**Template Regional Protocol for probang sampling and oro-pharyngeal fluid (OP Fluid)**

**collection and preservation**

|  **Subject** |  **Details/protocol** |
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| **Sample collection:** |  |
| 1. **Under which conditions this sample collection could be applied:**
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|  ⦁ Study objectives | * Where epithelial lesion samples are not available from ruminant animals (in advanced or convalescent cases), or where infection is suspected in the absence of clinical signs (carrier animals) or very early infection (preclinical stage)
* Post-outbreak investigations and for demonstration of historical infection
* FMD virus (serotype/genotype) characterization
* Vaccinated animals (often become infected with no clinical signs, i.e. neoteric subclinical infection)
* Primarily tested by RT-PCR/real-time RT-PCR
* Virus isolation if available in the lab (low rate of success; and requires treatment with TTE/Freon)
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|  ⦁ Target animal group | * Susceptible animals (primarily cattle & buffalo; sheep and goat can also be included provided specially designed probang cups are available)
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| ⦁Time frame for sampling | * Infection incubation period before onset of clinical signs;
* subclinical infection in the absence of FMD clinical signs
* 0–10 days after onset of clinical signs if no other samples are available;
* between 11 -28 days after onset of clinical signs if no other samples are available (transitional phase)
* > 28 days after onset of clinical signs (carrier phase)
 |
| ⦁Requirements on equipment/materials | * Probang cups (of suitable size for large and small ruminants), sterile plastic tubes with screw cap, ice and ice box (preferably liquid nitrogen), disinfectants, buckets (10-litre each for disinfection of probang cup)
* Transport medium/buffer (see annex)
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| 1. **Detailed protocol on sample collection:**
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| ⦁ Sampling procedure | * OP fluids should be collected before animal feeding time to avoid regurgitation and contamination of with ruminal contents to the sample collection. In China, OP fluids are collected 12 hours after animal feeding time.
* Step-wise procedure:

Restrain animals properly.Hold the mouth of the animal open by gentle pressure on the tongue with four fingers of the left hand (for right-handed people) in a gap between the lower right incisor teeth and the premolar teeth. Push the probang cup into the mouth, ensuring that the concave arm of the probang cup wire handle is towards the ground. Push the cup over the back of the tongue into the pharynx and then into the upper oesophagus. Judge its position by palpation of the upper oesophagus (or approximately to the depth just past the level of the eyes).Move probang up and down within the phaynx/oesophagus over a distance of 5-10 cm for 30 seconds or 5-6 times. Gently remove the cup from the animal’s mouth and try to retain as much material as possible in the probang cup.After collection, pour OP fluids containing cellular materials into the previously prepared tube containing equal volume of the transport medium and thoroughly mix by gentle shaking. Can pour the transport medium to the probang cup to rinse the OP fluids and pour back to the container tuber if OP fluids stick. Examine visually for quality. If samples are heavily contaminated with ruminal contents, discard and flush the animal’s mouth with water or PBS before repeated sampling*.* Samples from sheep tend to be small, mucoid and difficult to detach from probang cup. If so, insert the probang cup directly into a disposable universal 20 ml tubes or similar container into which has been dispensed 3 ml of buffer solution and gently mix, pour the sample and buffer into a previously labelled tubes for transport. Tightly close and properly label tubes and disinfect and clean the outside of the tubes. Keep OP fluid samples in ice boxes or ice pack (4°C) immediately to maintain the quality of the sample before delivery to the laboratory. Between collections from each animal, disinfect and wash probang cup thoroughly. In Thailand, wash probang cup between animals using three buckets system: First bucket: tap water; Second bucket: disinfectant; Third bucket: tap water.* In S Korea, decontaminate the Probang cup by soaking in a bucket containing 2% sodium carbonate solution for 3-5 minutes, then thoroughly rinsed with tap water.
* In China, after dip the probang cup in a bucket containing 0.5% citric acid or 2% sodium carbonate, thoroughly rinse the probang cup in running tap water.

*Special attention is needed for the following** If disinfectant is not flushed off the probang, then it will kill the virus from the next animal.
* Care must be taken not to introduce the probang too far down the oesophagus. This will cause the animal to regurgitate, and rumen fluid (pH <7) will ruin the sample.
* In these cases, sampling should be repeated once the mouth of the animal has been rinsed with water or PBS.
* Samples seen to contain blood are not desirable but may be suitable for molecular testing.
* For molecular testing the sample must be handled only in the laboratory (see below).
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| ⦁ Number of samples to collect | * Include all suspected animals and a number of in-contact animals. Repeat sampling at least once in a month is recommended given the intermittent success of identifying the viral genome and/or virus
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| ⦁ Labelling of samples | * Label each tube with date and place of collection, sample type, animal ID, sample ID, and include FMD infection & vaccination and animal movement history if possible.
* Immediately place in a cooler containing ice packs (liquid nitrogen is preferable
 |
| * Limitations
 | * Sampling success is dependent on the amount of fluid collected and the procedure adopted for passing the probang cup
* Care must be taken not to stimulate the gastric reflux. It will result in contamination with rumen contents. Care should also be taken not to cause bleeding as antibodies in the blood could affect virus isolation.
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| **Sample preservation:** |  |
| 1. **Guidelines for sample storage at the field units**
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| ⦁ Medium | Transport medium, pH 7.2~7.6 (see attached/consider adding buffer and/or antibiotic as per laboratory protocol) |
| ⦁ Container | sterile plastic tube with screw cap or vials |
| ⦁Temperature | OP fluids can be transported in dry ice in frozen condition or under liquid nitrogen vapour.The samples should be sent to the lab for testing as soon as possible. If the OP fluid samples couldn’t be sent to laboratory in time or processed in laboratory, place the samples in freezers at < - 20°C (preferably -80°C). |
| ⦁pH | 7.2-7.6 |
| ⦁Other important factors to consider | Probang samples often contain extremely low quantity of FMDV; so, sample handling and cold chain are critical to successful detection.  |
| 1. **Guidelines for sample preparation for shipment from the field unit to the national lab**
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| ⦁ Sample processing | It is recommended to wear gloves when storing and packaging samples for biosecurity reasonsDisinfect the outside of each tube before packing in the primary container (use 0.5% citric acid or 1% Virkon) |
| ⦁ Container | A sturdy, leakproof ice-box, or liquid nitrogen container can be used for transportation of samples |
| ⦁ Temperature | Cold condition (cooling conditions with ice pack or frozen condition by dry ice). |
| ⦁ Label | Place a label on the outside of the box to indicate the content, place of dispatch and place of destiny (include phone numbers of contact persons at both ends).Follow the regulation for transportation of infectious substance by air, road or rail |
| ⦁ Biosafety | Transportation of OP fluid samples should follow the biosafety and biosecurity principle in order to prevent the leaking and spread of hazard during transport. |
|  ⦁ Mode of transportation (speed/time) | By air, road or rail, the fastest way to the lab if possible |
| ⦁ Other important factors to consider | A list should accompany each shipment with details of all samples attached to the outside of the box. |
| 1. **Guidelines for sample storage at national and reference labs**
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| ⦁ Medium | Store in transport medium (see annex);For molecular diagnostic work the OP fluid in transportation medium may be mixed in a proportion of 1:1 in lysis buffer – e.g. 500 ul of OP Fluid + 500 ul of buffer (at least 200 ul total volume required for RNA extraction) and the rest may be stored at 80oC for long-term storage |
| ⦁Temperature | Better test the OP fluid samples as soon as possible.OP fluids must be stored under at -80oC for long-term storage. |
|  |
| ⦁Other important factors to consider | FMDV does not survive well at -20oC |
| **Guidelines for international transport** | Follow IATA guidelines |

**Appendix:**

* **Probang cup**

# For cattle: the cup is about 2.8 cm full diameter and 3.5 cm deep with a slightly sharpened edge, attached to a curved metal wire (70 cm in length).

# For calf: the cup is about 2.2 cm full diameter and 2.8 cm deep with a slightly sharpened edge, attached to a curved metal wire (70 cm in length).

# For goats and sheep: the cup is about 1-2 cm full diameter and 2-2.5 cm deep, attached to a curved metal wire (43 cm in length).

* **Disinfectants**

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| **Chemicals/Disinfectants** | **Concentration** | **User in the region** |
| Sodium carbonate | 2 % [w/v] in water | YTSAD, S. Korea |
| Speedyn | Iodophor as iodine 2.5%, W/V | RRL, Thailand |
| Citric acid | 0.5% [w/v] in tap water\*  | LVRI, China |
| Sodium carbonate | 2 % [w/v] in water | YASVI, China |

*\*OIE Terrestrial Manual 2018：* 0.5% [w/v] citric acid in tap water

* **OP fluid transport medium**

| **Users** | **Buffer** | **BSA** | **Phenol red** | **pH** | **Antibiotics** | **Eagle’s MEM** |
| --- | --- | --- | --- | --- | --- | --- |
| YTSAD, S. Korea | 0.08M PBS | 0.01% | 0.002% | 7.2 | Penicillin,1000 units/mlMycostatin,100 units/ml Neomycin 100 units/mlPolymyxin50 units/ml  | N/A  |
| RRL,Thailand  | 0.08M PBS | 1% | No | 7.4 | Penicillin 10,000 units/mlStreptomycin10,000 mg/mlKanamycin 10,000 mg/ml | N/A |
| LVRI, China | N/A | No | N/A | 7.4 | No | 1X Eagle’s MEM: 0.5% Lactalbumin hydrolysate/Earle’s medium (1:1) |
| AAHL,Australia | 0.08M PBS | 0.01% | 0.002% | 7.4±0.2 | penicillin, streptomycin and fungizone | N/A |
| YASVIChina | VTM for OP fluids and swab (nasal/oral)For 1 liter: sucrose, 68.46 g; HEPES, 5.96 g; KCl, 0.4 g; L-glutamic acid, 0.72 g; phenol red, 11.0 mg; CaCl 2 , 0.27 g; MgSO 4 .7H 2 O, 0.20 g; BSA, 5.0 g; gelatin, 5.0 g; vancomycin, 0.025 g; colistin, 200,000 units; amphotericin B, 1.0 mg. Adjust pH to 7.4. |  |  | 7.4 |  |  |

*\* OIE Terrestrial Manual 2018：0.08 M phosphate buffer containing 0.01%BSA, 0.002%, Phenol red, penicillin 1000 units/ml, mycostatin 100 units/ml, neomycin 100 units/ml, polymy* *polymyxin50 units/ml, pH 7.2.*