yielding the highest titre. It may be necessary to use different strains of the same genotype, and/or to test several pigs from an infected herd to obtain a definitive result.

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

Competitive, blocking and indirect techniques may be used on any suitable support and a number have been described (e.g. Colijn *et al*., 1997; Have, 1987; Leforban *et al*., 1990; Moser *et al*., 1996; Wensvoort *et al*., 1988a). The tests used should minimise cross-reactions with BVDV, BDV and other pestiviruses. However, the test system must ensure identification of all CSF infections, and at all stages of the immune response to infection. Most commercially available test systems are based on the immunodominant glycoprotein E2.

Antigen: The antigen should be derived from or correspond to viral proteins of one of the recommended CSFV strains. Cells used to prepare antigen must be free from any other *Pestivirus* infection.

Antisera: Polyclonal antisera for competitive or blocking assays should be raised in pigs or rabbits by infection with one of the recommended CSFV strains or with the lapinised C strain. MAbs should be directed against or correspond to an immunodominant viral protein of CSFV. Indirect assays should use an anti-porcine immunoglobulin reagent that detects both IgG and IgM.

The sensitivity of the ELISA should be high enough to score positive any serum from convalescent animals, i.e. at least 21 days post–inoculation that reacts in the neutralisation test. The ELISA may only be used with serum or plasma samples derived from individual pigs. If the ELISA procedure used is not CSF-specific, then positive samples should be further examined by differential tests to distinguish between CSF and other pestiviruses.

The complex-trapping blocking ELISA (Colijn *et al*., 1997) is a one-step method and is suitable for use in automated ELISA systems e.g. robots. The sera are tested undiluted. The test is fast and easy to perform, and detects antibodies against low virulence strains of CSFV at an early stage after infection. As the MAbs are specific for CSFV, the complex-trapping blocking ELISA will only rarely detect antibodies against BVDV, although BDV antibodies can be more problematic. Positive sera are retested for confirmation by the NPLA or FAVN.

~~Recently, a novel ELISA has been described that uses fused protein derived from viral peptides (Lin *et al*., 2005). The test claims to provide greater sensitivity and earlier detection of antibody than is obtained by conventional ELISAs, but, at this time, its reactivity with antibody induced by diverse strains of CSF is not known.~~

The use of marker vaccines depends on a discriminatory test able to distinguish between vaccinated and naturally infected animals. In combination with the E2 subunit vaccine, ELISAs detecting antibodies directed against the E^{rns} protein can be used as discriminatory tests. However, commercially available E^{rns}-specific ELISAs are less sensitive and specific than conventional CSF E2 antibody ELISAs. It is recommended to use the discriminatory tests on a herd basis and not for diagnostic analysis on samples of single animals (Anonymous, 2003; Floegel-Niesmann, 2001; Schroeder *et al*., 2012, in print).

More information on commercial kits for diagnosis can be obtained from the OIE Reference Laboratories. Although commercial test kits have been thoroughly validated before licensing, each lab must perform batch control with selected (positive and negative) reference sera prior to use.

C. REQUIREMENTS FOR VACCINES ~~AND DIAGNOSTIC BIOLOGICALS~~

This Section has been extensively revised and updated. Although some portions of the existing text have been incorporated, new text and deleted text have not been marked, in the interests of clarity.

1. Background

CSF has severe clinical and socio-economic consequences for pig production worldwide. The control of the disease is usually a national responsibility, and in many countries vaccination is carried out as part of a national control programme under the auspices of the veterinary authority.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements. Varying additional requirements relating to quality, safety and efficacy will apply in particular countries or regions in order for manufacturers to obtain an authorisation or licence for a veterinary vaccine.

Wherever CSFV is handled, the appropriate biosecurity procedures and practices should be used. The CSF vaccine production facility should meet the requirements for containment outlined in chapter 1.1.3.

Modified live vaccines (MLVs) based on several attenuated virus strains (e.g. C-strain, Thiverval, PAV-250, GPE-, K-strain) are most widely used, and many of them have proven to be both safe and efficacious. In addition, E2 subunit vaccines produced in baculovirus systems are available. Inactivated whole virus vaccines are presently not available.

Information regarding these vaccines can be found in review publications (Blome *et al.,* 2006; Ganges *et al.,* 2008; Geiser-Wilke & Moennig, 2004; Uttenthal *et al*., 2010; Vannie *et al.,* 2007; Van Oirschot, 2003)

New generations of marker vaccines are also being developed and one is in the process of licensing (Reimann *et al*, 2004).

Different strategies are available to differentiate infected from vaccinated animals (DIVA) by serological methods (e.g. ELISA) or genome detection methods (e.g. RT-PCR). A opinion published by the European Food Safety Authority (EFSA, 2008) demonstrated that the combination of a vaccine that uses the C-strain with RT-PCR to detect viral genome in slaughtered animals can be successfully used in a vaccination-to-live strategy (Li *et al.,* 2007; Zhao *et al*, 2008).

CSF vaccines are used in different epidemiological settings and situations. Most countries free of the disease have adopted a control strategy without prophylactic vaccination but established legal provisions for emergency vaccination scenarios. In endemic situations, vaccination is mainly used to lower the impact of the disease or as a first step in an eradication programme. During epidemic incidents in otherwise free areas, emergency vaccination can be an additional tool to control and eradicate the disease.

Moreover, oral vaccination of affected wild boar populations may be considered. These different scenarios and the different systems of pig production may require different vaccine characteristics or may influence the focus of requirements.

Limited antigen and vaccine banks exist and can be used for emergency situations.

The optimal CSF vaccine should have the following general characteristics: short- and long-term safety for target and non-target species (especially for oral vaccines), stability, rapid induction of a stable, preferably life-long immunity, efficacy against all strains and types of field viruses, full clinical protection and protection against carrier states, prevention of horizontal and vertical transmission. Furthermore, marker vaccines will have to be accompanied by reliable discriminatory tests.

2. Outline of production and minimum requirements for conventional live vaccines

a) Characteristics of the seed

CSF vaccines prepared in live animals do not follow OIE animal welfare principles. Their production and use should be discontinued.

i) *Biological characteristics of the master seed*

MLVs are produced from CSFV strains that have been attenuated by passage either in cell cultures or in a suitable host species not belonging to the family *Suidae*. Production is carried out in cell cultures, based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced, based on their ease of growth in cell culture, virus yield and stability.

The exact source of the underlying CSFV isolate, its sequence, and the passage history must be recorded.

ii) *Quality criteria*

Only MSVs that have been established as sterile, pure (free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials* and those listed by the appropriate licensing authorities) and immunogenic, should be used for vaccine virus (working seed viruses and vaccine batches) production. Live vaccines must be shown not to cause disease or other adverse effects in target animals injected in accordance with chapter 1.1.6 (section on *Safety tests* [for live attenuated MSVs]).

Identity of the MSV has to be confirmed using appropriate methods (e.g. through the use of specific MAbs or vaccine strain-specific genome detection methods).

iii) *Validation as vaccine strain*

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy agents (TSEs), consideration should also be given to minimising the risk of transmission by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

The vaccine virus in the final product should generally not differ by more than five passages from the master seed lot. The commercial vaccine should be produced in batches in lyophilised form as a homogeneous product.

iv) Emergency procedure for provisional acceptance of new MSV in the case of an epizootic (with pathogens with many serotypes, e.g. bluetongue virus, highly pathogenic avian influenza, FMD, etc.)

None.

b) Method of manufacture

i) *Procedure*

The virus is used to infect an established cell line. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with the requirements in chapter 1.1.6.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles). The harvest can be further processed by filtration and other methods. A stabiliser may be added as appropriate. The vaccine is homogenised before lyophilisation to ensure a uniform batch/serial.

ii) *Requirements for ingredients*

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.6.

iii) *In-process controls*

In process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests.

iv) *Final product batch/serial test*

Sterility

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

Identity

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the vaccine virus.

Residual moisture

The level of moisture contained in desiccated products should be measured as described in chapter 1.1.6.

Safety

Batch safety testing is to be performed unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions.

For batch/serial safety testing use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a tenfold dose of the vaccine. Observe the piglets daily for at least 14 days. The vaccine complies with the test if no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

Batch/ serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in pigs and titre of the modified live vaccine *in vitro*.

In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (see Section C.2.c.iii).

c) Requirements for authorisation /registration / licensing

i) *Manufacturing process*

For registration of a vaccine, all relevant details concerning preparation of MSV, manufacture of the vaccine and quality control testing (see Section C.2.a and b) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches originating from the same MSV, with a volume not less than 1/3 of the typical industrial batch volume.

The in-process controls are part of the manufacturing process.

ii) *Safety requirements*

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

Vaccines should be tested for any pathogenic effects on healthy pigs, and in sows to evaluate the safety in pregnant animals and their offspring.

Safety in young animals

Carry out the test for each recommended route of application using in each case piglets not older than the minimum age recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Use not fewer than eight piglets of 6–8 weeks of age that do not have antibodies against pestiviruses. Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the piglets daily for at least 14 days. The body temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 hours after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all piglets does not exceed 1.50°C, no piglet shows a temperature rise greater than 1.50°C for a period exceeding 3

consecutive days, and no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

Blood samples are taken at 7 days after vaccination and tested for leukopenia. The average white blood cell (WBC) count should exceed 7×10^6 cells/ml.

In addition, the vaccines in their commercial presentation should be tested for safety in the field (see chapter 1.1.6, section on *Field tests* [*safety and efficacy*]).

Safety test in pregnant sows and test for transplacental transmission

Carry out the test with vaccination by a recommended route using not fewer than eight healthy sows or gilts of the same age and origin, between the 55th and 70th days of gestation and that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

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

The test is invalid if the vaccinated sows do not seroconvert before farrowing. The vaccine virus complies with the test if no abnormalities in the gestation or in the piglets are noted. No sow or gilt shows notable signs of disease or dies from causes attributable to the vaccine.

Vaccine virus or antibodies against CSFV must not be present in blood samples from newborn piglets.

Non-transmissibility

Keep together for the test not fewer than 12 healthy piglets, 6–10 weeks old and of the same origin, that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by a recommended route to not fewer than 6 piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine.

Maintain not fewer than six piglets as contact controls. The mixing of vaccinated piglets and contact piglets is done 24 hours after vaccination.

After 45 days, euthanise all piglets. Carry out appropriate tests on the piglets to detect antibodies against CSFV and on the control piglets to detect CSFV in the tonsils. The vaccine complies with the test if antibodies are found in all vaccinated piglets and if no antibodies and no virus are found in the control piglets.

Reversion-to-virulence

The test for increase in virulence consists of the administration of the vaccine virus from the master seed lot or one or two passages above to piglets that do not have antibodies against pestiviruses.

This protocol is repeated five times. Administer to each of two healthy piglets free of antibodies to pestiviruses, 6–10 weeks old, by a recommended route, a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Collect an appropriate quantity of blood from each piglet daily between day 2 and day 7 after administration of the vaccine virus, and pool the samples taken on the same day. Then slaughter the piglets and take the tonsils of both of them, pool the tonsils and prepare a 10% suspension in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage. At the same time, the presence of CSF antigens is confirmed at each passage. Blood and pooled tonsillar tissue are used to inoculate two further pigs of the same age and origin by the same route as before.

Administer 2 ml of the pooled material (blood and tonsillar tissue) with the highest virus titre by a recommended route to each of two other piglets of the same age and origin. If no virus is found, repeat the administration once again with the same material and another two piglets. If no virus is found at this point, end the process here. If, however, virus is found, carry out a 2nd series of passages by administering 2 ml of positive material by a recommended route to each of two other piglets of the same age and origin.

Carry out this passage operation not fewer than four times (in total five groups from the start of the test should be vaccinated), verifying the presence of the virus at each passage in blood and tonsils. Care must be taken to avoid contamination by virus from previous passages.

The vaccine virus complies with the test if no indication of increasing virulence (monitored by clinical observations) of the maximally passaged virus compared with the unpassaged virus is observed.

If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

iii) *Efficacy requirements*

Protective dose

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge and is expressed by the number of 50% protective doses (PD_{50}) for pigs contained in the vaccine dose.

The test consists of a vaccination/challenge trial in piglets aged 6–10 weeks using different dilutions of the vaccine in question. An additional group of piglets of the same age an origin are used as controls. All animals have to be free from antibodies against pestiviruses prior to the trial. Each group of piglets except the control group is vaccinated with an appropriate dilution of the reconstituted vaccine (e.g. 1/40 and 1/160 using a suitable buffer solution.

Fourteen days after the single injection of vaccine, challenge the piglets by a suitable route with a dose of a virulent strain of CSFV that kills at least 50% of the non-vaccinated piglets in less than 21 days. Observe the piglets for 21 days and record the body temperature 3 days before challenge and daily after challenge for 21 days. The PD_{50} content of the vaccine is calculated from the number of animals protected in each group using the Spearman-Kärber method.

The test is invalid if fewer than 50% of the control piglets display typical signs of serious infection with swine fever virus, and die, and if fewer than 100% of the control piglets show clinical signs of disease within the 21 days following challenge.

The vaccine complies with the test if the minimum dose corresponds to not less than 100 PD_{50}.

Protection against transplacental infection

Use eight sows that do not have antibodies against pestiviruses, randomly allocated to either the vaccine group (n = 6) or the control group (n = 2).

Between the 34^{th} and 49^{th} day of gestation, all sows allocated to the vaccine group are vaccinated once with 1 dose of vaccine containing not more than the minimum titre stated on the label. Three weeks after vaccination, all 8 sows are challenged by a suitable route with a dose of virulent strain of CSFV that would be sufficient to kill at least 50% of non-vaccinated piglets in less than 21 days.

Just before farrowing, the sows are euthanised and their fetuses are examined for CSFV. Serum samples from sows and fetuses are tested for the presence of antibodies against CSFV. Isolation of CSFV is carried out from blood of the sows (collected 7 and 9 days after challenge and at euthanasia), and from homogenised organ material (tonsils, spleen, kidneys, lymph nodes) of the fetuses.

The test is valid if virus is found in at least 50% of the fetuses from the control sows (excluding mummified fetuses).

The vaccine complies with the test if no virus is found in the blood of vaccinated sows and in fetuses from the vaccinated sows, and antibodies against CSFV should not be found in the serum of the fetuses from the vaccinated sows

In addition, where appropriate, the vaccines should be tested for efficacy in the field (see chapter 1.1.6, section on *Field tests* [*safety and efficacy*]).

iv) *Duration of immunity*

As part of the authorisation procedure the manufacturer should demonstrate the duration of immunity of a given vaccine by either challenge or the use of a validated alternative test, at the end of the claimed period of protection.

At least ten vaccinated pigs are each inoculated with an amount of virus corresponding to 10^5 PID_{50} of a virulent strain of CSVF and observed for 3 weeks. The vaccinated animals have to remain healthy, only the controls should die.

The duration of immunity after vaccination against CSF shall not be less than 6 months.

v) *Stability*

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for authorisation.

The period of validity of a batch of lyophilised CSF vaccine should not be less than 1 year.

3. Requirements for other vaccines

3.1. Oral vaccine

3.1.1. Background

The most widely applied concept of oral bait vaccination of wild boar against CSF, including bait design and immunisation scheme was developed, evaluated, and optimsed by Kaden *et al.* (2010). The respective vaccines are conventional modified live vaccines. Immunisation occurs by uptake of the oral vaccine through the lymphoid tissues of the oral mucosa and tonsils, where expression of virus stimulates the immune system (Kaden *et al.,* 2000; 2002; 2003; 2004; Kaden & Lange, 2004; Rossi *et al.,* 2010).

Safety is of paramount consideration for oral vaccine use, not only for the target animals, but for the environment (see chapter 1.1.6) and other species which may come in contact with the vaccine.

3.1.2. Outline of production and minimum requirements for vaccines

In addition to the outline of production described for injectable vaccines above, the following specific requirements must be met:

a) Method of manufacture

i) *Final product batch/serial test*

After combining all of the ingredients, the final blend contains the definitive formulation that is usually used in liquid form. The last step of production of a batch/serial is filling the final blend into blisters/ capsules to be included in baits or filling directly into the bait. This final batch/serial is tested as described for the injectable vaccines, with the following differences.

ii) *Residual moisture test*

Does not apply if the oral vaccine is presented in liquid form

iii) *Safety*

Administer orally by syringe to each piglet a volume corresponding to ten oral doses as indicated by the manufacturer.

b) Requirements for authorisation/registration/licensing

In addition to the requirements described for injectable vaccines, the following specific requirements must be met.

- The bait

The bait is an integral part of the product and should ideally meet the following criteria:

- Designed for and attractive to the target species and adapted to the mode of distribution.
- Keep its form and shape under a wide range of temperature and weather conditions
- Ingredients are non-harmful, comply with animal feed standards and should not interfere with vaccine activity
- Feature a labelling system with a public warning and identification of the product

i) *Safety requirements*

For all the tests the liquid vaccine is administered orally with a syringe (not in the final bait formulation) to ensure that each animal receives the full dose.

Precaution hazards

The release of oral vaccines in the environment shall comply with the requirements in chapter 1.1.6.

ii) *Efficacy requirements*

Efficacy should be proven using the liquid vaccine administered by syringe to ensure that each animal receives the full dose. Proof-of-concept studies for the final formulation (vaccine integrated into bait) should be provided.

3.2. Biotechnology-based vaccines

3.2.1. Background

As described in Guideline 3.3 Section E *Subunit vaccines*, conventional, live attenuated CSF vaccines have a rapid onset of immunity and are effective at preventing transmission of infection (Van Oirschot, 2003), but have the disadvantage that it is not possible using serological methods (e.g. ELISA) to differentiate infected pigs from those that have merely been vaccinated. Commercial E2 subunit vaccines (Marker vaccine) have a slower onset of immunity and reduce but may not completely prevent viral shedding and transplacental infection. However, they enable a DIVA strategy to be followed thereby facilitating a 'vaccination to live' strategy.

The vaccine only elicits antibodies against the E2 glycoprotein and therefore antibodies against other CSFV antigens, such as the E^{RNS} antigen, can be used as markers of infection.

3.2.2. Outline of production

a) Characteristics of the seed

E2 subunit marker vaccine is prepared by the use of Baculovirus expressing the E2 antigen of CSFV. The vaccine therefore does not contain any CSFV while the baculo (vector) virus is chemically inactivated.

i) *Biological characteristic of the master seed*

Production is carried out in insect cell cultures, based on a seed-lot system.

Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield and stability.

The exact source of the isolate including its sequence and passage history should be recorded.

ii) *Quality criteria*

Only MSVs that have been established as sterile and pure (free of extraneous agents as described in chapter 1.1.7 and those listed by the appropriate licensing authorities), and immunogenic, shall be used for preparing the vaccine virus production.

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the MSV.

iii) *Validation as vaccine strain*

The vaccine prepared from the MSV is shown to be satisfactory with respect to safety and efficacy for the swine for which it is intended.

Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (TSEs) by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

The vaccine virus used to produce the final product should not differ by more than five passages from the material used for validating the seed lot. The commercial vaccine is inactivated for residual baculovirus and adjuvanted.

b) Method of manufacture

i) *Procedure*

The baculovirus is used to infect an established insect cell line. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with requirements in chapter 1.1.6.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus. The harvest can be further processed by filtration and other methods. Inactivation of residual baculovirus is performed preferably using a first order inactivant. The antigen is homogenised before formulation with adjuvant.

ii) *Requirements for ingredients*

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.6.

iii) *In-process controls*

Infectivity, sterility and antigenic mass are monitored. After inactivation a test for innocuity is carried out on every batch of antigen. The cells used to test for absence for residual live baculovirus are the same cell line used for production or potentially equally or more sensitive cells.

iv) *Final product batch/serial test*

Sterility

Must comply with chapter 1.1.6.

Identity

The identity test is performed by a specific MAbs based virus neutralisation against CSFV or an appropriate molecular identification. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source. This test may be combined with the potency test (see below).

Safety and prove of marker concept

Batch safety testing is to be performed unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.6.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions.

For batch/serial safety testing use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a double dose of the formulated vaccine. Observe the piglets daily for at least 14 days for local and systems reactions to vaccination. After 14 days they are each injected with a second single dose of vaccine.

Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. The vaccine should elicit antibodies against CSFV E2 but not against CSFV-E^{RNS} antigen,

Batch/ serial potency

Induction of specific anti-E2 antibodies in vaccinated pigs can be used to confirm the potency of each batch once the titre has been correlated with the results of the efficacy test.

c) Requirements for authorisation /registration/ licensing

i) *Manufacturing process*

See Section C.2.c.i.

ii) *Identity*

The identity test is performed by virus neutralisation using immune sera against CSFV. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source.

iii) *Safety requirements*

Safety in young animals

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in eight piglets of 6–8 weeks of age. Single dose and repeat dose tests using vaccines formulated to contain the maximum permitted payload should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reaction to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. It has to be proven that the vaccine does not elicit antibodies against CSFV-ERNS antigen,

Safety in pregnant sows

For the purpose of gaining regulatory approval a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration corresponding to the primary vaccination schedule (e.g. two injections) in eight pregnant sows. The sows are observed for local and systemic reactions to vaccination. The observation period must last until parturition to examine any harmful effects during gestation or on progeny. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. It has to be proven that the vaccine does not elicit antibodies against CSFV-ERNS antigen,

iv) *Efficacy requirements*

Protective dose

Vaccine efficacy is estimated in animals vaccinated according to the manufacturer's recommendation, following the methods described in Section C.2.c.iii.

Protection against transplacental infection

The trial vaccine should comply with the test described in Section C.2.c. iii.

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NB: There are OIE Reference Laboratories for Classical swine fever
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for classical swine fever

MEETING OF THE ENLARGED BUREAU GROUP OF THE OIE BIOLOGICAL STANDARDS COMMISSION
Paris, 18–19 February 2013

Status of the chapters identified for update and proposal for adoption in 2013

No.	Chapter title	Experts' draft	EBG recommendation	BSC decision
1.1.1	Collection of diagnostic specimens	RECEIVED	Approved to be sent to MCs as final version	Agree
1.1.2.	Transport of specimens of animal origin	RECEIVED	Approved to be sent to MCs as final version	Agree
1.1.3.	Standard for managing biorisk in the veterinary laboratory and animal facilities	RECEIVED	Recommend that the BSC consider proposing that the DG reconvene the AHG to look at the comments on this chapter and Guideline 3.5 with a view to redrafting the chapter. Remove mention in disease-specific chapters to risk assessment as performing a risk assessment.	Agree convene AHG, provide material beforehand.
1.1.5.	Principles and methods of validation of diagnostic assays for infectious diseases	RECEIVED	Approved to be sent to MCs as final version	Agree.
1.1.6.	Principles of veterinary vaccine production (re-write as a standard)	To be drafted by an AHG	Awaiting text	
1.1.10.	International standards for vaccine banks	Not yet received	Awaiting text	
2.1.3.	Bluetongue	RECEIVED	Chapter received but not yet reviewed	
2.1.4x	Crimean–Congo haemorrhagic fever	RECEIVED	Chapter too human oriented. Needs to be reviewed from an animal health perspective, and to include information on the situation in Europe. Are three serological tests necessary and when and for what purpose are they used in animals? Approach Collaborating Centre in CIRAD.	Agree
	Epizootic haemorrhagic disease	RECEIVED	Chapter received but not yet reviewed	
2.1.4.	Echinococcosis/Hydatidosis	Not yet received	Awaiting text	
2.1.6.	Heartwater	Not yet received	Awaiting text	
2.1.8.	Leishmaniosis	RECEIVED	Chapter received but not yet reviewed	

No.	Chapter title	Experts' draft	EBG recommendation	BSC decision
2.1.10.	Screwworm (*Cochliomyia hominivorax* and *Chrysomya bezziana*)	RECEIVED	Approved to be sent to MCs as final version	Agree
2.1.11.	Paratuberculosis (Johne's disease)	Not yet received	Awaiting text	
2.1.13.	Rabies (Vaccine section)	RECEIVED from AHG on Vaccine Quality	Approved to be sent to MCs as final version	Agree
2.1.14.	Rift Valley fever	RECEIVED chapter from AHG	Chapter received	Approved the AHG report and draft chapter for circulation for first round MC comment
2.1.20.	West Nile fever	RECEIVED	Approved to be sent to MCs as final version subject to clarification of some points from the authors.	Agree
2.2.1	Acarapisosis of honey bees	AHG revising all bee disease chapters (coor. by Ritter). Not yet received	Awaiting text	
2.2.2.	American foulbrood of honey bees	Not yet received	Awaiting text	
2.2.3.	European foulbrood of honey bees	Not yet received	Awaiting text	
2.2.4.	Nosemosis of honey bees	RECEIVED	Approved to be sent to MCs as final version subject to clarification of one point from the authors.	Agree
2.2.5.	Small hive beetle infestation (*Aethina tumida*)	RECEIVED	Approved to be sent to MCs as final version	Agree
2.2.6.	*Tropilaelaps* infestation of honey bees (*Tropilaelaps* spp.)	Not yet received	Awaiting text	
2.2.7.	Varroosis of honey bees	Not yet received	Awaiting text	
2.3.2.	Avian infectious bronchitis	RECEIVED	Approved to be sent to MCs as final version subject to clarification some points from the authors.	Agree
2.3.5.	Avian mycoplasmosis (*M. gallisepticum, M. synoviae*)	Not yet received	Awaiting text	

No.	Chapter title	Experts' draft	EBG recommendation	BSC decision
2.3.9.	Fowl cholera	Received first draft (but the vaccine section unchanged)	Diagnostic section received. Awaiting revision of vaccine section	
2.3.10.	Fowl pox	Not yet received	Awaiting text	
2.3.12.	Infectious bursal disease (Gumboro disease)	Not yet received	Awaiting text	
2.4.5.	Bovine genital campylobacteriosis	Asked to be moved to 2014	Awaiting text	
2.4.8.	Bovine viral diarrhoea	Not yet received	Awaiting text	
2.4.9.	Contagious bovine pleuropneumonia	RECEIVED	Chapter received but not yet reviewed	
2.4.15.	Malignant catarrhal fever	RECEIVED	Approved to be sent to MCs as final version.	Agree
2.4.16.	Theileriosis	RECEIVED	Chapter received but not yet reviewed	
2.4.18.	Trypanosomosis (Tsetse-transmitted)	RECEIVED	Approved to be sent to MCs as final version subject to clarification of some points from the authors.	Agree
2.5.3.	Dourine	RECEIVED	Approved to be sent to MCs as final version.	Agree
2.5.5.	Equine encephalomyelitis (Eastern & Western)	RECEIVED	Approved to be sent to MCs as final version.	Agree
2.5.6.	Equine infectious anaemia	RECEIVED	Approved to be sent to MCs as final version.	Agree
2.5.8.	Equine piroplasmosis	RECEIVED	Chapter received but not yet reviewed	
2.5.9.	Equine rhinopneumonitis	Received first draft (but vaccine section unchanged)	Diagnostic section received. Awaiting revision of vaccine section	Agree
2.5.10.	Equine viral arteritis	RECEIVED	Approved to be sent to MCs as final version.	Agree
2.5.11.	Glanders	RECEIVED	Approved to be sent to MCs as final version.	Agree
2.5.13.	Venezuelan equine encephalomyelitis	RECEIVED	Approved to be sent to MCs as final version.	Agree

No.	Chapter title	Experts' draft	EBG recommendation	BSC decision
2.6.1.	Myxomatosis	Not yet received	Awaiting text	
2.7.5.	Contagious agalactia	RECEIVED	Approved Diagnostic Section to be sent to MCs as final version subject to clarification of one point from the authors. Section C on hold	Agree
2.7.10.	Ovine pulmonary adenomatosis (adenocarcinoma)	RECEIVED	Chapter received but not yet reviewed	
2.7.11.	Peste des petits ruminants	RECEIVED	Approved to be sent to MCs as final version subject to clarification of some points from the authors.	Agree
2.8.3.	Classical swine fever (hog cholera)	RECEIVED vaccine section from AHG in 2013	Chapter (vaccine section) received	Approved the AHG report and draft vaccine section for circulation for first round MC comment
2.8.9.	Swine vesicular disease	RECEIVED	Approved to be sent to MCs as final version subject to clarification of some points from the authors.	Agree
2.9.1.	Bunyaviral diseases of animals (excluding Rift Valley fever)	RECEIVED	Received one of three reviews. Awaiting the other reviews.	
2.9.2.	Camelpox	RECEIVED	Chapter received but not yet reviewed	
2.9.4.	Cryptosporidiosis	Not yet received	Awaiting text	
2.9.5.	Cysticercosis	Not yet received	Awaiting text	
2.9.7.	*Listeria monocytogenes*	Not yet received	Awaiting text	
2.9.8.	Mange	RECEIVED	Approved to be sent to MCs as final version and ask authors to provide pictures.	Agree
2.9.11.	Verocytotoxogenic *Escherichia coli*	Not yet received	Awaiting text	

New chapters and chapters proposed for update in 2014

No.	Title
New chapter	Management of Veterinary Laboratories (to include sections on quality and biorisk management)
1.1.8	Minimum requirements for vaccine production facilities
1.1.9	Quality control of vaccine
1.1.6.	Principles of veterinary vaccine production (re-write as a standard)
2.1.9.	Leptospirosis
2.1.19.	Vesicular stomatitis
2.3.3.	Avian infectious laryngotracheitis
2.3.6.	Avian tuberculosis
2.4.3.	Bovine brucellosis
2.4.5.	Bovine genital campylobacteriosis
2.4.10.	Dermatophilosis
2.5.4.	Epizootic lymphangitis
2.7.2.	Caprine and ovine brucellosis (excluding *Brucella ovis*)
2.7.6.	Contagious caprine pleuropneumonia
2.7 9.	Ovine epididymitis (*Brucella ovis*)
2.8.5.	Porcine brucellosis
2.8.10.	Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or Teschen/Talfan disease)
2.9.10.	Toxoplasmosis

The following three chapters were added to this list:

2.4.1.	Bovine anaplasmosis
2.4.2.	Bovine babesiosis
2.4.16.	Theileriosis (already received)

MEETING OF THE ENLARGED BUREAU GROUP OF THE OIE

BIOLOGICAL STANDARDS COMMISSION

Paris, 18–19 February 2013

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1. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*

1.1. Update on progress since last meeting
1.2. Review of chapters proposed for adoption in May 2013: chapters already sent for first round of comments [opinion and recommendations]
1.3. Update on the status of the chapters identified for revision and proposal for adoption in 2014
1.4. Question from Code Commission re: nonhuman primate tests
1.5. Is the polymerase chain reaction for contagious equine metritis sufficiently validated to be a prescribed test?
1.6. Prescribed and Alternative Tests – proposal to abandon them (information and opinion)

For information: report of *ad hoc* Group on Vaccine Quality related to Classical Swine Fever and the proposed Manual text

2. Outcome: recommendations of the Enlarged Bureau Group to the BSC (table from point 1.2 adapted according to discussions)

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MEETING OF THE ENLARGED BUREAU GROUP OF THE OIE

BIOLOGICAL STANDARDS COMMISSION

Paris, 18–19 February 2013

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OIE EXPERT SURVEILLANCE PANEL ON EQUINE INFLUENZA VACCINE COMPOSITION

OIE Headquarters, 4 March 2013

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Conclusions and Recommendations

Influenza activity in 2012

During 2012, individual animal cases and outbreaks of equine influenza were reported by Argentina, Chile, France, Germany, Ireland, United Kingdom (UK), and United States of America (USA).

Sources of viruses characterised during 2012

Equine influenza A (H3N8) viruses were isolated and/or characterised from outbreaks in Argentina, Chile, France, Germany, Ireland, the UK, Uruguay and the USA. In quarantine stations equine influenza viruses were isolated and/or characterised from horses recently imported from Uruguay into Dubai, and from Belgium into Japan.

Field data

Equine influenza virus infections were confirmed in both vaccinated and unvaccinated horses. Vaccination breakdowns were observed in Thoroughbred yearlings in Kentucky, sport horses in France and racehorses in Ireland, as well as the horses imported into Dubai, and into Japan. Over 150 fully vaccinated horses were affected in three linked outbreaks in the Calvados area of France.

The viruses identified in the vaccine breakdowns belonged to both the Florida clade 1 and clade 2 lineages. Horses vaccinated with different vaccines were affected, including those updated in accordance with the 2004 recommendation to incorporate an A/eq/South Africa/04/2003-like virus. These vaccines had not been updated in accordance with the recommendations of 2010 and 2011 to include a virus from clade 2 for optimum protection.

Fatalities associated with influenza A virus infection were reported in France and Uruguay.

Characterisation of viruses isolated in 2012

Viruses isolated/identified in 2012 from outbreaks/cases in Argentina, Chile, Dubai, France, Germany, Ireland, Japan, the UK, Uruguay, and the USA were characterised genetically by sequencing of the haemagglutinin 1 (HA1) gene. Viruses isolated in Argentina, Dubai, Germany, Ireland, the UK, and the USA were also characterised antigenically by the haemagglutination inhibition (HI) assay using post-infection ferret antisera.

Genetic characterisation

All HA1 sequences obtained from viruses were of the American lineage (Florida sublineage). The viruses identified in Argentina, Chile and the USA were characterised as clade 1 viruses, as was a virus associated with an outbreak in Germany. All other viruses identified in France, Germany, Ireland and the UK were characterised as clade 2 viruses. The virus detected in a Belgian horse in a Japanese quarantine facility was characterised as a clade 2 virus. Influenza A viruses isolated in the quarantine facility in Dubai from horses imported from Uruguay were characterised as clade 1 viruses.

Novel HA amino acid substitutions were observed in viruses of both clades compared with isolates from 2011.

Antigenic characteristics

HI data and antigenic cartography analyses of HI data available for viruses isolated in 2012 indicate that the two clades of the Florida sublineage continue to co-circulate and evolve but are currently antigenically closely related to the recommended vaccine strains of that lineage.

Conclusions

No Eurasian viruses were isolated in 2012. Viruses isolated and characterised were from both clade 1 and 2 of the Florida sublineage. There was evident lack of vaccine effectiveness, against both clade 1 and clade 2 viruses. The detection of clade 1 and clade 2 viruses in quarantine facilities in Dubai and Japan illustrates the ongoing risk of international spread of influenza by infected vaccinated horses, the need for optimum protection and the requirement for vaccines to be updated with strains from both clades

Level of surveillance and updating of vaccines

The panel continues to emphasise the importance of increased surveillance and investigation of vaccination breakdown in different countries. Rapid submission of viruses to reference laboratories is essential if antigenic and genetic drift is to be monitored effectively on a global basis.

Vaccines should contain epidemiologically relevant viruses.

The updating of vaccines in a timely manner is necessary for optimum protection.

Recommendations

It is not necessary to include an H7N7 virus or an H3N8 virus of the Eurasian lineage in vaccines as these viruses have not been detected in the course of recent surveillance and are therefore presumed not to be circulating.

Vaccines for the international market should contain both clade 1 and clade 2 viruses of the Florida sublineage.
Clade 1 is represented by A/eq/South Africa/04/2003-like or A/eq/Ohio/2003-like viruses.
Clade 2 is represented by A/eq/Richmond/1/2007-like viruses.

A panel of viruses covering both clades is available from the OIE Reference Laboratories.

Manufacturers producing vaccines for a strictly national market are encouraged to liaise with reference laboratories. This will ensure utilisation of reference reagents in the selection of viruses for inclusion in vaccines that induce cross-reactive responses that are immunogenically relevant to the equine influenza viruses circulating nationally.

Reference reagents

Freeze-dried post-infection equine antisera to A/eq/Newmarket/1/93 (American lineage H3N8) and A/eq/South Africa/4/2003 (Florida clade 1, sublineage of the American lineage) are available from the European Directorate for the Quality of Medicines (EDQM). These sera have been assigned Single Radial Haemolysis values through an international collaborative study and can be used as primary reference sera for the assay.

Recent virus strains and small quantities of ferret sera for antigenic characterisation are available from the OIE reference laboratories.

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OIE EXPERT SURVEILLANCE PANEL ON EQUINE INFLUENZA VACCINE COMPOSITION

OIE Headquarters, 4 March 2013

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Annex 9

BSC Work Plan: February to September2013

Topic/Issue	Responsible(s)	Deadline
Manual of Diagnostic Tests and Vaccines for Terrestrial Animals		
Circulate the chapters approved by the Enlarged Bureau Group and the Commission as final versions for adoption in May 2013	SL	By mid- to end March 2013
Remind authors of the chapters identified by the Enlarged Bureau Group and the Commission for adoption in 2013 and 2014 but not yet received	SL	On going
Commission the chapters identified by the Enlarged Bureau Group and the Commission for proposal for adoption in 2014	SL	On going
Update all the disease-specific chapters of the *Manual* according to the new template	BSC/SST	Continuing implementation with the aim to finalise all these modifications for the publication of the paper version of the *Manual* in 2016
Ad hoc Groups		
Biosafety and Biosecurity in Veterinary Laboratories (Fourth Meeting)	SST: FD, Member of the BSC who will attend: VC, BS, PD	Dates: July 2013
High throughput sequencing and bioinformatics and computational genomics (HTS-BCG)	SST: EEV, FD, SL Member of the BSC who will attend: VC, PD	Dates:
Shortening the vaccine registration process when simply updating and incorporating relevant strains in equine influenza vaccines	SST: SM Member of the BSC who will attend: VC	Dates:
Vaccines to update chapter 1.1.6 *Principles of veterinary vaccine production*, and to draft two chapters: 1.1.8 *Minimum requirements for vaccine production facilities*, 1.1.9 *Quality control of vaccines*	SST: SM, FD	On hold: Collaborating Centre for Veterinary Medicinal Products offered to do. Commission asked they collaborate with other OIE Centres working on vaccines, to produce consensus documents. Once received, it will be determined whether they could be sent directly to Member Countries for comment or whether they could be used by an *ad hoc* Group as base documents for further elaboration.

Topic/Issue	Responsible(s)	Deadline
Meetings		
1-day seminar to be held during WAVLD meeting in Berlin in June 2013. Theme: validation or "(New Approaches to diagnosis: Applied Genomics). Need to finalise programme and speakers	SST & BSC	Cancelled