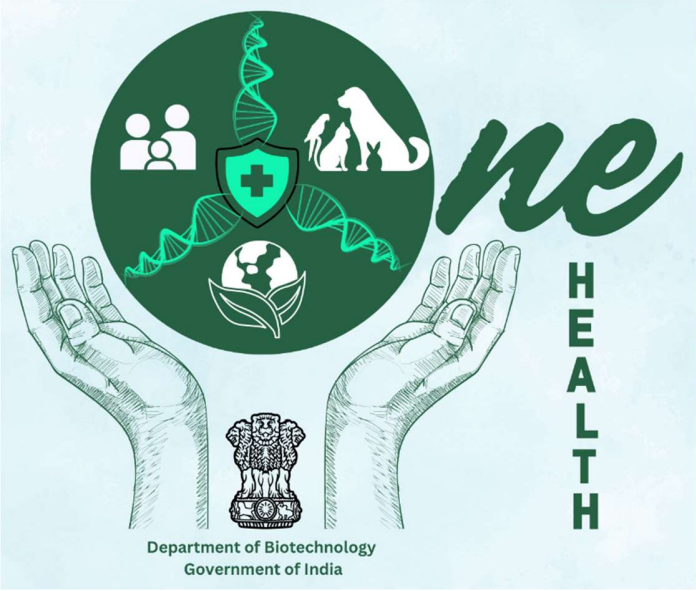
DEPARTMENT OF BIOTECHNOLOGY
MINISTRY OF SCIENCE & TECHNOLOGY , GOVERNMENT OF INDIA

NIAB
National Institute of Animal Biotechnology

Standard Operating Protocols for Screening of Zoonotic and Transboundary Diseases in Animals for the project entitled

'Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region'



	PREPARED BY	REVIEWED BY	APPROVED BY
NAME AND DESIGNATION	ONE HEALTH CONSORTIUM	PCMU	PMC
SIGN AND DATE			

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List of abbreviations

µg –	Microgram
µl –	Microlitre
AAU –	Assam Agricultural University
Ab –	Antibody
actA –	Actin filament protein A
AH&VD –	Directorate of Animal Husbandry & Veterinary
AIIMS –	All India Institute of Medical Sciences
ALOA test –	Agar Listeria according to Ottaviani and Agosti test
BGSA –	Bismuth Green Sulpha Agar
BOD –	Biochemical Oxygen Demand
bp –	Base pair
CAMP test –	Christie-Atkins-Munch-Petersen test
CAU –	Central Agricultural University
CCHF -	Crimean - Congo Haemorrhagic Fever
CYT –	Cysticercosis
DAH&VS –	Department of Animal Husbandry & Veterinary Sciences
DAHV&DD –	Department of Animal Husbandry, Veterinary & Dairy Development
DARD –	Department of Animal Resource and Development
DIL –	Disease Investigation Laboratories
DNA –	Deoxyribose Nucleic Acid
dNTP –	Deoxynucleotide Triphosphate
DV&AHS –	Department of Veterinary and Animal Husbandry Sciences
EDTA -	Ethylenediamine tetraacetic acid
ELISA –	Enzyme Linked Immunosorbent Assay
GADVASU –	Guru Angad Dev Veterinary and Animal Sciences
gm -	Gram
GMC –	Gandhi Medical College
HE agar –	Hektoen Enteric agar
hlyA –	Haemolysin A
HRP –	Horseradish Peroxidase
iELISA –	Indirect-ELISA
IgG –	Immuno-globulin G
InlC –	Invasion associated internalin gene
IVRI –	Indian Veterinary Research Institute
KCN broth –	Potassium Cyanide broth base
kg –	Kilogram
LIA –	Lysine decarboxylase
LSD –	Lumpy Skin Disease
M –	Molar
mAb –	Monoclonal Antibody
MAFSU –	Maharashtra Animal and Fishery Sciences University

ml – Millilitre
mM – Milli-molar
m PCR – Multiplex PCR
MR-VP test – Methyl Red-Voges Proskauer test
MTCC – Microbial Type Culture Collection and Gene Bank
NC – Negative Control
NIAB – National Institute of Animal Biotechnology
NIVEDI – National Institute of Veterinary Epidemiology and Disease Informatics
NP antigen – Nucleoprotein antigen
NRC on Meat – National Research Centre on Meat
OD – Optical Density
ODNC – Mean value of the Negative Control
ODPC – Mean value of the Positive Control
PALCAM agar – Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol agar
PBS – Phosphate Buffer Saline
PBST – Phosphate Buffer Saline – Tween20
PC – Positive Control
PCR – Polymerase Chain Reaction
PI – Percent Inhibition
plcA – Phosphatidylinositol-specific phospholipase C
pM – Pico-molar
PPE - Personal Protective Equipment
prfA – Positive regulatory factor-PrfA
PRRS – Porcine Reproductive and Respiratory Syndrome
QC – Quality Control
RMRC – Regional Medical Research Centre
RT – Room Temperature
S/P% - Sample/Positive control %
SBA – Sheep Blood Agar
sLPS – Smooth Lipo-polysaccharide
SOP – Standard Operating Protocol
TANUVAS – Tamil Nadu Veterinary and Animal Sciences University
Taq DNA Polymerase – *Thermus aquaticus* DNA Polymerase
Tb – Tuberculosis
TMB – 3,3',5,5'-Tetramethylbenzindine
TNMGRMR – Tamil Nadu Dr. M.G.R. Medical University
TSI agar – Triple Sugar Iron agar
UV transilluminator – Ultra-violet transilluminator
UVM-I broth – University of Vermont-I broth
WRTC – Wildlife Research & Training Centre

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Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region		
STANDARD OPERATING PROCEDURE		
TITLE: General SOP for animal sample collection		
SOP No: 1		
Version No.	**Effective Date**	**Next Revision Date**

Standard Operating Procedure

General SOP for animal sample collection

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Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region		
STANDARD OPERATING PROCEDURE		
TITLE: General SOP for animal sample collection		
SOP No: 1		
Version No.	**Effective Date**	**Next Revision Date**

1.1 General SOP for animal sample collection

1.1.1 Purpose

To obtain a standardized protocol for collection of samples from animals for disease diagnosis to safeguard good quality samples for testing with a focus on animal and human welfare.

To furnish guidance on the effective collection of samples for animal disease testing.

1.1.2 Scope

This SOP is applicable to all personnel participating in the acquisition of animal samples for disease testing within the specified facility or field.

1.1.3 Responsibilities

1. Veterinary Doctors and trained professionals are responsible for sample collection.
2. Laboratory technicians are tasked with processing and analyzing the samples which are to be collected.

1.1.4 Materials and Equipment

1. Gloves, masks, and hair covers.
2. 75% ethanol, surgical spirit and cotton.
3. Scalpel blade, disposable syringes, scissors, identification of labels / tags.
4. Sterile sample collection containers /tubes / vacutainers/plastic bags and swabs.
5. Ice packs or coolant for sample storage and transportation.
6. Transportation media.
7. Data sheets providing detailed information of collected samples.

1.1.5 Procedure

For successful and precise inference, proper collection of samples and their dispatch to the laboratory is necessary.

A. Pre-collection preparation

- Zoonotic pathogens constitute an occupational hazard for slaughterhouse workers, butchers and contact persons such as veterinarians, lab technicians, para vets, farmers, and farm workers. Hence care should be exercised in minimizing contact with suspected samples, and hence spill-over of zoonosis.
- Ensure comprehensive training for all the people involved in sample collection techniques that should adhere with the safety protocols.
- Ensure that all the sample collection containers are sterile and labelled.
- Before proceeding for sampling, identify the animals which have to be sampled, and record detailed history.

B. Sample Collection

- Select appropriate sample type based on the suspected disease and sampling guidelines.
- Employ aseptic methodologies during sample collection to minimize contamination risks.
- Ensure collecting an adequate volume to obtain accurate test results, based on type of investigation and disease to be diagnosed (As per Tabel 1).
- Properly label each sample container with distinct identification tags, including animal identification number, date, place, and sample type.
- Thereafter, document details pertaining to sample collection within the data log.

C. Sample Handling and Transportation

- Ensure that the samples are stored and transported using icepacks/ coolant
- Ensure that the samples are stored and transported using icepacks/coolant to

maintain the sample integrity.

- Samples collected on swabs have to be carried by keeping them moist in recommended storage medium.
- Fulfil all essential documentation requirements for sample transportation, encompassing regulatory obligations for hazardous materials as applicable.

D. Post-Collection Procedures

- Promptly transport/carry samples to the designated laboratory or processing facility without delay.
- Inform the laboratory personnel of sample arrival and provide relevant documentation.
- Upon receipt of the sample, the laboratory personnel must store the samples accordingly either in 4˚C, -20˚C or -80˚C until further usage.

E. Reporting and Documentation

- Maintain comprehensive records pertaining to sample collections; including animal species, type of sample collected, volume of sample collected, collection date, location, and handling protocols.
- Fill the sample submission forms properly without any errors.
- Ensure secure archival of all documentation, facilitating accessibility for further reference in the future.

F. Training and Communication

- Conduct training sessions for all personnel engaged in sample collection, ensuring proficient adherence to the prescribed procedures outlined in this SOP.
- Promptly share any updates or revisions to the SOP to pertinent staff members through effective communication channels.

G. Review and Revision

- Carry out regular evaluations of this SOP to include feedback, any other

guidelines and alterations in testing procedures.

- Revise regularly as it is required to uphold alignment with contemporary best practices and regulatory standards.

H. Compliance

- Strict compliance with this SOP is obligatory for all personnel engaged in animal sample collection for disease testing.

Table 1: List of targeted diseases, type of sample to be collected and respective testing methods for the purpose of estimating the prevalence of the indicated diseases for One Health program.

S. No.	Pathogen	Type of Sample	Test kit
1	**African Swine Fever**	Serum	INgezim diagnostic kit
2	**Brucellosis**	Serum	BruAlert kit
3	**Crimean - Congo Haemorrhagic Fever (CCHF)**	Serum	ID Screen® CCHF Double Antigen Multi-species ELISA kit
4	**Cryptosporidiosis**	Stool/Faeces	Monoscreen Ag ELISA kit for *Cryptosporidium*.
5	**Cysticercosis**	Serum	BT LAB Porcine Cysticercosis, CYT antibody, IgG ELISA kit (Cat. No. ED0017Po)
6	**Swine Influenza**	Serum	Ingezim Influenza Porcina 2.0, Spain, Product Ref. 11.FLU.K1) ID Screen Influenza A nucleoprotein swine indirect (FLUMPS-SW) from ID-Vet

7	**Japanese encephalitis**	NA	NA
8	**Listeriosis**	Faeces, blood, cerebrospinal fluid, aborted material/ fluids, milk	Bacterial culturing and PCR
9	**Lumpy Skin Disease**	Serum	ID Screen® Capripox Double Antigen Multi-species kit
10	**Nipah Virus Disease**	Serum	Indirect ELISA
11	**Porcine Reproductive and Respiratory Syndrome**	Serum	INgezim PRRS 2.0; Product ref. 11.PR2.K1
12	**Q Fever**	Serum	ID Screen® Q Fever Indirect Multi-species kit
13	**Salmonellosis**	Stool/faeces	Bacterial culturing, PCR and Widal test
14	**Scrub typhus**	NA	NA
15	**Tuberculosis**	Milk and nasal swabs	DNA isolation using Qiagen QIA-amp DNA Blood Mini Kit (Cat No: 51104) and Qiagen DNeasy Blood & Tissue Kit (Cat No: 69506) and TaqMan Real-Time PCR test

Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region		
STANDARD OPERATING PROCEDURE		
TITLE: SOPs for animal species-specific sample collection		
SOP No. 2		
Version No.	**Effective Date**	**Next Revision Date**

2.1 SOPs for animal species-specific sample collection

2.1.1 Purpose

To obtain a standardized protocol for collection of samples from animals, viz., Bovines (cattle, buffaloes), pigs, goats and sheep, to safeguard good quality samples for testing.

2.1.2 Scope

This SOP is applicable to all personnel participating in the acquisition of animal samples for testing within the specified facility or field.

2.1.3 Responsibilities

1. Veterinary doctors and trained professionals are responsible for sample collection.
2. Laboratory technicians are tasked with processing and analysing the samples which are to be collected.

2.1.4 Materials Required

1. Gloves, masks, coveralls, eye protection and hair cover.
2. 75% ethanol, surgical spirit, gauge and cotton.
3. Scalpel Blade, disposable syringes, scissors, labels / tags.
4. Omni nasal swabs.
5. Sterile white-capped sample containers, 15 ml centrifuge tubes, fecal loops, blood collection vacutainers (without EDTA coating), plastic biohazard bags and cotton swabs.
6. Plastic box/containers for discarding sharps.
7. Disposable syringes/vacutainers with needle having 16, 18 and 20 gauge.
8. Ice packs or coolant for sample storage and transportation.
9. Disinfectant – Sodium hypochlorite
10. Sterile 1X PBS
11. Data sheets providing detailed information of collected samples.

2.1.5 Procedure

A. SOPs for collection of serum samples from bovines (cattle/buffaloes)

a. Sample collection –

- Restrain the animal in a halter using rope(s) and secure lead to the stanchion with a quick-release knot with the head elevated and the jugular vein exposed.
- Place the thumb at the base of the jugular groove near the bottom part the neck, and select an area which has easy access to the vein.
- If necessary, clip the hair without injuring the animal.
- Swipe with antiseptic gauze to remove superficial dirt and debris. This may also assist in visualizing raised vein.
- Occlude jugular vein by applying pressure at the base of the jugular groove and visualize raised vein.
- With bevel up, insert needle (18-gauge vacutainer needle) firmly into skin and into vein at 20˚ angle.
- Stabilize needle and push the vacutainer tube (without EDTA) into the hub. If you have hit the vein, blood will flow freely the into tube. Multiple tubes can be filled by removing filled tube and replacing with fresh tube.
- **NOTE:** Do not pull the needle out of vein with vacutainer tube still attached as this will release vacuum in vacutainer.
- If you have missed the vein, carefully reposition the needle, with the vacutainer attached, until the vein penetrated. The vein is fairly deep and may roll away from needle. Typically, no more than two to three attempts should be made at a time to minimize distress to the animal and potential damage to the vein.
- All vacutainers should be filled with the required volume indicated with black coloured fill lines at the side of the label.
- Alternately, you can use needle and syringe. In this case, break the seal on the syringe by gently pulling back before using. Clear the air, and with the needle attached to the syringe, insert the needle firmly at 20˚ angle, and aspirate syringe to confirm insertion and collect blood.

- Once collection is complete, remove vacutainer tube (or the syringe), then, applying pressure over injection site, remove the needle.
- Clean the site of injection with 70% alcohol and apply pressure using cotton gauze to avoid bleeding. In order to ensure adequate haemostasis, apply pressure with gauze for 30 to 60 sec.
- Dispose of the needle in an approved sharps container.
- Put the label on the vacutainer/tube mentioning the serial number of the animal and other related details.
- Blood can also be collected *via* the coccygeal vein. The following procedure should be followed in this case –
  - Restrain the animal. Raise the tail vertically until it is horizontal to the ground.
  - Locate the groove lying in the ventral midline of the tail.
  - Swab the site with antiseptic.
  - Midway along the body of a coccygeal vertebra, insert the needle (18-gauge) perpendicularly to the surface of the skin to a depth of a few millimetres.
  - Withdraw blood sample and remove the needle.
  - Dispose of the needle in approved sharps container.
  - In order to ensure adequate haemostasis, apply pressure with gauze for 30 to 60 sec.

b. Sample processing –

- Serum, which is to be used in further testing has to be separated out from whole blood.
- The sample tubes should be kept in vertical position to allow the blood to clot for a minimum of 30 min. to 90 min. at room temperature.
- After the specimen has been allowed to fully clot, the tube is to be centrifuged ideally within 1 - 2 hr. of sample collection. If not, the sample must be stored at 4˚C.
- After clotting is complete, centrifuge the tubes at 1300 *g* for 15 min. using a fixed angle rotor.

- Carefully remove the tubes from the rotor, without disturbing the clotted blood.
- Pipette out the serum leaving a small amount on top of packed cells, into 1.5 - 2 ml microcentrifuge tubes, depending on the volume of the sample. Mix the sample by pipetting up and down (avoid foaming).

c. Sample storage –

- Aliquot the serum sample in 1.5 - 2 ml microcentrifuge tubes/cryovials.
- Aliquoting of sample should be performed in biosafety cabinet considering the infectious/hazardous nature of the sample.
- Sample should be aliquoted in appropriate volumes as mentioned below:

Volume of the sample collected	**Volume of the aliquots**	**Number of aliquots**
>2 ml	500 µl	4-6 (depending on the volume of sample)
2 ml	500 µl	4
1 ml	500 µl	2
<1 ml	150 - 250 µl	2

d. Precautions -

- Blood collection from animals should be performed by veterinary doctors, following all the protocols for sample collection.
- Always wear PPE to minimize the risk of contamination and infection.
- To avoid hematoma, appropriate gauge needle should be used. For serum separation, EDTA free vacutainer/tubes should be used for blood collection.

- Disinfect the site of injection before and after drawing blood using 70% ethanol.
- Use sterile single-use devices and needles.
- Minimize the stress experienced by the animal during blood collection to reduce the chances of haemolysis.
- After sample collection, the blood should be gently mixed, and the tubes should be gently placed in a slanted position.
- Never place original collected tube in freezer. Freeze only the labelled serum aliquot samples.
- Carefully wipe all the surfaces having blood droplets with 70% ethanol.
- Dispose of the sharps in a separate box/container. Waste should be collected and discarded after autoclaving.

B. SOPs for collecting faeces samples from bovines (cattle/buffaloes)

a. Sample collection and processing -

- Faecal samples should be fresh, preferably collected from the animal during the act of defecation or from the rectum using a faecal loop during physical examination.
- Collect the fresh faeces in a dry, sterile and sealed white-capped plastic container.
- Try to collect the sample from beginning, middle and end section of the faeces.
- Label the specimen container with serial number of the animal, date of collection and any other specific detail.
- Place the specimen vials in biohazard bag, seal the bag and transport the sample to the laboratory with ice packs.

b. Sample storage –

- Samples should be shipped to the laboratory at 4˚C along with ice packs.
- Samples should be stored at 2 – 4˚C, if they are to be processed immediately or otherwise at -20˚C.

c. Precautions -

- Wear all the PPE while collecting the sample.
- Handle the animal with utmost caution.
- Secure the sample container and close it tightly to avoid spillage.
- Transport the collected specimens to the laboratory within 72 hr.. If unable to transport the same day, keep the specimen refrigerated (4˚C) or frozen.

C. SOPs for collection of milk samples from bovines (cattle/buffaloes; for TB testing)

a. Sample collection -

- Animals should be restrained inside Travis before collection of milk sample.
- Wear nitrile gloves while milking.
- Clean the teat(s) thoroughly with gauze pads soaked in 70% ethyl alcohol.
- Allow the teat to dry completely.
- Squeeze the teat and discard the first milk to prevent contamination.
- Avoid sampling from infected/inflamed teat and from animals with mastitis.
- Collect ~50 ml milk directly into a sterile 50 ml centrifuge tubes in equal volume from all the available teats.
- Label the tubes properly, seal tightly and store in ice box containing frozen ice packs and transported to laboratory within 24 hr. for further processing.

b. Sample processing prior to DNA isolation (Zumárraga et al., 2012) -

- Centrifuge the raw milk at 1,000 g for 15 min at 4°C.
- Discard the supernatant carefully without disturbing the cell pellet and avoid mixing of fat layer into the pellet.

- Resuspend the pellet in 1 ml of 1X PBS and transfer into 1.5 ml centrifuge tube. Avoid mixing of fat layer while transferring suspended pellet from 50 ml centrifuge tube in to 1.5 ml tube. Centrifuge at 3,000 g for 5 min. and discard the supernatant.
- Re-suspended the pellet in 200 µl of sterile 1X PBS and heat to 70°C for 70 min. in dry bath to kill the infectious pathogens if any, present in the sample and then store at - 20°C until genomic DNA extraction.

c. Sample storage -

- Samples should be shipped to the laboratory at 4˚C along with ice packs.
- Samples should be stored at 2 - 4˚C, if they are to be processed immediately or otherwise at -20˚C.

d. Precautions –

- Wear all the PPE while collecting the sample.
- Handle the animal with utmost caution.
- Secure the sample container and close it tightly to avoid spillage.
- Transport the collected specimens to the laboratory immediately. If unable to transport the same day, keep the specimen refrigerated (4˚C) or frozen.

D. SOPs for collection of nasal swab samples from bovines (cattle/buffaloes)

a. Sample collection –

- Animals should be restrained inside Travis properly before sample collection.
- Clean the outside of nostril with a sterile kitchen tissue paper.
- Insert sterile OmniSwabs / Himedia cotton swab approximately 5 cm into the nostril and swab against nasal mucosa for 10-20 seconds. Samples should be collected from both the nostrils separately.

- Place both the nasal swabs directly in a sterile 15 ml centrifuge tube containing 1 ml of sterile 1X PBS.

b. Sample storage –

- Keep the samples in ice box containing frozen ice packs during transportation and subsequently freeze at -80°C within 12-24 hr.

c. Precautions –

- Wear all the PPE while collecting the sample.
- Handle the animal with utmost caution.
- Secure the sample container and close it tightly to avoid spillage.
- Transport the collected specimens to the laboratory immediately. If unable to transport the same day, keep the specimen refrigerated (4˚C) or frozen.

E. SOPs for collection of serum samples from Pigs

a. Sample collection –

- Restrain the animal with a snare, securely contained against a wall or corner; alternatively, the swine can be placed in a sling, while smaller pigs can be held or placed in v-trough.
- Clean with gauze as needed to remove superficial dirt and debris.
- Select the appropriate needle as mentioned below in the table:

Size of Pig	**Gauge**
Baby pigs (up to 25 kg)	20
Grower (25 to 70 kg)	18
Finisher/Breeders (>70 kg)	16

- For blood collection from jugular vein locate jugular furrow, and align with point of the shoulder and point of the manubrium. With bevel up, insert the needle perpendicular to the skin.
- If using a vacutainer, once needle is inserted, stabilize the needle and push the vacutainer tube into hub. If you have hit the vein, blood will flow freely into tube. Multiple tubes can be filled by removing filled tube and replacing with fresh tube.
- **NOTE:** Do not pull needle out of vein with vacutainer tube still attached as this will release the vacuum in the vacutainer.
- If you have missed the vein, you can carefully reposition needle, with vacutainer attached, until the vein is penetrated. The vessel is fairly deep and may roll away from needle. Typically, no more than two to three attempts should be made at a time to minimize distress to the animal and potential damage to the vein.
- Alternately, you can use needle and syringe. Break the seal on the syringe by gently pulling back before using. Clear the air, and with the needle attached to the syringe, insert the needle firmly at 90° angle, and aspirate syringe to confirm insertion and collect blood.
- Once collection is complete, remove the vacutainer tube, then, applying pressure over the injection site, remove the needle.
- Dispose of the needle in approved sharps container.
- In order to ensure adequate hemostasis, apply pressure for 30 to 60 sec.
- Alternately, blood can be collected from anterior vena cava, following the procedure below:
  - Using same technique as listed above, with a needle up to 4 inches in length.
  - Insert the needle alongside the breastbone at approximately 90°, directed slightly inwards towardsthe spine.
  - Insert straight in, and remove needle slowly in same manner in order to avoid lacerating the vessel.
  - In order to ensure adequate hemostasis, apply pressure with gauze for 30 to 60 sec.

b. Sample processing –

- Serum, which is to be used in further testing has to be separated out from whole blood.
- The sample tubes should be kept in vertical position to allow the blood to clot for a minimum of 30 - 90 min. at room temperature.
- After the specimen has been allowed to fully clot, the tube is to be centrifuged ideally within 1-2 hr. of sample collection. If not, the sample must be stored at 4˚C.
- After clotting is complete, centrifuge the tubes at 1300 *g* for 15 min. using a fixed angle rotor.
- Carefully remove the tubes from the rotor, without disturbing the clotted blood.
- Pipette out the serum leaving a small amount on top of packed cells, into 1.5-2 ml microcentrifuge tubes, depending on the volume of the sample. Mix the sample by pipetting up and down (avoid foaming).

c. Sample storage –

- Aliquot the serum sample in 1.5-2 ml microcentrifuge tubes/cryovials.
- Aliquoting of sample should be performed in biosafety cabinet considering the infectious/hazardous nature of the sample.
- Sample should be aliquoted in appropriate volumes as mentioned below:

Volume of the sample collected	Volume of the aliquots	Number of aliquots
>2 ml	500 µl	4-6 (depending on the volume of sample)
2 ml	500 µl	4
1 ml	500 µl	2
<1 ml	150 - 250 µl	2

d. Precautions -

- Blood collection from animals should be performed by veterinary doctors, following all the protocols for sample collection.
- Always wear PPE to minimize the risk of contamination and infection.
- To avoid the hematoma appropriate gauge needle syringe should be used. For serum separation, EDTA free vacutainer/tubes should be used for blood collection.
- Disinfect the site of injection before and after drawing blood using 70% ethanol.
- Use sterile single-use devices and needles.
- Minimize the stress experienced by the animal during blood collection to reduce the chances of haemolysis.
- After sample collection, the blood should be gently mixed, and the tubes should be gently placed in a slanted position.
- Never place original collected tube in freezer. Freeze only the labelled serum aliquot samples.
- Carefully wipe all the surfaces having blood droplets with 70% ethanol.
- Dispose of the sharps in a separate box/container. Waste should be collected and discarded after autoclaving.

F. SOPs for collection of serum samples from goats and sheep

a. Sample collection –

- Restrain animal with head elevated and jugular vein exposed.
- Stand the sheep with the animal's back against your legs. Alternatively, set the sheep on its rump with its back against your legs. Hold the head of the sheep at about a 30˚ angle to the side to extend neck and expose the jugular area.

- Collection is most easily performed with two handlers - one to restrain and one to collect the blood. With experience, one handler can hold and collect the blood – with sheep set between the legs, tuck the head under arm, and access the jugular vein from above.
- Clip a small area over the jugular groove, and swipe with antiseptic gauze to remove superficial dirt and debris. This may also assist in visualizing the raised vein.
- Occlude the jugular vein by applying pressure at the base of the jugular groove and visualize the raised vein.
- With the bevel up, insert the needle through the skin and into vein at a 20˚ angle.
- Using vacutainer method - once needle (18-gauge) is inserted, stabilize the needle and push the vacutainer tube into the hub. If you have hit the vein, blood will flow freely into the tube. Multiple tubes can be filled by removing filled tube and replacing with fresh tube.
- Using needle and syringe method - before use, break the seal on the syringe by gently pulling back the plunger. Clear the air, and with the needle attached to the syringe, insert the needle at 20° angle, and aspirate syringe to confirm insertion and collect blood.
- If you have missed the vein, you can carefully reposition the needle until the vein is penetrated. Vessel may be fairly deep and roll away from needle. Typically, no more than two to three attempts should be made at a time to minimize distress to the animal and potential damage to the vein.
- **NOTE:** When using the vacutainer, do not pull the needle out of the skin with the vacutainer tube attached, as this will cause the vacuum to be lost.
- Once the blood collection is complete, release the pressure to the vein (and detach the vacutainer tube if used), then, applying pressure over the injection site with gauze, remove the needle.
- Dispose of the needle in approved sharps container.
- In order to ensure adequate haemostasis, apply pressure with gauze for 30 to 60 seconds.
- Alternately, blood can be collected from cephalic vein and/or femoral vein.

b. Sample processing –

- Serum, which is to be used in further testing has to be separated out from whole blood.
- The sample tubes should be kept in vertical position to allow the blood to clot for a minimum of 30 - 90 min. at room temperature.
- After the specimen has been allowed to fully clot, the tube is to be centrifuged ideally within 1-2 hr. of sample collection. If not, the sample must be stored at 4˚C.
- After clotting is complete, centrifuge the tubes at 1300 *g* for 15 min. using a fixed angle rotor.
- Carefully remove the tubes from the rotor, without disturbing the clotted blood.
- Pipette out the serum leaving a small amount on top of packed cells, into 1.5 - 2 ml microcentrifuge tubes, depending on the volume of the sample. Mix the sample by pipetting up and down (avoid foaming).

c. Sample storage –

- Aliquot the serum sample in 1.5 - 2 ml microcentrifuge tubes/cryovials.
- Aliquoting of sample should be performed in biosafety cabinet considering the infectious/hazardous nature of the sample.
- Sample should be aliquoted in appropriate volumes as mentioned below:

Volume of the sample collected	Volume of the aliquots	Number of aliquots
>2 ml	500 µl	4-6 (depending on the volume of sample)
2 ml	500 µl	4
1 ml	500 µl	2
<1 ml	150 - 250 µl	2

d. Precautions -

- Blood collection from animals should be performed by veterinary doctors, following all the protocols for sample collection.
- Always wear PPE to minimize the risk of contamination and infection.
- To avoid hematoma, appropriate gauge needle should be used. For serum separation, EDTA free vacutainer/tubes should be used for blood collection.
- Disinfect the site of injection before and after drawing blood using 70% ethanol.
- Use sterile single-use devices and needles.
- Minimize the stress experienced by the animal during blood collection to reduce the chances of haemolysis.
- After sample collection, the blood should be gently mixed, and the tubes should be gently placed in a slanted position.
- Never place original collected tube in freezer. Freeze only the labelled serum aliquot samples.
- Carefully wipe all the surfaces having blood droplets with 70% ethanol.
- Dispose of the sharps in a separate box/container. Waste should be collected and discarded after autoclaving.

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3.1 Applicable to

The procedure applied to the detection of antibodies against **African Swine Fever disease** using **INgezim diagnostic iELISA kit.**

3.2 Objective

To provide step-wise guidelines on performing the assay to detect antibodies in the sera of pigs for African Swine Fever disease.

3.3 Scope

The SOP covers the details about the responsibilities of various lab personals, requirement of the equipment, materials, sample for diagnosis, procedures and general safety practices.

3.4 Responsibility

The laboratory personnel must be familiar with all the laboratory safety procedures and guidelines.

3.5 Equipment

a) ELISA microplate reader
b) Table top centrifuge
c) Bio safety cabinet
d) Water bath

3.6 Materials

a) 96-wells microtitration strip plates divided in 12 strips of 8 wells each.
b) Bottles with washing solution (25X concentrated).
c) Bottles with diluent ready-to-use.
d) Bottles with ready-to-use substrate.

e) Bottles with stop solution.
f) Kit controls; Ready-to-use Positive control serum and Negative control serum.
g) Centrifuge tubes (1.5 ml and 2 ml)
h) Glassware/plasticware for diluting solutions
i) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl)
j) Multichannel pipette 30-300 µl
k) Squirt bottle, manifold dispenser, or automated microplate washer
l) Absorbent paper for blotting the microtiter plate
m) 100 ml and 500 ml graduated cylinders
n) De-ionized or distilled water
o) Gloves and other PPE

3.7 Specimen collection, processing and transport

a) Blood samples are collected from pigs and serum is separated as described in section E of SOP 2.
d) Entry should be made in the log for the samples received.

3.8 Procedure

Test is to be carried out and results interpreted as per manufacturer's protocol for INgezim diagnostic iELISA kit.

a) Sera samples must be tested at a dilution of 1/20 (i.e., 5 µl of serum in 100 µl) in dilution buffer.
b) Dilute one part of the concentrated washing solution provided in the Kit with 24 parts of distilled or deionized water. Once ready, this solution remains stable between +2ºC and +8ºC.
c) Diluent, Control sera, Conjugate and Substrate: All are supplied ready to use. Bring all the reagents to room temperature before use.
d) Add 100 µl of positive control to two wells of the plate, 100 µl of the negative control to another two wells and 100µl of each of the dilutions of sera to be tested (prepared according to previous instructions) in the remaining wells of the plate. Seal the plate and incubate for 1 hr. ± 5 min. at 37ºC.

e) Wash three times following the described procedure in the kit manual.
f) Add 100 µl of conjugate ready to use. Seal the plate and incubate for 45 ± 5 min. at 37ºC.
g) Wash 5 times following the described procedure as mentioned in the instruction manual.
h) Add 100 µl of substrate solution to each well. Keep the plate for 15 ± 1 min. at room temperature.
i) Add 100 µl of stop solution to each well.
j) Read absorbance at 405 nm within 5 min. after the addition of stop solution.

3.9 Calculation of cut-off value

Calculate the S/P %:

$$S/P\% = \frac{\text{(absorbance of sample)} - \text{(absorbance of Negative control)}}{\text{(absorbance of Positive control)} - \text{(absorbance of Negative control)}} \times 100$$

3.10 Validity of the test

The assay will be valid only when:
a) OD of positive control is higher than 0.75
b) OD of negative control is lower than 0.25

3.11 Interpretation of the test

S/P % values equal or higher than 50% must be considered positive.
S/P % values lower than 45% must be considered negative.
S/P % between both values must be doubtful.

3.12 Precautions and general safety practices

a) Handling of the blood/serum samples should be done with utmost care.
b) Eating, drinking, smoking, applying cosmetics and handling of contact lenses is

prohibited while working in the laboratory.

3.13 After the test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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4.1 Applicable to

This procedure applies for detection of antibodies against **Brucellosis** by **BruAlert blocking ELISA kit.**

4.2 Objective

To describe step-wise requirements and procedure for detection of antibodies against *Brucella* species, by BruAlert blocking ELSIA kit.

4.3 Scope

The SOP covers the details about the responsibilities of various lab personnel, requirement of the equipment, materials, sample collection and processing, procedures, general safety practices and references.

4.4 Responsibility

It is the responsibility of the lab in-charge to ensure that he/she or person performing the test to be familiar with the lab safety procedures. The interpretation of the results must be done by the person trained in the procedure.

4.5 Equipment

a) ELISA microplate reader or Multimode reader
b) Table top mini centrifuge
c) Biosafety cabinet

4.6 Materials

a) Brucella sLPS coated microwells (96 wells)
b) Wash buffer
c) Sample diluent

d) HRP Conjugated brucella specific mAb
e) Substrate
f) Kit controls; Positive control and Negative control
g) Stop solution
h) Centrifuge tubes (1.5 ml and 2 ml)
i) Glassware/plasticware for diluting solutions
j) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl).
k) Multichannel pipette 30-300 µl
l) Squirt bottle, manifold dispenser, or automated microplate washer
m) Absorbent paper for blotting the microtiter plate
n) 100 ml and 500 ml graduated cylinders
o) De-ionized or distilled water
p) Gloves and other PPE

4.7 Sample collection, processing and transport

a) Blood samples are collected from bovines and goat & sheep and serum is separated as described in section A and F, respectively of SOP 2.
b) Entry should be made in the log for the samples received.

4.8 Procedure

Test is to be carried out and the results are interpreted as per manufacturer's protocol for BruAlert blocking ELSIA kit.

a) Equilibrate all reagents to room temperature (18-25°C).
b) Remove the required number of microwells from the foil sachet and insert into the strip holder. Along with the samples, six microwells are required, that is, for negative control (N), positive control (P) and quality control (QC) in duplicate. The remaining unused wells should be sealed tightly and replaced in the foil sachet.
c) Pipette 100 µl of positive, negative and quality controls to respective control wells.

d) Pipette 90 µl of sample diluents to the sample wells and 10 µl of sera samples to the respective sample wells.
e) Gently mix the contents of the wells, seal the plate and incubate for 1 hr. at room temperature (RT; 20-25°C).
f) Wash 4 times with PBST (PBS + 0.05% Tween-20; 300 µl per well).
g) Add 100 µl of conjugate to each well. Seal the plate and incubate for 1 hr. at RT.
h) Wash 4 times with PBST.
i) Add 100 µl of substrate (TMB) to each well. Keep the plate for 10 min. at RT.
j) Add 100 µl of stop solution to each well.
k) Read optical density (OD) at 450 nm within 5 min. after addition of stop solution.
l) Calculate the Percentage Inhibition (PI) value:

$$PI = 100 - \{(\text{Test sample OD}/\text{Negative control OD})\} \times 100$$

4.9 Interpretation of the test

a) PI value 40 and above is declared positive for brucellosis.
b) PI value below 40 is declared negative for brucellosis.

4.10 Precautions and general safety practices

a) Since brucellosis is a zoonotic disease, all samples should be considered as infectious and hazardous, handling of such samples should be done in biosafety cabinet with appropriate PPE.
b) Handling of the blood/serum samples should be done with utmost care.
c) Eating, drinking, smoking, applying cosmetics and handling of contact lenses is prohibited while working in the laboratory.
d) All laboratory personnel should immediately report development of any symptoms to the concerned lab in-charge.

4.11 After the test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be essentially maintained.

c) All samples should be discarded in 1% sodium hypochlorite and autoclaved to disinfect prior to disposal.

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5.1 Applicable to

This procedure applies for detection of **Crimean - Congo Haemorrhagic Fever Virus (CCHFV)** specific antibodies in serum samples by **ID Screen® CCHF Double Antigen Multi-species ELISA kit.**

5.2 Objective

To describe step wise requirements and procedure for detection of CCHF virus specific antibodies in sera by iELISA.

5.3 Scope

The SOP covers the details about the responsibilities of various lab personnel, requirement of the equipment, materials, sample for diagnosis, procedures and general safety practices.

5.4 Responsibility

It is the responsibility of the lab in-charge to ensure that he/she or person performing the test to be familiar with the lab safety procedures. The interpretation of the results must be done by the person trained in the procedure.

5.5 Equipment

a) ELISA microplate reader
b) BOD incubator
c) Table top centrifuge

5.6 Materials

a) Microplates coated with CCHFV recombinant nucleoprotein
b) Concentrated Conjugate (10X), freeze-dried
c) Reconstitution Buffer

d) Dilution Buffer 14
e) Wash Concentrate (20X)
f) Substrate Solution
g) Stop Solution (0.5 M)
h) Kit controls; Positive control and Negative control.
i) Centrifuge tubes (1.5 ml and 2 ml)
j) Glassware/plasticware for diluting solutions
k) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl).
l) Multichannel pipette 30-300 µl
m) Squirt bottle, manifold dispenser, or automated microplate washer
n) Absorbent paper for blotting the microtiter plate
o) 100 ml and 500 ml graduated cylinders
p) De-ionized or distilled water
q) Gloves and other PPE

5.7 Sample collection, processing and transport

a) Blood samples are collected from bovines and goat & sheep and serum is separated as described in section A and F, respectively of SOP 2.
b) Entry should be made in the log for the samples received.

5.8 Procedure

Test is to be carried out and the results interpreted as per manufacturer's protocol for ID Screen® CCHF Double Antigen Multi-species ELISA kit.

a) In the ELISA microplate, add:
   - 50 µL of Dilution Buffer 14 to each well.
   - 30 µL of the Negative Control to wells A1 and B1.
   - 30 µL of the Positive Control to wells C1 and D1.
   - 30 µL of each sample to be tested to the remaining wells.
b) Cover the plate and incubate 45 min. ± 4 min. at 21°C (± 5°C).
c) Prepare the Conjugate 1X by diluting the Concentrated Conjugate 10X to 1:10 in Dilution Buffer 14.

d) Empty the wells. Wash each well 5 times with at least 300 µL of the Wash Solution. Avoid drying of the wells between washes.
e) Add 50 µL of the Conjugate 1X to each well.
f) Cover the plate and incubate 30 min. ± 3 min. at 21°C (± 5°C).
g) Empty the wells. Wash each well 5 times with at least 300 µL of the Wash Solution. Avoid drying of the wells between washes.
h) Add 100 µL of the Substrate Solution to each well.
i) Cover the plate and incubate 15 min. ± 2 min. at 21°C (± 5°C) in the dark.
j) Add 100 µL of the Stop Solution to each well, in the same order as in step 'h' to stop the reaction.
k) Read and record the OD at 450 nm.

5.9 Calculation of the test

The S/P percentage is calculated by:

$$S/P\% = \frac{\text{OD value of the sample}}{\text{OD value of Positive control}} \times 100$$

5.10 Validity of the test

The assay will be valid only when:
a) The mean value of the Positive control OD (ODPC) is greater than 0.350.
b) The ratio of the mean values of the Positive and Negative controls (ODPC and ODNC) is greater than 3.

5.11 Interpretation of the test

Samples presenting a S/P percentage (S/P %):
- Less than or equal to 30% are considered negative.
- Greater than 30% are considered positive.

Result	Status
S/P% ≤ 30%	Negative
S/P% > 30%	Positive

5.12 Precautions and general safety practices

a) Handling of the blood/serum samples should be done with utmost care.
b) Eating, drinking, smoking, applying cosmetics and handling of contact lenses is prohibited while working in the laboratory.

5.13 After the test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be essentially maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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6.1 Applicable to

This procedure applies to the detection of antibodies against **Cryptosporidium** using the **Mono Screen Ag ELISA Cryptosporidium kit**.

6.2 Objective

The purpose of this procedure is to describe the step-by-step requirements and procedures for the diagnosis of Cryptosporidiosis using the appropriate diagnostic kit.

6.3 Scope

This SOP covers the responsibilities of various lab personnel, equipment requirements, materials, sample collection and processing, procedures, general safety practices, and references.

6.4 Responsibility

It is the responsibility of the lab in-charge to ensure that they or the person performing the test are familiar with the lab safety procedures. The interpretation of the results must be done by a person trained in the procedure.

6.5 Equipment

a) ELISA microplate reader
b) Table top centrifuge
c) Biosafety cabinet

6.6 Materials

a) Microplates: Two 96 well microtitration plates (8 X 12 wells microtiter plates). Rows A, C, E, G are coated with anti-Cryptosporidium specific antibodies (Crypto +ve), while rows B, D, F, H are coated with non-specific antibodies

(Crypto -ve).

b) Washing solution: One 100 ml bottle of 20X concentrated washing solution.
c) Dilution buffer: One 50 ml bottle of 5X concentrated dilution buffer.
d) Conjugate: One 25 ml bottle of anti-Cryptosporidium peroxidase conjugate (Horseradish peroxidase-labelled anti-Cryptosporidium monoclonal antibody).
e) Positive Control: 1 vial of 4 ml containing Cryptosporidium oocyst antigen.
f) Chromogen solution/Single component TMB: One 25 ml bottle of tetramethylbenzidine (TMB).
g) Stopping solution: One 15 ml bottle of 1 M phosphoric acid stop solution.
h) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl).
i) Multichannel pipette 30-300 µl
j) Dispenser tips.
k) Reagent reservoir for multichannel pipettes.
l) Squirt bottle, manifold dispenser, or automated microplate washer.
m) Absorbent paper for blotting the microtiter plate.
n) 100 ml and 500 ml graduated cylinders.
o) Distilled water.
p) Gloves and other PPE.

6.7 Sample Collection, Processing, and Transport

a) Blood samples are collected from bovines and goat & sheep and serum is separated as described in section A and F, respectively of SOP 2.
b) Entry should be made in the log for the samples received.

6.8 Procedure

The test may be carried out and the results interpreted as per manufacturer's protocol for Mono Screen Ag ELISA Cryptosporidium kit.

a) Bring all the reagents to room temperature before use.
b) Dilute the concentrated washing solution (20X) into washing solution (1X) with distilled water, ensuring no salt crystals remain.
c) Dilute the concentrated dilution buffer (5X) into Dilution Buffer (1X) with

distilled water.

d) Store the Washing solution (1X) and Dilution Buffer (1X) between +2°C and +8°C when not in use. Once diluted, these solutions remain stable for 6 weeks if kept between +2°C and +8°C
e) Dilute faecal/stool samples per volume into Dilution buffer (1X). This is a qualitative dilution only, which must allow the pipetting of faecal suspensions. Take 0.2-0.5 g of fresh or thawed frozen stool samples and add Dilution buffer (1 X) (approximately 1:4 dilution v/v). Do not centrifuge the faecal suspensions
f) Add 100 µl aliquots of the diluted samples to the wells as follows: sample 1 in wells A1 and B1, sample 2 in wells C1 and D1, etc. Proceed in the same manner for the positive control (e.g., G1 and H1).
g) Cover with a lid and incubate the plate at 21°C ± 3°C for 1 hr.
h) Wash the plate with the washing solution (1X). Discard the contents in the microplate by flipping it sharply over a container filled with an inactivating agent. Keep the microplate drain upside-down on a filter paper so as to eliminate all liquid. Add 300 µl of the washing solution (1 X) and then empty the plate once again by flipping it over above the containment vessel.
i) Repeat the entire operation two more times, taking care to avoid the formation of bubbles in the microwells. After the plate has been washed 3 times proceed to the next step.
j) Add 100 µl of the Conjugate solution to each well. Cover with a lid and incubate the plate at 21°C ± 3°C for 1 hr.
k) Wash the plate using washing solution (1X) three times.
l) Add 100 µl of the Chromogen solution (TMB) to each well on the plate. The chromogen solution must be absolutely colourless when it is pipetted into the wells. If a blue colour is visible, this means that the solution in the pipette has been contaminated.
m) Incubate for 10 min. at 21°C ± 3°C and keep the plate away from light. Do not cover.
n) Add 50 µl of Stop solution per well. The blue colour will change into a yellow colour.
o) Read the OD in the wells using an ELISA plate reader and using a 450 nm filter. Results must be read fairly soon after the stopping solution (within 10 min.) has been added since the chromogen may precipitate in wells with strong signals and

distort the results.

6.9 Determination of Cut-off Values and Assay Validation Criteria

a) The ΔOD of each sample is determined by subtracting the OD of the sample well with the OD of the negative control.
b) ΔOD value of sample = OD value of sample- OD value of negative control.
c) ΔOD sample = (OD Cryptosporidium specific antibodies - OD non-specific antibodies) i.e., [OD of the sample in well coated with anti-Cryptosporidium parvum antibodies (Crypto+) – OD of the same sample in well coated with negative control antibodies (Crypto-)]
d) The ΔOD of positive control sample is determined by subtracting the OD of positive control sample with the OD of negative control.
e) The test is valid only if the positive control antigens yield a difference in optical density at 10 min. that is greater than the values given on the QC data sheet. For example, OD value > 1.138 for the test kit Batch number CRY22G01.
f) The obtained value is then multiplied by 100 and divided with positive control to get the desired results.

S/P Value = (Delta OD sample/Delta OD positive control) x 100

6.10 Interpretation of the Test

Using the information provided with each batch of kit, determine each sample's status (positive or negative).
For example, for test kit Batch number CRY22G01:

If the S/P value is ≥ 7, the sample is positive.
If the S/P value is < 7, the sample is negative.

6.11 Precautions and General Safety Practices

a) All samples should be considered infectious and hazardous, and handling should

be done in a biosafety cabinet with appropriate PPE.

b) Handle stool/faecal samples with utmost care.
c) Eating, drinking, smoking, applying cosmetics, and handling contact lenses are prohibited in the laboratory.
d) All laboratory personnel should immediately report any symptoms to the lab in-charge.

6.12 After the Test

a) Sanitize all contact surfaces after completion of the work.
b) Maintain proper records of tested samples.
c) All samples should be autoclaved to disinfect prior to disposal.

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7.1 Applicable to

This procedure applies to the detection of antibodies against **Cysticercus** using the **BT LAB Porcine Cysticercosis, CYT Antibody, IgG ELISA kit.**

7.2 Objective

To describe the step-by-step requirements and procedures for the detection of serum antibodies to Cysticercus using the BT LAB Porcine Cysticercosis, CYT Antibody, IgG ELISA kit.

7.3 Scope

This SOP covers the responsibilities of various lab personnel, equipment requirements, materials, sample collection and processing, procedures, general safety practices, and references.

7.4 Responsibility

It is the responsibility of the lab in-charge to ensure that they or the person performing the test are familiar with the lab safety procedures. The interpretation of the results must be done by a person trained in the procedure.

7.5 Equipment

a) ELISA microplate reader
b) Tabletop centrifuge
c) Biosafety cabinet

7.6 Materials

a) Precoated ELISA plate
b) Kit controls; Positive control and Negative control

c) HRP Conjugate
d) Sample Diluent
e) Substrate solution A
f) Substrate solution B
g) Stop solution
h) Wash Buffer (25X)
i) Plate sealer
j) Zipper Bag
k) Centrifuge tubes (1.5 ml and 2 ml)
l) Glassware/plasticware for diluting solutions
m) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl).
n) Multichannel pipette 30-300 µl
o) Squirt bottle, manifold dispenser, or automated microplate washer
p) Absorbent paper for blotting the microtiter plate
q) 100 ml and 500 ml graduated cylinders
r) De-ionized or distilled water
s) Gloves and other PPE

7.7 Sample Collection, Processing, and Transport

a) Blood samples are collected from bovines and goat & sheep and serum is separated as described in section A and F, respectively of SOP 2.
b) Entry should be made in the log for the samples received.

7.8 Procedure

The test may be carried out and the results interpreted as per the manufacturer's protocol for the BT LAB Porcine Cysticercosis, CYT Antibody, IgG ELISA kit (Cat. No. ED0017Po).

a) Prepare all reagents, standard solutions, and samples as instructed in the manual.
b) Bring all reagents to room temperature before use.
c) Determine the number of strips required for the assay based on the number of samples. Insert the strips in the frames for use.

d) Set a blank well without any solution.
e) Add 50 µl negative control to each of the negative control wells and 50 µl positive control to each of the positive control wells. Add 40 µl sample diluent and then add 10 µl of serum sample to the sample well and mix well.
f) Cover with a plate sealer and incubate for 30 min. at 37°C.
g) Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml of wash buffer for 30 sec. to 1 min. for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer.
h) Blot the plate onto a paper towel or other absorbent material.
i) Add 50 µl of HRP conjugate to each well except the blank well.
j) Cover with plate sealer and incubate for 30 min. at 37°C.
k) Remove the sealer and wash again as described above.
l) Add 50 µl of substrate solution A to each well and then add 50 µl of substrate solution B to each well. Mix well and incubate the plate covered with a new sealer for 10 min. at 37°C in the dark.
m) Add 50 µl of stop solution to each well. The blue color will change to yellow immediately.
n) Determine the optical density (OD) value of each well immediately using a microplate reader at 450 nm within 15 min. after adding the stop solution.

7.9 Determination of Cut-off Values and Assay Validation Criteria

a) Average the readings from duplicate or triplicate samples. For calculation of the prevalence of porcine cysticercosis, CYT antibody, IgG, compare the sample wells with controls.
b) Quality Control

i. The average OD positive ≥ 1.00
ii. The average OD negative ≤ 0.10

7.10 Interpretation of the Test

a) Cut-off values = average of Negative Control value + 0.15

b) OD sample < Cut-off values is Negative
c) OD sample ≥ Cut-off values is Positive

7.11 Precautions and General Safety Practices

a) All samples should be considered infectious and hazardous; handling should be done in a biosafety cabinet with appropriate PPE.
b) Handle serum, plasma, urine/ascites/cerebrospinal fluid, cell culture supernatant, and tissue samples with utmost care.
c) Eating, drinking, smoking, applying cosmetics, and handling contact lenses are prohibited in the laboratory.
d) All laboratory personnel should immediately report any symptoms to the lab in-charge.

7.12 After the Test

a) Sanitize all contact surfaces after completion of the work.
b) Maintain proper records of tested samples.
c) All samples should be autoclaved to disinfect prior to disposal.

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8.1 Applicable to

The procedure applies to the detection of antibodies against **Swine Influenza** by using **Ingezim Influenza Porcina 2.0 kit and ID Screen Influenza A nucleoprotein swine iELISA kit.**

8.2 Objective

To provide step wise guidelines for detecting antibodies in the sera of pigs against Swine Influenza virus by using Ingezim Influenza Porcina 2.0 kit and ID Screen Influenza A nucleoprotein swine iELISA kit.

8.3 Scope

The SOP covers the details about the responsibilities of various lab personals, requirement of the equipment, materials, sample for diagnosis, procedures and general safety practices.

8.4 Responsibility

The laboratory personnel must be familiar with all the laboratory safety procedures and guidelines.

8.5 Equipment

a) ELISA microplate reader
b) Table top centrifuge

8.6 Materials

a) Microplates coated with purified NP antigen
b) Positive Control
c) Negative Control
d) Concentrated Conjugate (10X)

e) Dilution Buffer 2
f) Dilution Buffer 3
g) Wash Concentrate (20X)
h) Substrate Solution
i) Stop Solution (0.5 M)
j) 96-wells sample pre-dilution plates
k) Centrifuge tubes (1.5 ml and 2 ml)
l) Glassware/plasticware for diluting solutions
m) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl).
n) Multichannel pipette 30-300 µl.
o) Squirt bottle, manifold dispenser, or automated microplate washer
p) Absorbent paper for blotting the microtiter plate
q) 100 ml and 500 ml graduated cylinders
r) De-ionized or distilled water
s) Gloves and other PPE

8.7 Sample collection, processing and transport

a) Blood samples are collected from pigs and serum is separated as described in section E of SOP 2.
b) Entry should be made in the log for the samples received.

8.8 Procedure

8.8.1 Test and interpretation as per manufacturer's protocol for Ingezim Influenza Porcina 2.0 kit.

A. Protocol

a) Prior to starting the test, bring all reagents to room temperature (22-25ºC) (except conjugate). Sera samples must be assayed at 1/100 dilution (i.e., 5 µl of serum with 495 µl of diluent).
b) Add the samples, diluted as specified in the previous instructions, to the wells of the plate. Add 100 µl of the positive and negative control serum to their

respective wells. We recommend running samples and control in duplicate. Incubate the plate for 1 hr. at 37ºC.

c) Wash 4 times as per the procedure described as mentioned in the instruction manual.
d) Add 100 µl of the conjugate (prepared according to the previous instructions) to each well. Incubate the plate for 1 hr. at room temperature (+18 - 25ºC).
e) Wash 5 times as per the procedure described as mentioned in the instruction manual.
f) Add 100 µl of the substrate to each well. Keep the plate in darkness for 10 min. at room temperature.
g) Add 100 µl of the stop solution to each well.
h) Read the OD of each well at 450 nm within 5 min. after the addition of the stop solution.

B. Calculation of the test

S/P ratio is calculated for each sample.

S/P = (Sample OD/Positive controls OD)

C. Validity of the test

The assay will be valid only when:

a) OD value of Positive control ≥1
b) $\frac{\text{OD value of Negative control}}{\text{OD value of Positive control}}$ <0.2

D. Interpretation of the test

a) Samples with S/P values ≥ 0.2 are positive to antibodies to influenza A viruses.
b) Samples with S/P values < 0.2 are considered negative to antibodies to Influenza A viruses.
c) Titer of the samples:

The titre of a sample must be calculated as follows:
T(Titer) = 2222 x [(S/P)1.93]
For example:
OD Negative control= 0.162
OD Positive control = 1.111
OD Sample = 0.921
Validation = 0.162/ 1.111=0.145 (< 0.2)
S/P ratio = 0.921 / 1.111=0.829
Sample titre =2222 x (0.829)1.93=1547

8.8.2 Test and interpretation as per manufacturer's protocol for ID Screen Influenza A nucleoprotein swine iELISA kit.

A. Protocol

a) Allow all reagents to come to room temperature (21°C ± 5°C) before use.
b) Homogenize all reagents by inversion or vortexing.
c) Samples are tested at a final dilution of 1:100 in Dilution Buffer 2. In a pre-dilution plate, add:
- 5 µl of the Negative Control to wells A1 and B1,
- 5 µl of the Positive Control to wells C1 and D1,
- 5 µl of each sample to be tested,
- 245 µl of Dilution Buffer 2 to each well.
Note: It is recommended to respect the indicated order to be able to visually control addition of samples to each well.
d) In the ELISA microplate, add:
- 50 µl of Dilution Buffer 2.
- 50 µl of the pre-diluted controls and samples as prepared above.
e) Cover the plate and incubate 1 hr. ± 6 min. at 37°C (± 2°C).
f) Prepare the Conjugate 1X by diluting the Concentrated conjugate 10X to 1:10 in Dilution Buffer 3.
g) Empty the wells. Wash each well 3 times with at least 300 µl of the Wash Solution 1X. Avoid drying of the wells between washes.
h) Add 100 µl of the Conjugate 1X to each well.

i) Cover the plate and incubate 30 min. ± 3 min. at 21°C (± 5°C).
j) Empty the wells. Wash each well 3 times with at least 300 µl of Wash Solution 1X. Avoid drying of the wells between washes.
k) Add 100 µl of the Substrate Solution to each well.
l) Cover the plate and incubate 15 min. ± 2 min. at 21°C (± 5°C) in the dark.
m) Add 100 µl of the Stop Solution to each well, in the same order as in step no. 'k' to stop the reaction.
n) Read and record the O.D. at 450 nm.

B. Validity of the test

The test is valid if:
a) The mean OD value of the Positive Control (ODPC) is greater than 0.250 (ODPC > 0.250)
b) The ratio of the mean values of the Positive and Negative Controls (ODPC and ODNC) is greater than 3.

C. Calculation and Interpretation of the test

For each sample, calculate the S/P ratio and antibody titer as follows:

a) S/P ratio:

$$\text{S/P Value} = \frac{\text{OD sample -OD Negative Control}}{\text{OD Positive Control-OD Negative Control}}$$

b) Antibody titer:
$\mathbf{log\ 10}\ (\boldsymbol{titer}) = \mathbf{1.2 \times log\ 10}\ (\ \boldsymbol{S}\ /\ \boldsymbol{P}\ ) + \mathbf{3.500}$
$\boldsymbol{titer} = \mathbf{10\ log10}(\boldsymbol{titer})$

c) Results are interpreted as follows:

S/P value	ELISA antibody titer	Immune status
S/P ≤ 0.4	TITER ≤ 1053	Negative
S/P > 0.4	TITER > 1053	Positive

8.9 Precautions and general safety practices

a) Handling of the blood/serum samples should be done with utmost care.
b) Eating, drinking, smoking, applying cosmetics and handling of contact lenses is prohibited while working in the laboratory.

8.10 After the test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be essentially maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region
STANDARD OPERATING PROCEDURE
TITLE: SOP for detection of *Listeria* species by bacterial isolation and PCR test
SOP No. 9

Version No.	Effective Date	Next Revision Date

9.1 Applicable to

This procedure applies to the isolation and identification of ***Listeria* species**, particularly *L. monocytogenes* and *L. ivanovii*, using **bacterial isolation and PCR** methods.

9.2 Objective

The purpose of this procedure is to outline the step-by-step requirements and protocols for the isolation and identification of *Listeria* species from various samples.

9.3 Scope

This SOP covers the responsibilities of laboratory personnel, equipment requirements, materials, sample collection and processing, procedures for bacterial culture and PCR, interpretation of results, and general safety practices.

9.4 Responsibility

It is the responsibility of the laboratory in-charge to ensure that personnel performing the tests are trained in the specific procedures outlined in this SOP. All results must be interpreted by qualified personnel.

9.5 Equipment

a) BOD Incubator
b) Electronic weighing balance
c) Biosafety cabinet
d) Thermal cycler
e) Electrophoresis apparatus
f) Gel Documentation system/UV transilluminator

9.6 Materials

a) Sterile serological pipettes (10 ml and 50 ml)

b) Pipette dispenser/pipette controller
c) Micropipette 10 - 100 µl
d) Sterile culture plates
e) UVM-I or Half Fraser Broth
f) PALCAM agar plate
g) PCR primers (*prs*, *isp*)
h) PCR tube (0.2 ml)
i) Agar for DNA gel electrophoresis
j) Gloves

9.7 Sample for the test

a) For animals: Blood (approx. 5 ml), cerebrospinal fluid vaginal secretions, aborted material (preferably placental bits), faecal matter, and milk samples.
b) Foods: 25 gm or ml of food samples (Meat, milk, and their products)

9.8 Procedure

9.8.1 Enrichment of sample:

- For solid sample such as aborted material, tissue etc. homogenization or maceration is recommended. Take approx. 5 gm of sample and inoculate into the 45 ml of UVM-I or Half Fraser Broth.
- For liquid sample such as blood, milk etc. add 5 ml of sample to the 45 ml of UVM-I or Half Fraser Broth. Incubate at 37˚C for 24 hr.
- After incubation transfer 0.1 ml of UVM-I or Half Fraser to the UVM-II or Full Fraser broth and further incubate at 37˚C for 24 - 48 hr.

9.8.2 Isolation and identification of organism by Bacterial Culture Method:

a) This is a presumptive test for the presence or absence of *Listeria* spp.
b) Streak a loopful from enriched Full Fraser Broth onto PALCAM agar plate. Incubate at 37°C for 24 - 48 hr.
c) Observe for characteristic of *Listeria* spp colonies.

d) Observe the plates daily and sub-culture colonies as early as possible before storing the agar plate in the refrigerator. Pick isolated colonies and transfer to Brain Heart Infusion Broth and preserve at 4˚C.

9.8.3 ALOA Test

a) Suspected isolates from bacterial isolation can be confirmed by ALOA test.
b) ALOA test is performed using ALOA Agar plates. Spot inoculate on ALOA agar plate (HiMedia or any other) and incubate at 37˚C for 48hr.
c) Observe the colonies on the agar plate.

9.8.4 Hemolysis test

a) Suspected isolates from bacterial isolation can be confirmed by hemolysis test.
b) Streak the isolates on 5% sheep blood agar, incubate at 37ºC for 24h and observe for hemolysis.

9.8.5 Differentiation of *L. monocytogenes* and *L. ivanovii* by CAMP test:

a) Streak overnight grown *Staphylococcus aureus* and *Rhodococcus equi* sheep blood agar (SBA) (Annexure) plates having 5% sheep blood. The streaking is specific and can be done as in the Figure 1.

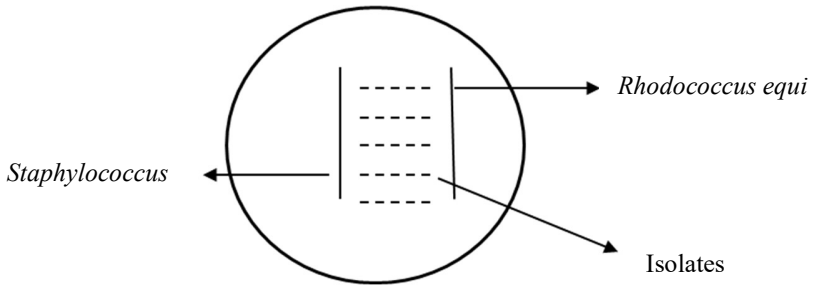

Figure 1: Streaking for CAMP test on 5% sheep blood agar. All dotted lines are horizontal streaking lines of suspected pathogenic strains of *Listeria* spp.

b) Streak the test strains of presumptive hemolytic *Listeria* at 90˚ angle to *S. aureus* and *R. equi* strain with a distance of 3 mm from these strain streaking line.
c) Inoculate these plates and incubated at 37˚C for 24 hr. and then examine for enhancement of hemolytic zone between the test strain and *S. aureus* or *R. equi*, if any, owing to the synergistic effect of their haemolysis.

9.8.6 Biochemical differentiation

a) Perform the Gram staining using kit, for the typical colonies of presumptive *Listeria* spp.
b) Further, following biochemical tests can be performed to differentiate among *Listeria* spp:
- Catalase
- Oxidase
- CAMP with; *S. aureus*, *R. equi*, PI-PLC
- Fermentation of; L-Rhamnose, D-Mannitol, D-Xylose, alpha-D-Methyl mannoside.

9.8.7 Detection of *Listeria monocytogenes* by Polymerase Chain Reaction (PCR)

A. Genus and species-specific multiplex PCR

a) The isolates showing characteristic biochemical properties can be further subjected to genus and species-specific multiplex PCR targeting *prs* (Genus specific) and *isp* (Species specific) genes.
b) The standard pathogenic strains of *L. monocytogenes* 4b (MTCC 1143) is used as the positive control for PCR. The primers are as follows:

Target gene	Primer sequence	Product size (bp)
prs	Forward 5'-AGCTGAAGAGATTCCGAAAGA 3' Reverse 5'-TTCACCAAGAAGAGCTGCAA -3'	844

isp	Forward 5'-TGCAGCGAATGCTCTTAGTG-3' Reverse 5'-AGCCAAGCACGGCTACTTTA -3'	713

c) Prepare the PCR mix by mixing 2.5 µL of 10X PCR buffer, 2.0 µL of 10 mM dNTP mix, 2.0 µL of 50 mM $MgCl_2$, 1.0 µL of 10 pM of each of the four primers, 1 U of Taq DNA polymerase, a single colony of the bacterium, made up to 25 µL of nuclease-free water.
d) Tap the PCR tube (0.2 ml) containing the reaction mixture thoroughly with your finger and then flash spin in a microcentrifuge to settle reactants at the bottom.
e) Place the tubes in the thermal cycler and perform the reaction with a preheated lid.
f) Set up cycling conditions of: Initial denaturation of 95°C for 5 min.; 40 cycles of denaturation of 95°C for 30 sec., 53°C for 1 min., 72°C for 2 min.; final extension of 72°C for 10 min.
g) Visualize the amplified DNA (844 bp and 713 bp) using 1.2% agarose gel electrophoresis and UV trans-illuminator. Record digitally by gel documentation system.

B. Serotyping PCR

a) A multiplex PCR can be performed for molecular serotyping. This PCR divides *L. monocytogenes* strains into four distinct molecular serogroups: 1/2a, 1/2c, 3a, 3c; 1/2c, 3c; 1/2b, 3b, 4b, 4d, 4e and 4b, 4d, 4e.
b) Target gene and primer sequences along with the respective product size are mentioned below:

Target gene	Primer sequence	Product size (bp)
***lmo*0737**	Forward 5'-AGGGCTTCAAGGACTTACCC-3' Reverse 5'- ACGATTTCTGCTTGCCATTC-3'	691
***lmo*1118**	Forward 5'-AGGGGTCTTAAATCCTGGAA-3'	906

	Reverse 5'-CGGCTTGTTCGGCATACTTA-3'	
ORF2819	Forward 5'-AGCAAAATGCCAAAACTCGT-3' Reverse 5'- CATCACTAAAGCCTCCCATTG-3'	471
ORF2110	Forward 5'-AGTGGACAATTGATTGGTGAA-3' Reverse 5'-CATCCATCCCTTACTTTGGAC-3'	597

c) The standard pathogenic strains of L. monocytogenes 4b (MTCC 1143) is used as the positive control for PCR.

d) Prepare PCR mix by mixing 2.5 µL of 10X PCR buffer, 4.0 µL of 10 mM dNTP mix, 4.0 µL of 50 mM $MgCl_2$, 1.0 µL of 10 pM of each of the eight primers, 2 U of Taq DNA polymerase.

e) Tap the PCR tube (0.2 ml) containing the reaction mixture thoroughly with your finger and then flash spin in a microcentrifuge to settle the reactants at the bottom.

f) Place the tubes in the thermal cycler and perform the reaction with a preheated lid.

g) Set up cycling conditions of: Initial denaturation of 95°C for 5 min.; 40 cycles of denaturation of 95°C for 30 sec., 56°C for 1 min., 72°C for 2 min.; final extension of 72°C for 10 min.

h) Visualize the amplified DNA using 1.2% agarose gel electrophoresis and UV trans-illuminator and record digitally by gel documentation system.

C. Multiplex PCR targeting virulence associated genes

a) A multiplex PCR is performed for the detection of virulence associated genes viz., haemolysin (*hlyA*), phosphatidylinositol-specific phospholipaseC (*plcA*), actin filament protein (*actA*), positive regulatory factor ‑ PrfA (*prfA*) and invasion associated internalin gene (*InlC*).

b) The primers for the mPCR targeting virulence associated genes are listed below:

Target gene	Primer sequence	Product size (bp)
***plc* A**	Forward CTGCTTGAGCGTTCATGTCTCATCCCCC Reverse CATGGGTTTCACTCTCCTTCTAC	1484
***prf* A**	Forward CTGTTGGAGCTCTTCTTGGTGAAGCAATCG Reverse AGCAACCTCGGTACCATATACTAACTC	1060
***hly* A**	Forward GCAGTTGCAAGCGCTTGGAGTGAA Reverse GCAACGTATCCTCCAGAGTGATCG	456
***act* A**	Forward CGCCGCGGAAATTAAAAAAAGA Reverse ACGAAGGAACCGGGCTGCTAG	839
InlC	Forward AATTCCCACAGGACACAACC Reverse CGGGAATGCAATTTTTCACTA	517

c) The standard pathogenic strains of *L. monocytogenes* 4b (MTCC 1143) is used as the positive control for PCR.

d) Prepare PCR mix by mixing 2.5 µL of 10X PCR buffer, 2.0 µL of 10 mM dNTP mix, 3.0 µL of 25 mM $MgCl_2$, 1.0 µL of 10 pM of each of the ten primers, 5 U of Taq DNA polymerase, a single colony of *Listeria* and sterilized milli Q water to make p the reaction volume.

e) Tap the PCR tube (0.2 ml) containing the reaction mixture thoroughly with your finger and then flash spin in a microcentrifuge to settle the reactants at the bottom.

f) Place the tubes in the thermal cycler and perform the reaction with a preheated lid.

g) Set up cycling conditions of: Initial denaturation of 95°C for 5 min.; 35 cycles of denaturation of 94°C for 15 sec., 60°C for 36 sec, 72°C for 1 min. 30 sec.; final extension of 72°C for 10 min.

h) Visualize the amplified DNA using 1.2% agarose gel electrophoresis and UV trans-illuminator and record digitally by gel documentation system.

D. Multiplex PCR for detection of Lineages of *Listeria monocytogenes*

a) A multiplex PCR is performed for detection of genus *Listeria* species *Listeria monocytogenes* and the three known Lineages of *L. monocytogenes* i.e. Lineage 1 (L1), Lineage 2 (L2), Lineage 3 (L3).

b) The primers for the PCR are listed below:

Target Gene	Primer sequences	Product size (bp)
prs	F - 5′-AGCTGAAGAGATTGCGAAAGA-3′	844
	R - 5′-TTCACCAAGAAGAGCTGCAA-3′	
isp	F - 5′-TGCAGCGAATGCTCTTAGTG-3′	713
	R - 5′-AGCCAAGCACGGCTACTTTA-3′	
L1	F - 5′-GGCGCATTCAAATCCAAGAG-3′	384
	R - 5′-GTGGTTGCTTGGTACAATGAG-3′	
L2	F - 5′-CAGAAAATGGCTGGGGATTA-3′	476
	R - 5′-GCGGAACATTGGTCTGAACT-3′	
L3	F - 5′-GTAAGCGAGCTTTAGGAGAGTT-3′	261
	R - 5′-CGTATATGCCTAAACCTACACCA-3′	

c) Prepare PCR mix by mixing 2.5 µL of 10X PCR buffer, 5.0 µL of 10 mM dNTP mix, 5.0 µL of 50 mM MgCl2, 1.0 µL of 10 pM of each of the ten primers, 5 U of Taq DNA polymerase.

e) Tap the PCR tube (0.2 ml) containing the reaction mixture thoroughly with your finger and then flash spin in a microcentrifuge to settle reactants at the bottom.

f) Place the tubes in the thermal cycler and perform the reaction with a preheated lid.
g) Set up cycling conditions of: Initial denaturation of 95°C for 5 min.; 40 cycles of denaturation of 95°C for 30 sec., 56°C for 1 min., 72°C for 2 min. 30 sec.; final extension of 72°C for 10 min.
h) Visualize the amplified DNA using 1.2% agarose gel electrophoresis and UV trans-illuminator and record digitally by gel documentation system.

9.9 Interpretation of the results

9.9.1 Isolation and identification of organism by Bacterial Culture Method

a) The typical greyish green, glistening, iridescent and pointed colonies of about 0.5 mm diameter surrounded by a diffuse black zone of aesculin hydrolysis can be presumed as of *Listeria*, as shown in Figure 2.

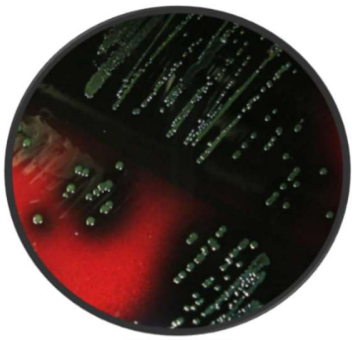

Figure 2 – *Listeria* colonies on PALCAM agar.

9.9.2 ALOA Test

a) Isolates showing green coloured colonies on agar plate will be *Listeria* spp.

b) Isolates showing green colour colonies with halo will be pathogenic spp. (either *L. monocytogenes* or *L. ivanovii*).

Figure 3 – *Listeria* colonies with halo formation on ALOA agar.

9.9.3 Hemolysis test

a) Pathogenic spp of Listeria exhibit weak beta-hemolysis.

9.9.4 Differentiation of *L. monocytogenes* and *L. ivanovii* by CAMP test:

a) To differentiate between *L. mononcytogenes* and *L. ivanovii,* their hemolysis patterns are analysed.
b) *L. mononcytogenes* strain produces hemolysis synchronously with *S. aureus* and *R. equi,* while *L. ivanovii* strains produces hemolysis with *R. equi* only.

9.9.5 Biochemical differentiation

a) Presence of Gram-positive coccobacilli can be presumed as *Listeria* spp.
b) Further biochemical tests as mentioned in Table 2 can be performed to differentiate the *Listeria* species.

	L. monocytogenes	*L.innocua*	*L.ivanovii*	*L.seeligeri*	*L.welshimeri*	*L.grayi*	*L. marthii*	*L. rocoucrtiae*
Hemolysin	+	-	+	+	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-
CAMP with								
S. aureus	+	-	-	-	-	-	-	-
R. equi	+	-	+	-	-	-	-	-
PI-PLC	+	-	+	-	-	-	-	-
Fermentation of:								
L-Rhamnose	+	+/-	-	-	+/-	+/-	-	?
D-Mannitol	-	-	-	-	-	-	-	?
D-Xylose	-	-	+	+	+	-	-	?
alpha-D-Methyl mannoside	+	+	-	-	+	+	?	?
Mice Virulence	+	-	+	-	-	-	-	-

Table 2: Biochemical tests to differentiate Listeria spp. (+ = positive reaction, - = negative reaction, ? = not known).

9.9.6 Detection of *Listeria monocytogenes* by Polymerase Chain Reaction (PCR)

A. Genus and species-specific multiplex PCR

a) Detection of *Listeria* by genus- (*prs* gene) and species (*isp* gene)- specific multiplex PCR. Specific amplification of 844 bp *prs* gene and 713 bp *isp* gene confirms the presence of *Listeria* spp.

B. Serotyping PCR

a) The PCR products showing bands of amplicon size corresponding to the primers used indicate sample positive for particular serotype.

C. Multiplex PCR targeting virulence associated genes

a) The PCR products showing bands of amplicon size corresponding to the primers used indicate sample positive for particular serotype.

D. Multiplex PCR for detection of Lineages of *Listeria monocytogenes*

a) The PCR products showing bands of amplicon size corresponding to the primers used indicate sample positive for particular serotype.

9.10 Precautions and General Safety Practices

a) Since listeriosis is a zoonotic disease, all samples should be considered infectious and hazardous. Handling of such samples must be conducted in a biosafety cabinet with appropriate PPE.
b) Handling of blood/serum samples should be performed with utmost care to prevent exposure.
c) Eating, drinking, smoking, applying cosmetics, and handling contact lenses are prohibited while working in the laboratory.
d) Laboratory personnel should immediately report any symptoms to the concerned lab in-charge.

9.11 After the Test

a) After completion of work, sanitize all contact surfaces thoroughly.
b) Maintain proper records of tested samples.
c) All samples should be autoclaved to disinfect prior to disposal.

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Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region		
STANDARD OPERATING PROCEDURE		
TITLE: SOP for detection of antibodies against Lumpy Skin Disease (LSD) by ELISA test		
SPO No. 10		
Version No.	**Effective Date**	**Next Revision Date**

10.1 Applicable to

This procedure applies for detection of antibodies against **Lumpy Skin Disease Virus** by **ID Screen® Capripox Double Antigen Multi-species kit**.

10.2 Objective

To describe step wise requirements and procedure for detecting antibodies against Lumpy Skin Disease virus by ID Screen® Capripox Double Antigen Multi-species kit.

10.3 Scope

The SOP covers the details about the responsibilities of various lab personals, requirement of the equipment, materials, sample collection and processing, procedures, general safety practices and references.

10.4 Responsibility

It is the responsibility of the lab in-charge to ensure that he/she or person performing the test to be familiar with the lab safety procedures. The interpretation of the results must be done by the person trained in the procedure.

10.5 Equipment

a) ELISA microplate reader or Multimode reader
b) BOD incubator
c) Table top mini centrifuge
d) Biosafety cabinet

10.6 Materials

a) Microplate coated with capripox virus purified antigen

b) Concentrated conjugate (10X)
c) Kit controls; Positive control and Negative control
d) Dilution Buffer 19
e) Dilution Buffer 12
f) Wash Concentrate (20X)
g) Substrate Solution
h) Stop Solution (0.5 M)
i) Centrifuge tubes (1.5 ml and 2 ml)
j) Glassware/plasticware for diluting solutions
k) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl)
l) Multichannel pipette 30-300 µl
m) Squirt bottle, manifold dispenser, or automated microplate washer
n) Absorbent paper for blotting the microtiter plate
o) 100 ml and 500 ml graduated cylinders
p) De-ionized or distilled water
q) Gloves and other PPE

10.7 Sample collection, processing and transport

a) Blood samples are collected from bovines and serum is separated as described in section A of SOP 2.
b) Entry should be made in the log for the samples received.

10.8 Procedure

The test may be carried out and the results interpreted as per manufacturer's protocol for ID Screen® Capripox Double Antigen Multi-species kit.

a) Allow the reagents to come to room temperature, 21°C (± 5°C), before use.
b) Thoroughly mix all reagents by inversion or vortexing and add:
   - 50 µl of Dilution Buffer No. 19 to each microwell.
   - 50 µl of the Negative Control to wells A1 and B1.
   - 50 µl of the Positive Control to wells C1 and D1.
   - 50 µl of each sample to be tested on the remaining wells.

c) Cover the plate and incubate 90 ± 5 min. at 21°C (± 5°C).
d) Empty the wells. Wash each well 5 times with at least 300 µl of the Wash Solution.
e) Avoid drying of the wells between washes.
f) Prepare the Conjugate 1X by diluting the Concentrated Conjugate 10X to 1/10 in Dilution Buffer No. 12.
g) Add 100 µl of the Conjugate 1X to each well.
h) Cover the plate and incubate 30 ± 3 min. at 21°C (± 5°C).
i) Empty the wells. Wash each well 5 times with at least 300 µl of the Wash Solution. Avoid drying of the wells between washes.
j) Add 100 µl of the Substrate Solution to each well.
k) Cover the plate and incubate 15 ± 2 min. at 21°C (± 5°C) in the dark.
l) Add 100 µl of the Stop Solution to each well in order to stop the reaction.
m) The stop solution should be added in the same order as in step 10, 12.
n) Read and record the OD at 450 nm.

10.9 Determination of cut-off values and assay validation criteria

a) The test is valid if:
The mean OD value of positive control (ODPC) is greater than 0.350
The ratio of the mean values of the positive and negative controls (ODPC and ODNC) is greater than 3.
b) S/P percentage (S/P%) is calculated by (Sample serum/Positive serum)

$$S/P\% = \frac{\text{OD value sample- OD value negative control}}{\text{OD value Positive control- OD value Negative Control}} \times 100$$

10.10 Interpretation of the test

a) Sample with S/P% of less than 30% are considered negative.
b) Sample with S/P% greater than or equal to 30% are considered positive.

10.11 Precautions and general safety practices

a) All samples should be considered as infectious and hazardous, and handling of such samples should be done in biosafety cabinet with appropriate PPE.
b) Handling of the blood/serum samples should be done with utmost care.
c) Eating, drinking, smoking, applying cosmetics and handling of contact lenses is prohibited while working in the laboratory.
d) All laboratory personnel should immediately report development of any symptoms to the concerned lab in-charge.

10.12 After the test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be essentially maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region		
STANDARD OPERATING PROCEDURE		
TITLE: SOP for detection of antibodies against Nipah Virus Disease by ELISA test		
SOP No. 11		
Version No.	**Effective Date**	**Next Revision Date**

11.1 Applicable to

This procedure applies for the detection of antibodies to **Nipah Virus** in the sera of pigs by **NiVsG and mcsF iELISA kit** developed by the **Pirbright Institute**.

11.2 Objective

To describe step wise requirements and procedure for detection of Nipah Virus specific antibodies in serum samples by iELISA.

11.3 Scope

The SOP covers the details about the responsibilities of various lab personals, requirement of the equipment, materials, sample for diagnosis, procedures and general safety practices.

11.4 Responsibility

It is the responsibility of the lab in-charge to ensure that he/she or person performing the test to be familiar with the lab safety procedures. The interpretation of the results must be done by the person trained in the procedure.

11.5 Equipment

a) ELISA microplate reader
b) BOD incubator
c) Table top centrifuge
d) Heat block
e) Biosafety cabinet

11.6 Materials

a) Centrifuge tubes (1.5 ml and 2 ml)
b) ELISA plates (Nunc MaxiSorp™ flat-bottom, Fisher 44-2404-21)
c) Coating buffer (carb/bicarb, 0.05 M, pH9.6) (Merck C3041-50CAP)
d) Recombinant NiV sG and NiV mcsF proteins - Supplied by TPI
e) NiV sG protein stock concentration = 3.3 mg/mL (5 µl volume)
f) NiV mcsF protein stock concentration = 7.5 mg/mL (5 µl volume)
g) PBS (0.01 M)
h) Marvel dry skimmed milk powder
i) Tween 20
j) Goat anti-porcine IgG Ab, HRP conjugate (Merck AP166P) – Supplied by TPI
k) TMB (Merck T444)
l) 1M sulphuric acid
m) Glassware/plasticware for diluting solutions
n) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl).
o) Multichannel pipette 30-300 µl
p) Squirt bottle, manifold dispenser, or automated microplate washer
q) Absorbent paper for blotting the microtiter plate
r) 100 ml and 500 ml graduated cylinders
s) De-ionized or distilled water
t) Gloves and other PPE

11.7 Sample collection, processing and transport

a) Blood samples are collected from pigs and serum is separated as described in section E of SOP 2.
b) Entry should be made in the log for the samples received.

11.8 Procedure

The test is to be carried out and the results interpreted as per manufacturer's protocol for iELISA kit for determination of antibodies against infection.

a) Dilute NiVsG protein to 1 µg/mL or NiVmcsF 0.5 µg/ml in 0.05 M carbonate/bicarbonate buffer (pH9.6) for coating plates.
   - 5 µL of concentrated protein + 16,495 µL Coating Buffer
   - 2 µL of concentrated protein+ 29,998 µL Coating Buffer
b) Pipette 100 µl per well of NiVsG coating dilution to appropriate labelled plates. Pipette 100 µl per well of NiVmcsF coating dilution to appropriate labelled plates.
c) Cover, and incubate overnight at 4˚C.
d) Flick plates to remove coating buffer/antigen mix.
e) Block plates with 200 µL/well, 5% skim milk in PBS for 2hr., at 37˚C (no Tween20 in block).
   - 120 mL needed in total for all plates.
f) Flick plates to remove blocking buffer.
g) Wash 3X with 300 µL/well PBS-Tween 20 0.05% (PBST). Tap plate on paper towel to remove excess liquid.
h) Add 100 µL sera diluted 1:400 in 2.5% skim milk in PBST in duplicate to the corresponding wells and incubate at 37˚C for 1hr.
   - Set this up in 96-well plate during blocking stage. Add 1.25 µL each sera to 498.75 µL solution, then add 100 µL per well in duplicate (according to plate plans given below).
i) Wash plate 3X with 300 µL/well PBST. Tap plate on paper towel to remove excess liquid.
j) Add 100 µL goat-ant-pig IgG HRP conjugate at a dilution of 1:10,000 in 2.5% skim milk in PBST and incubate at 37˚C for 1hr.
   - 6 µL in 59,994 µL 2.5% skim milk in PBST
k) Wash 4X with 300 µL/well PBST. Tap plate on paper towel to remove excess liquid.
l) Add 100 µL TMB solution and cover with foil. Leave at RT for 5min. or until see colour change.
m) DO NOT FLICK PLATE TO REMOVE TMB. Directly add 100 µL stop solution (1 M H_2SO_4) to wells in the same order as TMB was added.
n) Read absorbance at 450 nm.

Plate plans

Plate 1 – Uncoated

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pig 1		Pig 9		Pig 17		Pig 25		Pig 33		Pig 41	
B	Pig 2		Pig 10		Pig 18		Pig 26		Pig 34		Pig 42	
C	Pig 3		Pig 11		Pig 19		Pig 27		Pig 35		Pig 43	
D	Pig 4		Pig 12		Pig 20		Pig 28		Pig 36		Pig 44	
E	Pig 5		Pig 13		Pig 21		Pig 29		Pig 37		Pig 45	
F	Pig 6		Pig 14		Pig 22		Pig 30		Pig 38		Pig 46	
G	Pig 7		Pig 15		Pig 23		Pig 31		Pig 39		Negative Control	
H	Pig 8		Pig 16		Pig 24		Pig 32		Pig 40		Positive Control	

Plate 2 – Uncoated

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pig 47		Pig 55		Pig 63		Pig 71					
B	Pig 48		Pig 56		Pig 64		Pig 72					
C	Pig 49		Pig 57		Pig 65		Negative Control					
D	Pig 50		Pig 58		Pig 66		Positive Control					
E	Pig 51		Pig 59		Pig 67							
F	Pig 52		Pig 60		Pig 68							
G	Pig 53		Pig 61		Pig 69							
H	Pig 54		Pig 62		Pig 70							

Plate 3 – NiV sG coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pig 1		Pig 9		Pig 17		Pig 25		Pig 33		Pig 41	
B	Pig 2		Pig 10		Pig 18		Pig 26		Pig 34		Pig 42	
C	Pig 3		Pig 11		Pig 19		Pig 27		Pig 35		Pig 43	
D	Pig 4		Pig 12		Pig 20		Pig 28		Pig 36		Pig 44	
E	Pig 5		Pig 13		Pig 21		Pig 29		Pig 37		Pig 45	
F	Pig 6		Pig 14		Pig 22		Pig 30		Pig 38		Pig 46	
G	Pig 7		Pig 15		Pig 23		Pig 31		Pig 39		Negative Control	
H	Pig 8		Pig 16		Pig 24		Pig 32		Pig 40		Positive Control	

Plate 4 – NIV sG coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pig 47		Pig 55		Pig 63		Pig 71					
B	Pig 48		Pig 56		Pig 64		Pig 72					
C	Pig 49		Pig 57		Pig 65		Negative Control					
D	Pig 50		Pig 58		Pig 66		Positive Control					
E	Pig 51		Pig 59		Pig 67							
F	Pig 52		Pig 60		Pig 68							
G	Pig 53		Pig 61		Pig 69							
H	Pig 54		Pig 62		Pig 70							

Plate 5 – NiV F coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pig 1		Pig 9		Pig 17		Pig 25		Pig 33		Pig 41	
B	Pig 2		Pig 10		Pig 18		Pig 26		Pig 34		Pig 42	
C	Pig 3		Pig 11		Pig 19		Pig 27		Pig 35		Pig 43	
D	Pig 4		Pig 12		Pig 20		Pig 28		Pig 36		Pig 44	
E	Pig 5		Pig 13		Pig 21		Pig 29		Pig 37		Pig 45	
F	Pig 6		Pig 14		Pig 22		Pig 30		Pig 38		Pig 46	
G	Pig 7		Pig 15		Pig 23		Pig 31		Pig 39		Negative Control	
H	Pig 8		Pig 16		Pig 24		Pig 32		Pig 40		Positive Control	

Plate 6 – NIV F coated

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pig 1		Pig 9		Pig 17		Pig 25		Pig 33		Pig 41	
B	Pig 2		Pig 10		Pig 18		Pig 26		Pig 34		Pig 42	
C	Pig 3		Pig 11		Pig 19		Pig 27		Pig 35		Pig 43	
D	Pig 4		Pig 12		Pig 20		Pig 28		Pig 36		Pig 44	
E	Pig 5		Pig 13		Pig 21		Pig 29		Pig 37		Pig 45	
F	Pig 6		Pig 14		Pig 22		Pig 30		Pig 38		Pig 46	
G	Pig 7		Pig 15		Pig 23		Pig 31		Pig 39		Negative Control	
H	Pig 8		Pig 16		Pig 24		Pig 32		Pig 40		Positive Control	

11.9 Calculation of the test

Cut off determination using the formula:

$$\text{Cut off} = X_{neg} + 0.13\,\overline{X_{pos}} \quad (2)$$

11.10 Interpretation of the test

Mean of negative control	0.186
Mean of positive control	1.684
Cut off	0.4

11.11 Precautions and general safety practices

a) Handling of the blood/serum samples should be done with utmost care.
b) Eating, drinking, smoking, applying cosmetics and handling of contact lenses is prohibited while working in the laboratory.

11.12 After the test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be essentially maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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Version No.	Effective Date	Next Revision Date

12.1 Applicable to

This procedure applies to detection of antibodies against **Porcine Reproductive and Respiratory Syndrome (PRRS) virus** in serum samples by iELISA kit.

12.2 Objective

To describe step wise requirements and procedure for the detection of Porcine Reproductive and Respiratory Syndrome virus-specific antibodies in serum samples by iELISA.

12.3 Scope

The SOP covers the details about the responsibilities of various lab personals, requirement of the equipment, materials, sample for diagnosis, procedures and general safety practices.

12.4 Responsibility

It is the responsibility of the lab in-charge to ensure that he/she or person performing the test to be familiar with the lab safety procedures. The interpretation of the results must be done by the person trained in the procedure.

12.5 Equipment

a) ELISA microplate reader
b) Table top centrifuge
c) Water bath
d) Biosafety cabinet

12.6 Materials

a) 96 Well Microtitration plates

b) Blotting paper
c) Test serum
d) Kit controls; Positive control serum and Negative control serum
e) Peroxidase conjugate
f) 25X Washing Solution
g) Diluent for serum
h) Substrate
i) Stop Solution
j) Centrifuge tubes (1.5 ml and 2 ml)
k) Glassware/plasticware for diluting solutions
l) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl)
m) Multichannel pipette 30-300 µl
n) Squirt bottle, manifold dispenser, or automated microplate washer
o) Absorbent paper for blotting the microtiter plate
p) 100 ml and 500 ml graduated cylinders
q) De-ionized or distilled water
r) Gloves and other PPE

12.7 Sample collection, processing and transport

a) Blood samples are collected from pigs and serum is separated as described in section E of SOP 2.
b) Entry should be made in the log for the samples received.

12.8 Procedure

The test is to be carried out and the results interpreted as per manufacturer's protocol for INgezim PRRS 2.0 iELISA kit for determination of antibodies against PRRS virus.

a) Prior to starting the test, bring all reagents to room temperature (22 - 25°C). Sera must be tested at a dilution 1/20 in dilution (i.e., 5 µl of serum in 100 µl of dilution buffer).
b) Add 100 µl of positive control to two wells of the plate, 100 µl of the negative control to another 2 wells and 100 µl of each of the dilutions of sera to be tested

(prepared according to previous instructions) in the remaining wells of the plate. Seal the plate and incubate for 30 min. at room temperature (18 - 25°C).

c) Wash 3 times following the procedure previously described.
d) Add 100 µl of the ready to use conjugate to each well. Seal the plate and incubate for 30 min. at room temperature (18 - 25°C)
e) Wash 5 times following the described procedure.
f) Add 100 µl of the substrate solution to each well. Keep the plate at room temperature for 15 min. In order to speed up this process, it is advisable to use a multichannel pipette. Add 100 µl of the stop solution to each well. We recommend adding this reagent following the same order in which the substrate was added
g) Read the OD of each well at 450 nm.

12.9 Validity of the test

The test is considered valid when the OD of the positive control minus the OD of the negative control is higher than 0.35 and the negative control OD at 450 nm is lower than 0.35

12.10 Calculation and Interpretation of the test

a) S/P ratio:

$$\text{S/P Value} = \frac{\text{OD sample - OD Negative Control}}{\text{OD Positive Control - OD Negative Control}}$$

b) Negative S/P value must be considered with value 0
c) Sample with a S/P higher or equal than 0.4 should be considered positive.
d) Samples with a S/P lower than 0.4 should be considered negative.

12.11 Precautions and general safety practices

a) Handling of the blood/serum samples should be done with utmost care.
b) Eating, drinking, smoking, applying cosmetics and handling of contact lenses is prohibited while working in the laboratory.

12.12 After the test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be essentially maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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13.1 Applicable to

This procedure applies to the detection of antibodies against ***Coxiella burnetti*** **(causative agent of Q-fever)** using the **ID Screen® Q Fever Indirect Multi-species kit.**

13.2 Objective

To describe the step-by-step requirements and procedures for the detection of antibodies against *Coxiella burnetti* (causative agent of Q-fever) using the ID Screen® Q Fever Indirect Multi-species kit.

13.3 Scope

This SOP covers the responsibilities of various lab personnel, equipment requirements, materials, sample collection and processing, procedures, general safety practices, and references.

13.4 Responsibility

It is the responsibility of the lab in-charge to ensure that they or the person performing the test are familiar with the lab safety procedures. The interpretation of the results must be done by a person trained in the procedure.

13.5 Equipment

a) ELISA microplate reader or Multimode reader
b) BOD incubator
c) Table top mini centrifuge
d) Biosafety cabinet

13.6 Materials

a) Microplate coated with phase-I and II *Coxiella burnetii* antigens
b) Concentrated conjugate (10X)
c) Kit controls: Positive control and Negative control
d) Dilution Buffers 2 and 3
e) Wash Concentrate (20X)
f) Substrate Solution
g) Stop Solution (0.5 M)
h) Centrifuge tubes (1.5 ml and 2 ml)
i) Glassware/plasticware for diluting solutions
j) Micropipettes (1 ml, 200 µl, 10 µl) and Tips (1 ml, 200 µl, 10 µl)
k) Multichannel pipette 30 - 300 µl
l) Squirt bottle, manifold dispenser, or automated microplate washer
m) Absorbent paper for blotting the microtiter plate
n) 100 ml and 500 ml graduated cylinders
o) Deionized or distilled water
p) Gloves and other PPE

13.7 Sample Collection, Processing, and Transport

a) Blood samples are collected from bovines and goat & sheep and serum is separated as described in section A and F, respectively of SOP 2.
b) Entry should be made in the log for the samples received.

13.8 Procedure

a) Allow all reagents to come to room temperature (21°C ± 5°C) before use.
b) Thoroughly mix all reagents by inversion or vortexing.
c) Samples are tested at a final dilution of 1:50 as follows:
d) In a 96-well pre-dilution microplate, add:
   - 5 µl of the Negative Control to wells A1 and B1.
   - 5 µl of the Positive Control to wells C1 and D1.
   - 5 µl of each sample to be tested in the remaining wells.

- 245 µl of the Dilution Buffer 2 to each well.

e) In the ELISA microplate, transfer:
- 100 µl of the pre-diluted Negative Control to wells A1 and B1.
- 100 µl of the pre-diluted Positive Control to wells C1 and D1.
- 100 µl of each pre-diluted sample to be tested in the remaining wells.

f) Cover the plate and incubate 45 min. ± 4 min. at 21°C (± 5°C).
g) Empty the wells. Wash each well three times with at least 300 µl of the Wash Solution. Avoid drying of the wells between washes.
h) Prepare the Conjugate 1X by diluting the Concentrated Conjugate 10X to 1:10 in Dilution Buffer 3.
i) Add 100 µl of the Conjugate 1X to each well.
j) Cover the plate and incubate 30 min. ± 3 min. at 21°C (± 5°C).
k) Empty the wells. Wash each well 3 times with at least 300 µl of the Wash Solution. Avoid drying of the wells between washes.
l) Add 100 µl of the Substrate Solution to each well.
m) Cover the plate and incubate 15 min. ± 2 min. at 21°C (± 5°C) in the dark.
n) Add 100 µl of the Stop Solution to each well, in the same order as in step 'l', to stop the reaction.
o) Read and record the OD at 450 nm.

13.9 Determination of cut-off values and assay validation criteria

The test is valid if:

a) The mean OD value of the Positive Control (ODPC) is greater than 0.350; ODPC > 0.350
b) The ratio of the mean values of the Positive Control OD to the Negative Control OD (ODPC to ODNC) is greater than 3
c) For each sample, calculate the S/P percentage (S/P%)

$$S/P\% = \frac{\text{OD value sample- OD value negative control}}{\text{OD value Positive control- OD value Negative Control}} \times 100$$

13.10 Interpretation of the test

Serum or Plasma	
Result	**Status**
S/P% ≤ 40%	Negative
40% < S/P% ≤ 50%	Doubtful
50% < S/P% ≤ 80%	Positive
S/P% > 80%	Strong Positive

13.11 Precautions and General Safety Practices

a) Since, Q-fever is a zoonotic disease, all samples should be considered infectious and hazardous, and handling should be done in a biosafety cabinet with appropriate PPE.
b) Handle blood/serum samples with utmost care.
c) Eating, drinking, smoking, applying cosmetics, and handling contact lenses are prohibited in the laboratory.
d) All laboratory personnel should immediately report any symptoms to the lab in-charge.

13.12 After the Test

a) Sanitize all contact surfaces after completion of the work.
b) Maintain proper records of tested samples.
d) All samples should be autoclaved to disinfect prior to disposal.

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Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region
STANDARD OPERATING PROCEDURE
TITLE: SOP for detection of *Salmonella* bacteria using bacterial isolation, PCR and Widal test
SOP No. 14

Version No.	Effective Date	Next Revision Date

14.1 Applicable to

This procedure applies to the **detection of *Salmonella* spp.** using **bacterial isolation method, PCR, and Widal test** protocols in the laboratory.

14.2 Objective

To describe the step-by-step requirements and procedures for the detection of *Salmonella*.

14.3 Scope

This SOP covers the responsibilities of various lab personnel, equipment requirements, materials, sample collection and processing, procedures, general safety practices, and references.

14.4 Responsibility

It is the responsibility of the lab in-charge to ensure that they or the person performing the test are familiar with the lab safety procedures. The interpretation of the results must be done by a person trained in the procedure.

14.5 Equipment

a) BOD incubator
b) Biosafety cabinet
c) Thermal cycler
d) Gel Documentation system
e) DNA Electrophoresis apparatus

14.6 Materials

a) MacConkey agar

b) Hektoen enteric (HE) agar
c) TSI agar
d) Bismuth green sulpha agar (BGSA)
e) Urea broth
f) Lysine decarboxylase (LIA)
g) TSI agar
h) Urease
i) Lysine decarboxylase broth
j) Phenol red dulcitol broth
k) KCN broth
l) Malonate broth
m) Phenol red lactose broth
n) Phenol red sucrose broth
o) Methyl red test
p) Simmons citrate
q) 10X PCR buffer
r) $MgCl_2$ (50 mM)
s) dNTP mix (10 mM)
t) *InvA* Forward primer (TCG TGA CTC GCG TAA ATG GCG ATA)
u) *InvA* Reverse primer (GCA GGC GCA CGC CAT AAT CAA TAA)
v) Taq DNA Polymerase (1 U/µl)
w) Forward primer (SdfI/ Via B/ Spy)
x) Reverse primer (SdfI/ Via B/ Spy)

14.7 Sample Collection, Processing, and Transport

a) Stool/faeces samples are collected from bovines as described in section B of SOP 2.

14.8 Procedure

Test: Isolation and Identification of *Salmonella* (Culture, PCR, and Widal test) Test kit

14.8.1 Bacterial isolation and identification

A. Isolation:

a) Inoculate a matchstick head-size sample of stool in 10 ml of buffered peptone water, and incubate at 37°C for 18 - 24 hr..
b) Inoculate a loopful of culture from the pre-enriched samples in selenite-F broth and aerobically incubate overnight at 37°C for 18 - 24 hr.
c) Spin the top layer (1 ml) of an overnight culture at 20,000 g for 5 min.
d) Spread a 1 µl loop to subculture from the pellet by spreading on selective agar plates to achieve characteristic colonies.
e) Pinkish-white or red colonies surrounded by a red halo in are indicative of *Salmonella* spp. in bismuth green sulpha agar (BGSA).

B. Identification:

a) TSI agar:

(i) Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate triple sugar iron (TSI) slant by streaking slant and stabbing butt.
(ii) Incubate TSI slants at 35°C for 24 hr. ± 2 hr. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar).

b) MR-VP test:

(i) Inoculate medium with small amount of growth from each TSI slant suspected to contain *Salmonella*. Incubate 48 ± 2 hr. at 35°C. For VP test, transfer 1 ml of the 48 hr. culture to the test tube and incubate the remainder of MR-VP broth an additional 48 hr. at 35°C. Add 0.6 ml α-naphthol and shake well. Add 0.2 ml 40% KOH solution and shake. To intensify and speed the reaction, add a few crystals of creatine. Read the results after 4 hr.; development of pink-to-ruby red colour throughout the medium is a positive test. Most cultures of *Salmonella* are VP-negative, indicated by the absence of development of pink-to-red colour throughout the broth.

(ii) For MR test, to 5 ml of 96 hr. MR-VP broth, add 5 - 6 drops of methyl red indicator. Most *Salmonella* cultures give a positive test, indicated by diffuse red colour in the medium. A distinct yellow colour is a negative test. Discard, as not *Salmonella*, cultures that give positive KCN and VP tests and negative methyl red tests.

c) Simmons citrate test:

(i) Inoculate this agar, using needle containing growth from TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate 96 hr. ± 2 hr. at 35°C.

d) Urease test (conventional):

(i) With a sterile needle, inoculate growth from each TSI slant culture into tubes of urea broth. Since occasionally, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include an uninoculated tube of this broth as control. Incubate 24 hr. ± 2 hr. at 35°C.

14.8.2 Detection of *Salmonella* bacteria by PCR

Test Protocol: Use the DNA isolated from bacterial culture as template

A. Salmonella genus identification by PCR:

(i) Set up PCR as follows:

Reagent Name	µl per reaction
10X PCR buffer	2.5
$MgCl_2$ (50 mM)	1.0
dNTP MIX (10 mM)	1.0
InvA Forward primer TCGTGACTCGCGTAAATGGCGATA	1.0
InvA reverse primer GCAGGCGCACGCCATAATCAATAA	1.0
Taq DNA Polymerase (1 U/µl)	1.0
DNA template	4.0
D/W	13.5

(ii) Thermal cycler Conditions:

Temp (° C)	Time (min. or sec.)	Cycle (s)
94	5 min.	1
94	30 sec.	35
56	1 min.	
72	1 min. 30sec.	
72	10 min.	1
4	Hold	

(iii) Run by agarose gel (1.5%) electrophoresis stained with ethidium bromide (0.5 µg/ml) and visualize (483 bp) and document using UV gel documentation system.

B. Serotype identification by multiplex PCR:

(i) Set up PCR as follows:

Reagent Name	µl per reaction
10X PCR buffer	2.5
$MgCl_2$ (50mM)	3.0
dNTP mix (10mM)	1.0
Forward primer (*SdfI* / *Via B* / *Spy*)	1.0 µl of each
Reverse primer (*SdfI* / *Via B* / *Spy*)	1.0 µl of each
Taq DNA Polymerase (1 U/ µl)	3.0
DNA template	5.0
D/W	3.5

(ii) Primers for Serotype Identification:

Serotype	Target gene	Primer Sequence	Product size
S. enteritidis	*Sdfl*	Forward - TGT GTT TTA TCT GAT GCA AGA GG	304 bp
		Reverse - TGA ACT ACG TTC GTT CTT CTG G	
S. typhi	*Via B*	Forward - CAC GCA CCA TCA TTT CAC CG	738 BP
		Reverse - AAC AGG CTG TAG CGA TTT AGG	
S. typhimurium	*Spy*	Forward - TTG TTC ACT TTT TAC CCC TGA A	401 bp
		Reverse - CCC TGA CAG CCG TTA GAT ATT	

(iii) Thermal cycler conditions:

Temp (˚C)	Time (min. or sec.)	Cycle (s)
94	5 min.	1
94	30 sec.	35
57	1 min.	
72	1min. 30sec.	
72	10 min.	1
4	Hold	

(iv) Run by agarose gel (1.5%) electrophoresis, stained with ethidium bromide (0.5 µg/ml) and visualize and document using UV gel documentation system.

C. Testing by Widal protocol

(i) Prepare a serial dilution of the serum @ 1:40 to 1:320
(ii) Add an equal volume of *Salmonella* antigen. This can be done as a Slide method or as a Tube method.
(iii) When running in tubes, incubate for 12 hr. or overnight.
(iv) The highest dilution of serum exhibiting agglutination is noted. If it ends at 1:320, then that is the titre.

14.9 Interpretation of the test

14.9.1 Bacterial culture and identification:

(a) Based on the characteristic colony morphology on selective media following interpretations are made:

Test or substrate	Result		*Salmonella* spp. Reaction[a]
	Positive	Negative	
Glucose (TSI)	Yellow butt	Red butt	+
Lysine decarboxylase (LIA)	Purple butt	Yellow butt	+
H_2S (TSI and LIA)	Blackening	No blackening	+
Urease	Purple-red colour	No colour change	-
Lysine decarboxylase broth	Purple colour	Yellow colour	+
Phenol red dulcitol broth	Yellow colour and/or gas	No gas; no colour change	+[b]
KCN broth	Growth	No growth	-
Malonate broth	Blue colour	No colour change	-[c]
Indole test	Red colour at surface	Yellow colour at surface	-
Polyvalent flagellar test	Agglutination	No agglutination	+
Polyvalent somatic test	Agglutination	No agglutination	+
Phenol red lactose broth	Yellow colour and/or gas	No gas; no colour change	-[c]
Phenol red sucrose broth	Yellow colour and/or gas	No gas; no colour change	-

Voges-Proskauer test	Pink-to-red colour	No colour change	-
Methyl red test	Diffuse red colour	Diffuse yellow colour	+
Simmons citrate	Growth; blue colour	No growth; no colour change	v

14.9.2 PCR:

(a) *Salmonella* spp: PCR amplification of 483 bp.
(b) *S. enteritidis*: PCR amplification of 304 bp.
(c) *S. typhi*: PCR amplification of 738 bp.
(d) *S. typhimurium*: PCR amplification of 401 bp.

14.9.3 Widal test:

The test is positive if:

(a) The "O" antigen titre is greater than 1:160.
(b) The "H" antigen titre is greater than 1:160.
(c) A fourfold increase in the titre (e.g., from 1:40 to 1:160).

14.10 Precautions and General Safety Practices

a) Since salmonellosis is a zoonotic disease, all samples should be considered infectious and hazardous. Handling of such samples should be done in a biosafety cabinet with appropriate PPE.
b) Handling of the Stool/faeces samples should be done with utmost care.
c) Eating, drinking, smoking, applying cosmetics, and handling of contact lenses is prohibited while working in the laboratory.

d) All laboratory personnel should immediately report the development of any symptoms to the concerned lab in-charge.

14.11 After the Test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region
STANDARD OPERATING PROCEDURE
TITLE: SOP for detection of Tuberculosis using DNA isolation and performing TaqMan Real-Time PCR test
SOP No. 15

Version No.	Effective Date	Next Revision Date

15.1 Applicable to

This procedure applies to the detection of **tuberculosis** using **DNA isolation and performing TaqMan Real-Time PCR** based testing in laboratory.

15.2 Objective

To describe the step-by-step requirements and procedures for the detection of nucleic acid of tubercle bacilli.

15.3 Scope

This SOP covers the responsibilities of various lab personnel, equipment requirements, materials, sample collection and processing, procedures, general safety practices, and references.

15.4 Responsibility

It is the responsibility of the lab in-charge to ensure that they or the person performing the test are familiar with the lab safety procedures. The interpretation of the results must be done by a person trained in the procedure.

15.5 Equipment

a) BOD incubator
b) Biosafety cabinet
c) Nano Drop Lite spectrophotometer
d) Dry bath/Incubator
e) Vortex
f) Thermal cycler
g) Centrifuge
h) Gel Documentation system
i) DNA Electrophoresis apparatus

15.6 Materials

a) Nuclease free water.
b) Ethanol, Absolute.
c) Isopropyl alcohol, 70%.
d) 10X PBS.
e) Disinfectant – Sodium hypochlorite.
f) 1.5 ml and 2 ml Eppendorf tubes.
g) DNase - free filter tips (20 µl, 200 µl, 1000 µl)
h) Pipettes (2 - 20 µl, 20 - 200 µl, 100 - 1000 µl)
i) Qiagen QIA-amp DNA Blood Mini Kit (Cat No: 51104)
j) Qiagen DNeasy Blood & Tissue Kit (Cat No: 69506)

15.7 Sample collection, processing and transportation

a) Milk and nasal swab samples collected from cow and buffaloes as described in section C and section D of SOP 2.

15.8 Procedure

15.8.1 Isolation of DNA from milk samples by using QIA-amp Blood DNA isolation kit protocol

a) Bring samples to room temperature (15 – 25°C)
b) Perform all centrifugation steps at room temperature (15 – 25°C).
c) Add ethanol to buffer AW1 and AW2 as indicated on the bottle.
b) Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube. Add 200 µl cell suspension obtained from milk sample to the microcentrifuge tube.
c) Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 sec. **Note**: Do not add QIAGEN Protease or proteinase K directly to Buffer AL
d) Incubate at 56°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

e) Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 sec.
f) After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
g) Carefully apply the mixture to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g for 1 min.
h) Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate.
i) Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g for 1 min.
j) Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.
k) Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at 20,000 x g for 3 min.
l) Place the QIAamp Mini spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
m) Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate.
n) Carefully open the QIAamp Mini spin column and add 100 µl Buffer AE or nuclease free water.
o) Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000x g for 1 min. to elute DNA.
p) The quantity and quality of the DNA should be assessed by using a Nano Drop Lite spectrophotometer.
q) Genomic DNA with 260/280 nm ratio between 1.80 - 1.90 is considered pure.
r) Label the isolated DNA samples and store at -20° C until further analysis.

15.8.2 Isolation of DNA from nasal swab samples

a) Bring samples to room temperature (15 – 25°C).
b) Perform all centrifugation steps at room temperature (15 – 25°C).

c) Mix all buffer before use and add ethanol to buffer AW1 and AW2 as indicated on the bottle.
d) Pipet 200 µl from the 1 ml sterile 1X PBS swab solution into a 1.5 ml centrifuge tube.
e) Add 180 µl Buffer ATL and 20 µl Proteinase K into the centrifuge tube, mix by vortexing.
f) Incubate at 56°C until completely lysed, vortex occasionally during incubation.
g) Vortex for 15 sec before proceeding with next step.
h) Add 200 µl Buffer AL. Mix by vortexing, and incubate at 56°C for 10 min.
i) Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.
j) Pipet the mixture into the DNeasy Mini spin column placed in a 2 ml collection tube.
k) Centrifuge at ≥ 6000 x g for 1 min. Discard flow-through and collection tube.
l) Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at ≥ 6000 x g. Discard flow-through and collection tube.
m) Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g to dry the DNeasy membrane. Discard flow-through and collection tube.
n) Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 200 µl Buffer AE directly onto the DNeasy membrane.
o) Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥ 6000 x g to elute DNA.
p) The quantity and quality of the DNA should be assessed by using a Nano Drop Lite spectrophotometer.
q) Genomic DNA with 260/280 nm ratio between 1.80 - 1.90 is considered pure.
r) Label the isolated DNA samples and store at -20° C until further analysis.

15.9 Precautions and General Safety Practices

a) Since tuberculosis is a zoonotic disease, all samples should be considered infectious and hazardous. Handling of such samples should be done in a biosafety cabinet with appropriate PPE.

b) Handling of the milk and nasal swab samples should be done with utmost care.
c) Eating, drinking, smoking, applying cosmetics, and handling of contact lenses is prohibited while working in the laboratory.
d) All laboratory personnel should immediately report the development of any symptoms to the concerned lab in-charge.

15.10 After the Test

a) After completion of the work, all contact surfaces must be sanitized.
b) A proper record of tested samples should be maintained.
c) All samples should be autoclaved to disinfect prior to disposal.

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List of all the PIs/Co-PIs

Table 1 as per the sanction order / Progress Report

Compo-nent	Institute	Investigators (as per original sanction order)	Revised and approved list of investigators
	Coordination and Monitoring Unit	Director, NIAB Consultant	Director, NIAB Consultant
1.	*NIAB, Hyderabad, Telangana*	Principal Investigators Dr. Nagendra R. Hegde Co-Principal Investigators Dr. Ravi Kumar Gandham Dr. Pankaj Suman	Principal Investigators Dr. Nagendra R. Hegde Co-Principal Investigators Dr. Girish Radha Radhakrishnan Dr. Madhuri Subbiah Dr. Pankaj Suman
2.	*ICAR – NMRI, Hyderabad, Telangana*	Principal Investigator Dr. SB Barbuddhe Co-Principal Investigators Dr. Deepak Rawool Dr. Vishnuraj MR Dr. Laxman Chatlod Dr. Yogesh Gadekar	Principal Investigator Dr. SB Barbuddhe Co-Principal Investigator Dr. Deepak Rawool Dr. Vishnuraj MR Dr. Laxman Chatlod Dr. Yogesh Gadekar
3.	*MAFSU, Nagpur, Maharashtra*	Principal Investigator Dr. Nitin Kurkure Co-Principal Investigators Dr. Sandeep Chaudhari Dr. Shilpshri V Shinde Dr. Sunil W Kolte Dr. Megha P Kaore	Principal Investigator Dr. Nitin Kurkure Co-Principal Investigator Dr. Sandeep Chaudhari Dr. Shilpshri V Shinde Dr. Sunil W Kolte Dr. Megha P Kaore
4.	*TANUVAS, Chennai, Tamil Nadu*	Centre Coordinator Dr. G Dhinakar Raj Principal Investigator Dr. P Azhahianambi Co-Principal Investigators Dr. RP Aravindh Babu Dr. TMA Senthilkmar Dr. K Karthik Dr. A Raja	Centre Coordinator Dr. C. Soundara Rajan Project Director Dr. KG Tirumurugaan Principal Investigator Dr. P Azhahianambi Co-Principal Investigators Dr. RP Aravindh Babu Dr. TMA Senthilkumar Dr. K Karthik

			Dr. A Raja
5.	*GADVASU, Ludhiana, Punjab*	Centre Coordinator Dr. JPS Gill Principal Investigator Dr. Jasbir Singh Bedi Co-Principal Investigators Dr. Simranpreet Kaur Dr. Randhir Singh Dr. Pankaj Dhaka	Centre Coordinator Dr. JPS Gill Principal Investigator Dr. Jasbir Singh Bedi Co-Principal Investigators Dr. Simranpreet Kaur Dr. Randhir Singh Dr. Pankaj Dhaka Dr. Deepali Kalambhe Dr. HK. Sharma Dr. JH. Chaudhary Dr. Abhishek Gaurav
6.	*ICAR – IVRI, Izatnagar, Bareilly, Uttar Pradesh*	Principal Investigator Dr. Pronab Dhar Co-Principal Investigators Dr. Himani Dhanze Dr. Hira Ram	Principal Investigator Dr. KP Singh (Recently superannuated) Dr. Hira Ram (Recently approved PI) Co-Principal Investigators Dr. Himani Dhanze Dr. Saminathan Dr. M. SumanKumar Dr. GK Sharma
7.	*ICAR – RCNEH, Umiam, Meghalaya*	Principal Investigator Dr. Arnab Sen Co-Principal Investigators Dr. Sandeep Ghatak Dr. Samir Das Dr. Arun Prince Milton	Principal Investigator Dr. Sandeep Ghatak Co-Principal Investigators Dr. Samir Das Dr. Arun Prince Milton
8.	*AAU, Guwahati, Assam*	Principal Investigator Dr. NN Barman Co-Principal Investigators Dr. Saidul Islam Dr. Pankaj Deka Dr. Biswajit Datta Dr. SaratSonowal Dr. Rupam Dutta	Principal Investigator Dr. NN Barman Co-Principal Investigators Dr. Saidul Islam Dr. Pankaj Deka Dr. Biswajit Datta Dr. SaratSonowal Dr. Rupam Dutta Dr. Arfan Ali
9.	*CAU, Aizawl*	Principal Investigator Dr. Tapan Kumar Dutta Co-Principal Investigators Dr. P Roychoudhury	Principal Investigator Dr. Tapan Kumar Dutta Co-Principal Investigators Dr. P Roychoudhury Dr. H Lalrinkima

		Dr. H Lalrinkima Dr. RS Arya Dr. JM Gali	Dr. RS Arya Dr. JM Gali
10.	*NRC on Pig, Guwahati, Assam*	Principal Investigator Dr. Swaraj Rajkhowa Co-Principal Investigators Dr. Ajay Kumar Yadav Dr. SR Pegu Dr. Rajib Deb Dr. J Doley Dr. Souvik Pal	Principal Investigator Dr. Swaraj Rajkhowa Co-Principal Investigators Dr. SR Pegu Dr. Rajib Deb Dr. J Doley Dr. Souvik Pal
11.	*ICAR – NIVEDI, Bangalore, Karnataka*	Principal Investigator Dr. KP Suresh Co-Principal Investigators Dr. Divakar Hemadri Dr. SS Patil Dr. V Balamurugan	Principal Investigator Dr. KP Suresh Co-Principal Investigators Dr. Divakar Hemadri Dr. SS Patil Dr. V Balamurugan
12.	*AIIMS, New Delhi*	Principal Investigator Dr. Vikram Saini Co-Principal Investigators Dr. Shyam S Chauhan	Principal Investigator Dr. Vikram Saini Co-Principal Investigators Dr. Shyam S Chauhan
13.	*AIIMS, Jodhpur, Rajasthan*	Principal Investigator Dr. Kuldeep Singh	Principal Investigator Dr. Kuldeep Singh Co-Principal Investigators Dr. Pankaj Bhardwaj Dr. Ravi Shekhar Gadepalli Dr. Vidhi Jain Dr. Gopal Bohra Dr. Deepak Kumar
14.	*ICMR-RMRC, Gorakhpur, Uttar Pradesh*	Principal Investigator Dr. Rajni Kant Srivastava Co-Principal Investigators Dr. Kamran Zaman Dr. Rajeev Singh	Principal Investigator Dr. Rajeev Singh Co-Principal Investigators Mr. Rohit Beniwal
15.	*Gandhi Medical College,*	Principal Investigator Dr. K Nagamani	Principal Investigator Dr. K Nagamani Co-Principal Investigator

	Hyderabad, Telangana	Co-Principal Investigator Dr. GJ Archana	Dr. GJ Archana
16.	*TNMGRMU, Chennai, Tamil Nadu*	Centre Coordinator Dr. Sudha Seshayyan Principal Investigator Dr. Pushkala Subramanian Co-Principal Investigator Dr. G Srinivas	Centre Coordinator Dr. Sudha Seshayyan Principal Investigator Dr. Pushkala Subramanian Co-Principal Investigator Dr. Swathandran Hamsavardhini
17.	*ICMR-RMRC, Dibrugarh, Assam*	Principal Investigator Dr. Siraj Ahmed Khan Co-Principal Investigators Dr. Pramit Ghosh	Principal Investigator Dr. Siraj Ahmed Khan Co-Principal Investigators Dr. Pramit Ghosh
18.	*Nazareth Hospital, Shillong, Meghalaya*	Principal Investigator Dr. Michael GG Mawlong Co-Principal Investigator Dr. Karen Ranee	Principal Investigator Dr. Michael GG Mawlong Co-Principal Investigator Dr. Karen Ranee
19.	*WWF-I, Guwahati WRTC, Maharashtra*	Principal Investigator Dr. Parikshit Kakati Co-Principal Investigators Dr. Amit Sharma	Principal Investigator Dr. Shirish Upadhye
20.	*DIL, DAHV&DD, Itanagar, Arunachal Pradesh*	Principal Investigator Dr. Omit Tapir Co-Principal Investigators Dr. Gyamnya Baki Garam	Principal Investigator Dr. Badal Biswakarma Co-Principal Investigators Dr. Gyamnya Baki Garam
21.	*AH&VD, Khanapara, Assam*	Principal Investigator Dr. Jiten Ch. Bordoloi Co-Principal Investigators Dr. Moonmi Khaund	Principal Investigator Dr. Barnalee Saikia Co-Principal Investigators Dr. Moonmi Khaund Dr. Manoshi Phukan Dr. Olivia Gohain
22.	*DV&AHS, Imphal, Manipur*	Principal Investigator Dr. Ng Ibotombi Singh	Principal Investigator Dr. Sabitri Maibam Co-Principal Investigators

		Co-Principal Investigators Dr. Binodkumari Devi Dr. Sabitri Maibam	Dr. Binodkumari Devi Dr. Amita Devi
23.	*AH&VD, Shillong, Meghalaya*	Principal Investigator Dr. DI Kjam Co-Principal Investigators Dr. H Kylla	Dr. P Blahwar (recently transferred) Dr. Laureata Dkhar (approval request letter pending from DBT) Dr. H. Kylla
24.	*AH&VD, Aizawl, Mizoram*	Principal Investigator Dr. M Zohmingthangi Co-Principal Investigators Dr. Esther Lalzoliani Dr. C Neihthangpuii Dr. Lalrinhlui Dr. Lalramdintluanga	Dr. M Zohmingthangi (recently transferred) Dr. Esther Lalzoliani Ralte (approval request letter pending from DBT, earlier she was a co-PI) Dr. C. Neithangpuli Dr. Lalrinhlui Dr. Zohlimpuia Dr. Lalramdintluanga
25.	*DAH&VS, Kohima, Nagaland*	Principal Investigator Dr. Keduzol Ltu Co-Principal Investigators Dr. Khrisamhazo Rhetso Dr. Samen Jamir Dr. Tsilise Sangtam Dr. Pamingchuba	Principal Investigator Dr. Keduzol Ltu Co-Principal Investigators Dr. Khrisamhazo Rhetso Dr. Samen Jamir Dr. Tsilise Sangtam Dr. Pamingchuba
26.	*DAH&VS, Tadong, Sikkim*	Principal Investigator Dr. Sem Sem Bhutia Co-Principal Investigators Dr. Tilak B Ghatani Ms. Yangchen Gurung Dr. Ekta Pradhan Dr. Rinchen Ongmu Bhutia Dr. Hissey Lepcha	Principal Investigator Dr. Zeruiah Bhutia Co-Principal Investigators Dr. Seema Rai
27.	*DARD, Agartala, Tripura*	Principal Investigator Dr. Mrinal Kanti Dutta Co-Principal Investigators Dr. Bina Saikia	Principal Investigator Dr. Bina Saikia

28.	*AIIMS, Bhopal*	(Not included)	Principal Investigator Dr. Megha Katare Pandey

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