WORLD ORGANISATION FOR ANIMAL HEALTH
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OIE Virtual Training Series on Rabies Serology for SAARC Region

11-13 October 2021

Summary of questions & answers

Note: Answers as provided by Dr Shrikrishna Isloor and Dr Sharada of KVAFSU. For further queries, please directly contact Dr Isloor at kisloor@gmail.com or rdlkvafsucva@gmail.com

Day 1 (2021/10/11)

1. For large animals, the whole head is being submitted to my laboratory. Is there any SOP to collect head of large animals in field?
   - Even for large animals, the occipital foramen approach can be employed whenever the carcasses are presented. As far as the collection of head from the large animals is concerned, a horizontal deep incision be made at the site anterior to the first cervical vertebrae but posterior to the occipital protruberance. The head may be completely severed / decapitated by extending this deep incision. The decapitated head need to be put in a suitable ziplock cover (preferably 2 such covers to be used) and transported in cold chain to the nearest diagnostic facility
   - Details for occipital foramen method of brain sampling can be accessed here.

2. Do you suggest brain sample collection at the field level outside a biosafety cabinet?
   - Yes, it can be done by the occipital foramen approach which is very simple and user friendly. Please refer the presentation and videos from the virtual training organized in November 2020 for more details.
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3. What do we do if we find negative result for rabies though the lateral flow assays (LFA)? Do we need to conduct further tests to reconfirm before issuing the result to the client?
   - LFA though not a referred test for diagnosis, it is the only field based user friendly test for diagnosis of Rabies. Hence, results by LFA need to be confirmed by direct

fluorescent antibody (DFA) and/or PCR. Completely or partially putrified brain samples are not fit for testing by DFA but partially putrified brain samples can be tested by LFA. Even if negative on LFA, consider testing further by DFA.

4. How long can we keep prepared growth and maintenance media in stock?
   - In our laboratory, normally the growth medium is prepared just before use by adding FBS / serum to the maintenance medium. Whereas we preserve maintenance medium at 4 °C for up to two weeks.

5. Can we use Eagle's MEM instead of DMEM?
   - Eagles MEM can be used but ensure that first the cells get adapted to this medium before bulk usage.

6. In my laboratory, we use the following protocol for medium (for MSR and BHK cells): autoclaving Nissui's Eagles MEM along with TPB in DD water, and then after sterilization of this basic medium, we make growth or maintenance media as required by adding the remaining sterile ingredients to it. Can we prepare the media here like this too?
   - Generally, we do not autoclave ingredients of cell culture media, since heating may inactivate the biological activity. Distilled water and TPB are sterilized by autoclaving.
   - However as for the preparation of Nissui's Eagles MEM for cell culture is concerned, follow the manufacturer's instructions as to autoclave / filter sterilize the same as they have both types of media.

Day 2 (2021/10/12)

7. What is the concentration of trypsin EDTA used?
   - It is 0.25 % Trypsin EDTA

8. What should the cell density be for revival and sub-culture?
   - 1 million cells per ml for revival and 0.5 million cells per ml for subculture

9. Can Maintenance medium be used in the washing step after thawing?
   - Growth medium is preferred for washing since the serum concentration is higher in this which will maintain and support the survival of cells better

10. What is the maximum passage level for cells to be used for tests? What is the limit for subculturing a cell line?
    - Avoid sub-culturing beyond 60-65 times. With subculturing, cells age and with ageing cells may lose some of the original properties and develop genetic aberrations. Usually, in our laboratory, we will go up to 65 subculturing.

11. Do we do manual jerk shaking after addition of trypsin to the flask?
    - We may do gentle manual jerking for detachment of the cell monolayer.

12. What % of EDTA is used (with 0.25% trypsin)?
    - It is 0.02 % EDTA.

13. Can we use dry bath incubator instead of a water bath?
    - Avoid using a dry bath, as heat dissipation / conduction is efficient and faster in a water bath. Efficient and faster heat dissipation is important during thawing of frozen cells.

14. Can we incubate without CO_2 using some other reagents in the media?
    - If you use non-porous capped Tissue culture flasks, then CO_2 supplementation is not mandatory. 5% CO_2 supplementation supports cells to undergo faster proliferation and is supportive to cells – this mimics *in-vivo* cell proliferation.

15. Can we discard spent media in sodium hypochlorite solution?
    - Yes, you can. We use NaOH solution.

16. We keep the white lights of the room switched on while the UV lighting on inside the biosafety cabinet. Do you recommend keeping the whole room dark during/shortly after using the UV light, to avoid photo reactivity?
    - There is no need to turn off the lights in the room but within the BSC ensure that white light is turned off and that shutters are closed.

17. Can we use larger size flasks (larger than T25) for growing cell lines, to have a greater stock of working cell lines?
    - Yes, you can choose the flask size based on your needs.

18. Please clarify the storing temperatures for conjugates and other reagents used for the rabies test.
    - It is important to aliquot conjugates and other reagents on delivery and refreeze. Repeated thawing and refreezing will affect the reagents. It is also important to make aliquots of serum. As serum samples for international movements of pet animals have to be tested (for anti-rabies vaccinal antibodies) in a batch, such test serum samples be frozen on arrival at the laboratory.

19. How long can rabies virus strains be stored at -80 °C without change in the aliquot strength?
    - There will be a gradual reduction in the titre over a period though temperature is maintained at -80 °C throughout. We have been preserving the positive brain tissues for the past 8 years without losing its infectivity. Whereas we have preserved BHK21 cell culture harvest for a period of 5 years at -80 °C but encountered significant reduction in the titre although it was infective.

Day 3 (2021/10/13)

- Can we use 1 IU as unit of reference serum? Does 2 IU reference serum should always give inhibition up to 1:8 dilution?
- There is no hard and fast rule that it has to be 2 IU, but we need to ensure that after comparing its performance with WHO reference serum. You may also start with 1 IU. Yes, if 2 IU reference serum used in the first well of a column in the microtitre plate with 1:2 dilution of serum, then, the next well (second well) in the same column corresponds to 1:4 dilution of serum and titre of serum will be 1 IU. Accordingly, the third well in that column corresponds to 1:8 dilution of serum and titre of the serum will be 0.5 IU. Universally, 0.5 IU of anti rabies vaccinal anti body titre is considered sufficient to confer protection against rabies. Therefore, 2 IU reference serum at 1:8 dilution corresponds to 0.5 IU, the cut off.

20. How do we calculate the minimal variation?
    - Determine the standard deviation of IUs of the reference serum samples after 20 repetitions. The lower the deviation, the higher the precision of test.

21. What is the maximum IU detectable by RFFIT?
    - IU refers to International units. The neutralizing antibody titre is expressed in IU/mL. You may go as high as the dilution can be made, but in our lab, we go up to 1:256 (corresponds to 64 IU) dilution (H row, the last row in the microtitre plate.
22. Regarding microchips, details may not be present in rural dogs/pets in parts of India.
    - Microchip numbers are required for international movement of pets, but they are not required for routine sero-monitoring of anti-rabies antibodies to monitor mass dog vaccination campaigns.

23. Should I cover the plate with film during microscopy?
    - Do not cover the plate while reading the results as there is no biosafety concern, since the cells are fixed and virus inactivated after fixing with 70 % chilled acetone.

24. Can we use FITC green light during examination, or is it mandatory to use a blue filter?
    - Contact your microscope supplier to provide the filter suitable for FITC conjugate.

25. Is there any non-specific fluorescence during RFFIT?
    - Beginners may get confused between specific and non-specific fluorescence. If washing is not done properly, one may have non-specific fluorescence. Non-specific binding of conjugates is very low with most of the conjugates as they are monoclonal antibody based.

26. If the titer is below than 0.5 IU i.e. below 1:2 dilution (1st well), then what can we say about the exact figure (e.g. may be 0.27 IU or something else)?
    - At this rate of dilution, you can't specify anything below 0.5 IU. If you design the layout and dilutions differently, can answer this. At the most we can say that titre of antibodies is less than 0.5 IU
27. The diagnostic antibody is N protein based while the protective antibody is G protein

based. Is there a correlation between these?

- There is paucity of information correlating N and G protein based diagnostics. However, both N and G proteins can be assessed for diagnosis, but now it is invariably N protein which is assessed in laboratories. For immunodiagnostic purposes N protein is targeted as it is immunodominant and usually found in abundance.

28. What do we do if different observers read the fluorescence different?
    - You may have to repeat the test. Evaluators' competency needs to be proven. It is easy to say a sample is positive if you see even one viral inclusion; it is more difficult to say it is negative as you need to assess the complete well.

29. Is there a chance that RFFIT values less than 0.5 are still protective for rabies?
    - RFFIT does not detect the CMI response. So RFFIT could be <0.5 IU with some immunity present. KVAFSU is now researching this topic.

30. Do we need to decontaminate the exhaust air coming from the biosafety cabinet to keep the external-to-the-lab-environment safe from viral aerosols?
    - Some biosafety cabinets have a burn-off capacity, so even if any zoonotic pathogens pass through, they will be inactivated. This is not necessary for rabies virus. HEPA filters will effectively filter the air. It is recommended to have biosafety cabinets tested every year – maintenance is very important.

31. Can we use sodium hypochlorite instead of sodium hydroxide (NaOH)? Does sodium hydroxide have some advantages over hypochlorite?
    - NaOH has been shown to be the best to inactivate the pathogens especially in presence of organic materials

32. How do you manage biological waste and its safe disposal after lab activity is finished?
    - It is important to decontaminate biological materials (e.g. tissue papers used for cleaning), e.g. with 2% NaOH. You can also secondarily decontaminate by autoclaving before discard. The discard is sent to an outsourced agency which manages for its final disposal.

33. What type of commercially-available ELISA kit is used for rabies identification?
    - Indirect quantitative ELISA kits targeting N protein are available commercially.

34. Can we send the serum samples from lab personnel to KVAFSU to test their immune status?
    - KVAFSU-CVA laboratory personnels get their serum samples tested at the WHO Collaborating Centre for human rabies in Bangalore India (NIMHANS). Since the KVAFSU became an OIE RL last year, the government in India also asked them to test human samples (brain/serum). Dr Isloor will get back to participants on this point.

35. Is it required to have an MTA (material transfer agreement) to send samples to a reference laboratory?
    - Yes, it is required. KVAFSU-CVA RDL can share the MTA format. Note that the MTA is required even within India (even if just in Bangalore!)