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

STANDARD OPERATING PROCEDURE MANUAL

INDEX

SR NO.	Contents	PAGE NO.
1.	Cover Page	1
2.	Project brief	2-3
3.	Case algorithm	4
4.	Scrub Typhus	5-17
5.	Salmonellosis	18-27
6.	Q-fever	28-35
7.	Brucellosis	36-42
8.	Japanese Encephalitis	43-54
9.	Listeriosis	55-69
10.	Cryptosporidiosis	70-76
11.	CCHF	77-85
12.	Annexure 1	86-89
13.	Annexure 2	90-91

Project brief

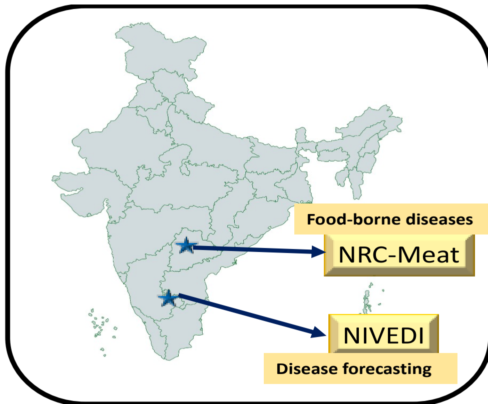
Title of the Project: Establishment of a Consortium for One Health to address Zoonotic and Transboundary Diseases in India, including the Northeast Region (Multi-Institutional).

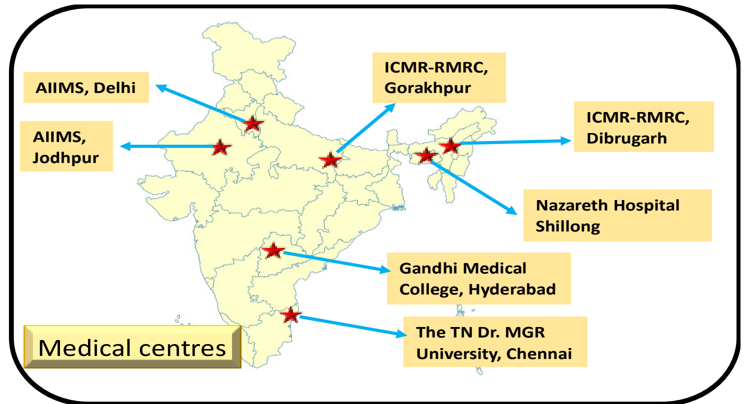
- One Health Approach" was conceptualised for improvement of human, animal and environmental health by conducting various programmes, policies and research.
- India's diverse agro-climatic conditions, agrarian practices, close interaction with animals and ecological niches acts as perfect recipe for the spread of zoonosis.
- Zoonotic and transboundary animal diseases impose a huge burden on lower- and middle-income countries, including India.
- In consortium, institutions from both North-eastern region and from outside the northeast, will work coherently to initiate or improve surveillance of selected diseases.

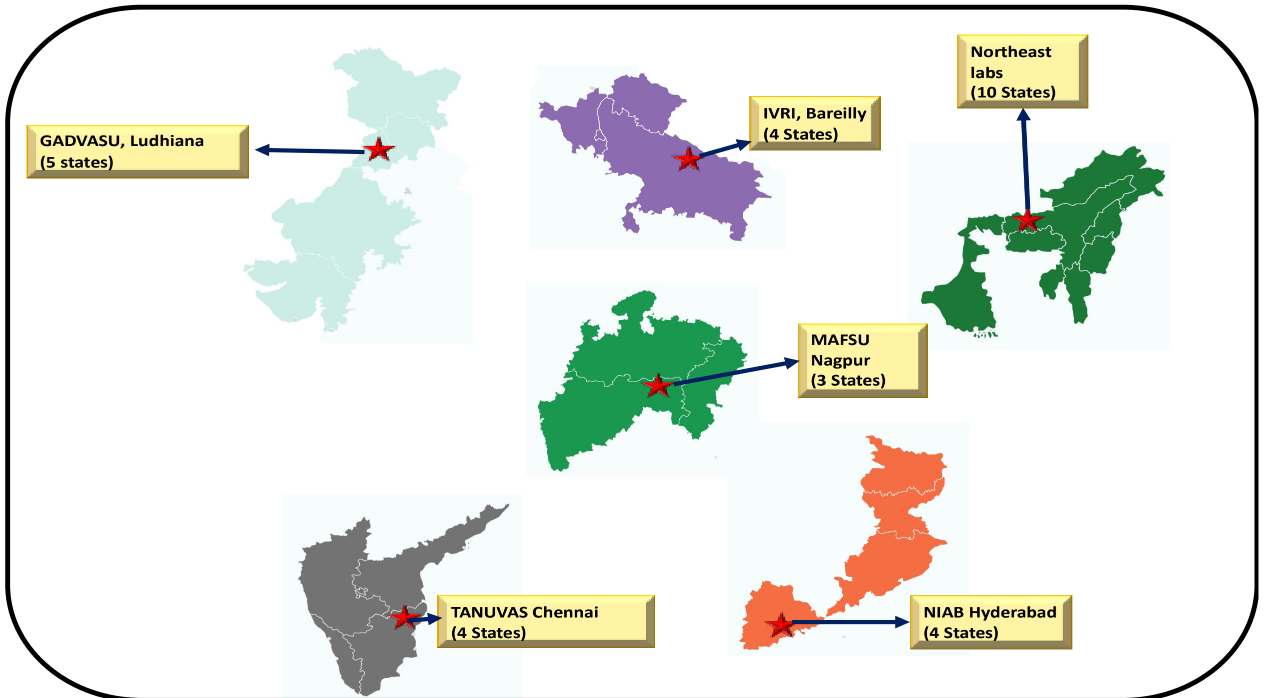
Project coordinating centre:

National Institute of Animal Biotechnology, Hyderabad, Telangana

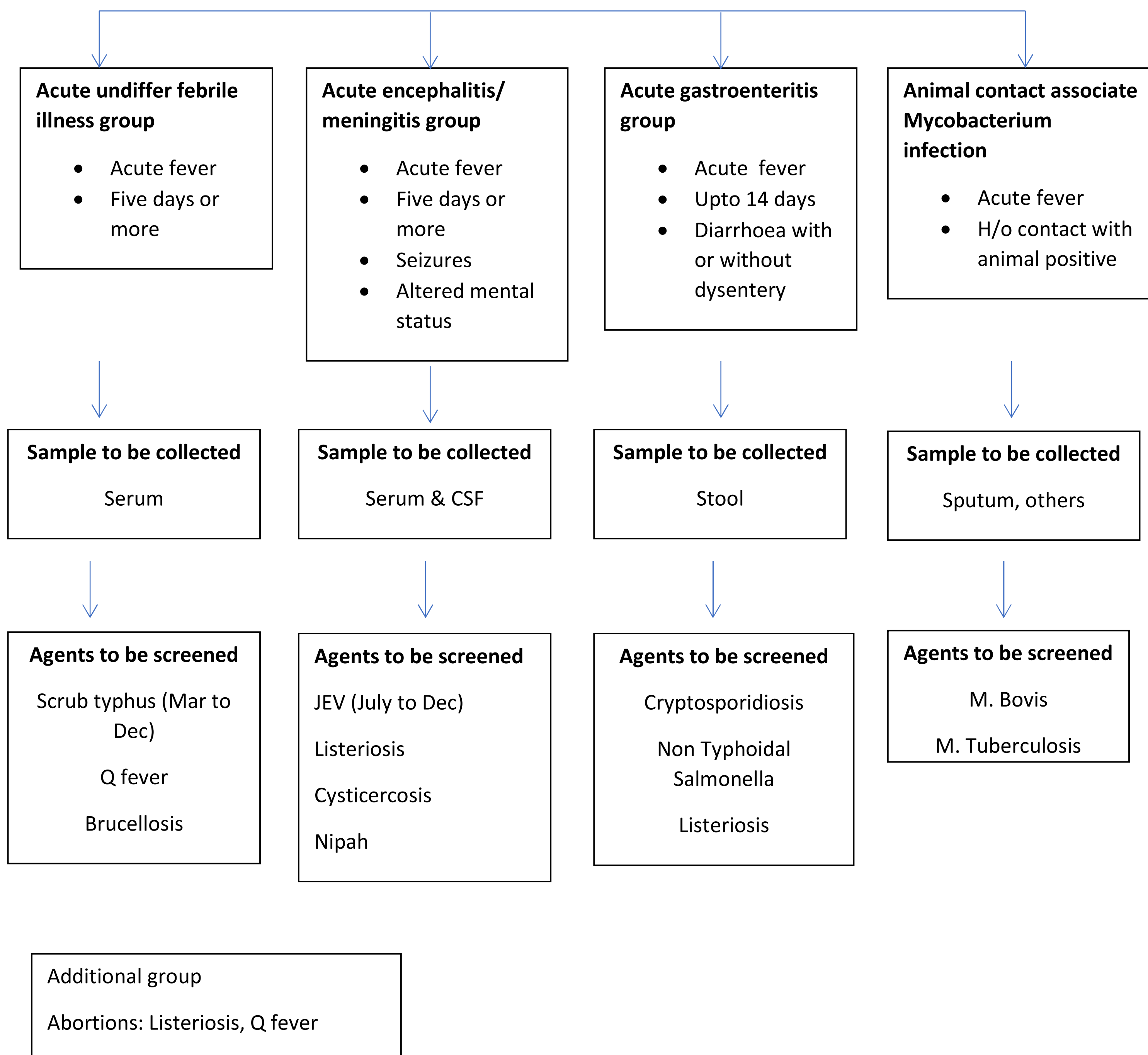
Partner institutes in the project







Categorisation of cases for Medical Centres



1. Scrub Typhus

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology laboratories, Medical centre's Consortium for one health project

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP-1	**Scrub Typhus**	**1.0**
Effective Date: 17-02-2022		

The content in this standard operating procedures are to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1.1. Introduction:

Scrub typhus, caused by the bacteria Orientia tsutsugamushi and transmitted by Leptotrombidium mites, is responsible for a potentially fatal tropical infection which is a grossly under-recognized public health problem in India [1,2]. About a million cases of scrub typhus are reported annually and the disease is associated with high mortality [3]. The disease is known to be endemic to the geographically confined area of the Asia-Pacific region termed as the 'tsutsugamushi triangle', which covers South and Southeast Asia, Northern Australia, and the islands of the Indian and Pacific Oceans [1].

There has been a resurgence of scrub typhus across India in the recent years; and scrub typhus has re-emerged as a major cause of acute undifferentiated febrile illnesses (AUFI) with high morbidity and mortality [5,6]. This disease is known to occur in diverse ecological settings in India with large numbers of cases being reported from Tamil Nadu, Andhra Pradesh, Karnataka, and Kerala in the South, Himachal Pradesh, Uttaranchal, Jammu, and Kashmir in the North, Meghalaya, Assam, and Nagaland in the North-East, West Bengal and Bihar in the East, and Maharashtra and Rajasthan in the West [7].

Scrub typhus commonly manifests with fever, breathlessness, cough, headache, nausea/ vomiting, and altered sensorium. In some areas of the country, scrub typhus accounts for up to 35–50% of acute undifferentiated febrile illnesses requiring hospital admissions [8,9]. About a third of the scrub typhus cases requiring hospitalization have multi-organ dysfunction with pulmonary, hepatic, cardiac, neurological, or renal complications leading to high fatality rates [6,10]. Studies from India reveal that the case fatality rate (CFR) of scrub typhus ranges from 1.3% to 33.5% depending on the organ involvement and complications present [11,12,13].

Although scrub typhus is a public health threat in India, its national burden and distribution remain unclear due to the paucity of data and appropriate surveillance systems. However, estimating the burden of scrub typhus in India can lead to better control and management strategies.[14].

1.2. Purpose: Hospital based serosurveillance of Scrub Typhus **by** IgM ELISA in human serum samples.

1.3. Case definition according to WHO

1. Definition of suspected/clinical case: Acute undifferentiated febrile illness of five days or more with or without eschar should be suspected as a case of rickettsial infection (if eschar is present, fever of less than five days duration should be considered as scrub typhus). Other presenting features may be headache and

2. Definition of probable case: A suspected clinical case showing titres of 1:80 or above in OX2, OX19 and OXK antigens by Weil-Felix test and an optical density (OD) > 0.5 for IgM by ELISA is considered positive for members of typhus and spotted fever groups of Rickettsiae.

3. Definition of confirmed case: A confirmed case is the one in which (a) Rickettsial DNA is detected in eschar samples or whole blood by PCR, or (b) Rising antibody titres on acute and convalescent serum samples detected by indirect immune fluorescence assay (IFA).

1.4. Materials and Methods:

1.4.1. Study Design: Cross sectional study

Inclusion Criteria: All Acute febrile illness patients who satisfy the criteria of Case

Exclusion Criteria: All Acute febrile illness patients who are diagnosed with Dengue, Malaria, Typhoid, Leptospira are excluded

1.4.2. Type of sample: Serum

Sample Size: 186 Serum samples

1.5. Methodology:

All Serum Samples will be subjected to Scrub Typhus **IgM** ELISA **(InBios International).**

Principle:

The Scrub Typhus Detect IgM ELISA system is a quantitative ELISA for the detection of IgM antibodies to O.tsutsugamushi (OT) in serum. Wells of each plate have been coated with unique recombinant antigen mix. During testing, the serum samples are diluted in InBios sample diluents and applied to each well. After incubation and washing, the wells are treated with polyclonal goat anti-human IgM antibodies labelled with the Horseradish peroxidise (HRP). After a second incubation and washing, the wells are incubated with the tetramethylbenzidine (TMB) substrate. An acidic stopping solution is then added and the degree of enzymatic turnover of the substrate is determined by absorbance measurement at 450nm. The absorbance measured is directly proportional to the concentration of IgM antibodies to OT present. End users are required to determine a robust locally relevant cut-

off using appropriate controls from each endemic location including but not limited to normal and unrelated disease specimens

1.6. **PROCEDURE:**

1.6.1. Primary sample: The test can be performed on serum only. The kit is not optimized for testing whole blood or plasma

1.6.2. Materials (Not provided in the kit)

a. Microplate Reader capable of absorbance measurement at 450nm

b. Biological or High-grade water

c. 37^0C incubator without CO_2 supply or humidification

d. Plate washer

e. multi-channel pipettors

f. Timer

1.7. Materials provided in the kit:

1. **Scrub Typhus ELISA plate:**
   One strip holder in ziplock foil, containing 96 polystyrene microtiter wells coated with OT-derived recombinant antigens in each well. Stable at 2-8^0C until the expiration date

2. **Sample Dilution Buffer for Scrub Typhus:**
   Two bottles, 25 ml each, to be used for preparing sample dilutions. A slight precipitate may form. Mix gently before use. Stable at 2-8^0C until the expiration date.

3. **Scrub Typhus IgM Positive Control**
   One vial, 50 µl. The positive control will aid in monitoring the integrity of the kit. Stable at 2-8^0C until the expiration date. Before use, quickly centrifuge the vial so that contents can be collected at the bottom.

4. **Scrub Typhus Negative control**
   One vial, 50 µl. The negative control will aid in monitoring the integrity of the kit. Stable at 2-8^0C until the expiration date. Before use, quickly centrifuge the vial so that contents can be collected at the bottom.

1. **Ready to use Enzyme Conjugate-HRP for Scrub Typhus IgM**

   One bottle, 12 ml of a pre-diluted conjugate to be used as is in the procedure below. Stable at 2-8^0C until the expiration date. (Note: The conjugate should be kept in alight protected bottle at all times as provided).

2. **10 X Wash Buffer**

   One bottle, 120 ml of 10X concentrate Wash buffer to be diluted and used n all the washing steps of this procedure. Stable at 2-8^0C until the expiration date.

   Note: See preparation of reagents in test procedure to prepare 1X Wash buffer.

3. **EnWash**

   One bottle, 20 ml of EnWash to be used in between the washing steps after enzyme conjugate-HRP and before liquid TMB addition. Stable at 2-8^0C until the expiration date.

4. **Liquid TMB Substrate**

   One bottle, 12ml of liquid substrate to be used in this procedure. Stable at 2-8^0C until the expiration date.

   Note: The substrate should be kept in a light protected bottle at all times

5. **Stop Solution**

   One bottle, 6ml to be used to stop the reaction. Stable at 2-8^0C until the expiration date.

   Caution: strong acid, wear protective gloves, lab coat and safety goggles. Dispose of all materials according to safety rules and regulations.

   Note: All reagents and controls must be allowed to reach room temperature (20-25 C) and mixed thoroughly by gentle inversion prior to use

2.0. Precautions

GENERAL PRECAUTIONS

1. A Thorough understanding of this package insert is necessary for successful use of the product. Reliable results will only be obtained by using precise laboratory techniques and accurately following the package insert.

2. Wear protective clothing, eye protection and disposable gloves while performing the assay. Wash hands thoroughly afterwards.

3. Do not eat, drink, smoke or apply cosmetics where immunodiagnostics materials are being handled.

4. Do not pipette by mouth.

5. Use a clean disposable pipette tip for each reagent, standard, control or specimen.

6. Cover working area with disposable absorbent paper.

2.1. SAMPLE PRECAUTIONS

1. All human source material used in the preparation of controls has been tested using FDA-approved methods for antibody to human immunodeficiency virus 1 and 2 (HIV 1 and 2), Hepatitis c (HCV) as well as hepatitis surface antigen and found to be negative. However, no test method can offer complete assurance and all human controls and antigen should be handled as potentially infectious material. The centres for disease control and prevention and the national institute of health recommended that potentially infectious agents be handled at the biosafety level 2.

2. This test must be performed on serum only. The use of whole blood, plasma, or another specimen matrix has not been established.

3. It is advised that icteric or lipaemic sera, or sera exhibiting hemolysis or microbial growth not be used.

4. Do not heat inactivate sera.

5. Dispense reagents directly from bottles using clean pipette tips. Transferring reagent may result in contamination.

6. To avoid cross contamination, a new pipette tip must be used for dispensing each control and test sera.

2.2. KIT REAGENTS PRECAUTIONS

1. All reagents must be equilibrated to room temperature (20-25 ^{o}C) before commencing the assay. The assay will be affected by temperature changes.

2. Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.

3. Unusual microwells must be resealed immediately and stored in the presence of desiccant. Failure to do this may cause erroneous results.

4. substrate system: a. As the liquid TMB substrate is susceptible to contamination from metal ions, do not allow the substrate system to come into contact with metal surfaces. b. Avoid prolonged exposure to direct light. c. Some detergents may interfere with the performance of the liquid TMB substrate. d. The liquid TMB substrate may have a faint blue color. This will not affect the activity of the substrate or the results of the assay.

5.Do not mix lots of any kit company within an individual assay microtitre plate.

6.Do not use any component beyond the expiration date shown on its label.

7.Avoid exposure of the reagents to excessive heat or direct sunlight during storage and incubation.

8.Some reagents may form a slight precipitation: mix gently before use.

9.Incomplete washing will adversely affect the outcome and assay precision.

10. To minimize potential assay drift due to variation in the substrate incubation time, care should be taken to add the stopping solution into the wells in the same order and speed used to add the liquid TMB substrate solution.

11. Avoid microbial contamination of reagents, especially of the ready to use enzymes conjugate –HRP.

12. Do not use a humidified chamber for 37oC incubations, as this may affect assay performances.

2.3. Kits Storage and Stability:

It is essential that all reagents/material is stored at 2-8^{0}C.

2.4. Procedure:

Bring all kit reagents and specimen to room temperature (25 C) before use. Thoroughly mix the reagents and samples before use by gentle invasion.

Preparation of reagents before performing test

(a) 1X Wash Buffer - Dilute wash buffer concentrate to 1X using Biological or High-grade water (Mix the provided 120 m l of 10X Wash buffer with 1080 ml of Biological or High-

Grade water). After it is diluted to 1X, store at room temperature for a maximum of six months.

Note: Discard the 1X wash buffer if any microbial growth is observed.

(b) Microtiter Wells - Select the number of coated wells required for the assay. The remaining wells should be placed back into the pouch, sealed with desiccant, and stored at 2-8^{0}C until ready to use or expiration date.

ASSAY PROCEDURE

1. Allow all reagents to reach room temperature (25oC) and mix thoroughly by gentle inversion before use. Positive and Negative controls should be assayed in duplicates. Test samples may be assayed in singlet.

2. Determine number of sera to be tested.

3. Organize sera according to the "Example for sera applications" Provided below or any preferred arrangement. Dilutions can be made either in tubes or in ELISA-type plastic wells (untreated plastics; not provided)

"Example for sera applications

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC										
B	PC	PC										
C	S1	S7										
D	S2	S8										
E	S3	S9										
F	S4	S10										
G	S5	S11										
H	S6	S12										

4. Dilute test sera to 1/100 by using the provided sample dilution buffer for scrub typhus (e.g 4ul serum plus 396ul sample dilution buffer for scrub typhus). Mix well.

5. Apply 100ul per well of the 1/100 diluted test sera and controls to marked scrub typhus ELISA plate.

6. Cover the plate with parafilm or plate covers just on the well opening surface, so the bottom of the plate is not covered. Incubate the plate at 37o c for 30 minutes in an incubator.

7. After the incubation is complete, wash the strips 6 times with the 1x wash buffer using an automatic plate washer. Use 300ul per well of 1x wash buffer in each wash cycle for all plate washing.

8. Add 100ul per well of ready to use enzyme–HRP conjugate for scrub Typhus IgM into all wells by multi-channels.

9. Cover the plate the parafilm just on the well opening surface, so the bottom of the plate is not covered. 10. Incubate the plate at 37o c for 30 minutes in an incubator.

11. After the incubation, wash the plate 6 times with an automatic plate washer using 1x wash buffer,300ul per well.

12. Add 150ul per well of Enwash into all wells by a multi-channel pipettor.

13. Incubate the plate at room temperature (20-25o c) for 5 minutes without any cover on the plate.

14. After the incubation, wash the plate 6 times with an automatic plate washer using 1x wash buffer, 300ul per well.

15. Add 100ul per well of liquid TMB substrate into all wells by multi-channel pipettor.

16. Incubate the plate at room temperature (20-25o c)in a dark place (or container) for 10 minutes without any cover on the plate.

17. After the incubation, add 50ul per well to stop solution into all wells by multi-channel pipettor and incubate at room temperature (20-25oC) for 1 minutes without any cover on the plate.

18. After the incubation, read the optical density (OD) at 450nm with a microtiter plate reader

17. Incubate at room temperature **in Dark** for 10 minutes.

18.Stop the reaction exactly after 10 minutes with 100 µl Stop Solution.

19.Measure the absorbance at 450 nm within 10 minutes after termination of reaction.

2.5. QUALITY CONTROL

Test Validity:

Absorbance value of Positive Control> 0.5000

Absorbance value of Negative Control> 0.2000

Discrimination capacity (R PC/NC) ≥ 5.00

Non fulfilment of these criteria is an indication of deterioration of reagents or an error in the test procedure and the assay must be repeated.

2.6. RESULT:

INTERPRETATION OF RESULTS:

CUT OFF = 0.500

OD value < Cut Off – Non-Reactive

OD value > Cut Off - Reactive

Any Reactive sample must be repeated to verify the results.

Values near the Cut off are considered to be doubtful and the assay must be repeated in triplicate or more.

2.7. Limitations:

(a) In Bios Scrub Typhus Detect IgM ELISA kit has not been validated with sera from HIV/Ot coinfected population and is not recommended for this population.

(b) All positive ELIS test results are presumptive and require confirmation by the clinician.

(c) Testing should only be performed on patients with clinical symptoms. This test is not intended for screening the general population. The positive predictive value depends on the likelihood of the disease being present.

(d) Serological cross reactivity across the mycobacterium group may be present.

(e) Positive results should be interpreted in the context of clinical and other laboratory findings and may not indicate active Scrub Typhus

(f) Assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient.

(g) The reagents supplied in this kit are optimized to measure OT-derived antigen reactive antibody levels in the serum.

(h)Repeated freezing and thawing of reagents supplied in the kit and of specimens must be avoided. Do not freeze the liquid TMB substrate

(i) hemolysed and lipemic specimens may give false values and should not be used

(j)The assay performance characteristics have not been established for visual result determination

(k)Results from immunosuppressed patients must be interpreted with caution.

(l) Generally primary responders exhibit mainly monotypic antibody responses; however, during successive infections the antibody responses broadens to include heterotypic reactivity to other related bacteria in the same or different antigenic groups.

(m) Serum and plasma comparisons: the assay described here has been optimized with serum. Care should be taken on the quality of sample. Particulate, lipemic, hemolysed and aged samples should not be used. Use of freshly drawn sample is preferred.

2.8. Safety Precautions:

1. Handle all specimens as potentially infectious.
2. Avoid contact with eyes, skin and mucous membrane.
3. Wear disposable gloves and face mask while doing the procedure.

3.0. REFERENCES

1. Bonell A, Lubell Y, Newton PN, Crump JA, Paris DH. Estimating the burden of scrub typhus: A systematic review. PLoS Negl.Trop Dis. 2017 Sep 25; 11(9):e0005838. https://doi.org/10.1371/journal.pntd. 0005838 PMID: 28945755; PMCID: PMC5634655
2. Behera B, Biswal M, Das RR, Dey A, Jena J, Dhal S, et al. Clinico-epidemiological analysis of scrub typhus in hospitalised patients presenting with acute undifferentiated febrile illness: A hospital-based study from Eastern India. Indian J Med Microbiol. 2019 Apr-Jun; 37(2):278–280. https://doi.org/10. 4103/ijmm.IJMM_19_147 PMID: 31745031
3. Taylor AJ, Paris DH, Newton PN. A Systematic Review of Mortality from Untreated Scrub Typhus (Orientia tsutsugamushi). PLoS Negl Trop Dis. 2015 Aug 14; 9(8):e0003971. https://doi.org/10.1371/ journal.pntd.0003971 PMID: 26274584; PMCID: PMC4537241.

4. Walker DH. Scrub Typhus—Scientific Neglect, Ever-Widening Impact. N Engl J Med. 2016 Sep 8; 375 (10):913–5. https://doi.org/10.1056/NEJMp1608499 PMID: 27602663.
5. Kispotta R, Kasinathan A, Kumar Kommu PP, Manikandan M. Analysis of 262 Children with Scrub Typhus Infection: A Single-Center Experience. Am J Trop Med Hyg. 2020 Nov 9. https://doi.org/10. 4269/ajtmh.20-1019 Epub ahead of print. PMID: 33219642.
6. Varghese GM, Trowbridge P, Janardhanan J, Thomas K, Peter JV, Mathews P, Abrahamet al. Clinical profile and improving mortality trend of scrub typhus in South India. Int J Infect Dis. 2014 Jun; 23:39– 43. https://doi.org/10.1016/j.ijid.2014.02.009 Epub 2014 Mar 21. PMID: 24661931.
7. Xu G, Walker DH, Jupiter D, Melby PC, Arcari CM. A review of the global epidemiology of scrub typhus. PLoS Negl Trop Dis. 2017 Nov 3; 11(11):e0006062. https://doi.org/10.1371/journal.pntd.0006062 PMID: 29099844; PMCID: PMC5687757.
8. India. Indian Pediatr. 2019 Apr 15; 56(4):304–306. PMID: 31064899.
9. Abhilash KP, Jeevan JA, Mitra S, Paul N, Murugan TP, Rangaraj A, et al. Acute Undifferentiated Febrile Illness in Patients Presenting to a Tertiary Care Hospital in South India: Clinical Spectrum and Outcome. J Glob Infect Dis. 2016 Oct-Dec; 8(4):147–154. https://doi.org/10.4103/0974-777X.192966 PMID: 27942194; PMCID: PMC5126753.
10. Sharma N, Biswal M, Kumar A, Zaman K, Jain S, Bhalla A. Scrub Typhus in a Tertiary Care Hospital in North India. Am J Trop Med Hyg. 2016 Aug 3; 95(2):447–51. https://doi.org/10.4269/ajtmh.16-0086 Epub 2016 Jun 13. PMID: 27296391; PMCID: PMC4973198.
11. Narvencar KPS, Rodrigues S, Nevrekar RP, Dias L, Dias A, Vaz M, et al. Scrub typhus in patients reporting with acute febrile illness at a tertiary health care institution in Goa. Indian J Med Res. 2012 Dec; 136(6):1020–4. PMID: 23391799
12. Takhar RP, Bunkar ML, Arya S, Mirdha N, Mohd A. Scrub typhus: A prospective, observational study during an outbreak in Rajasthan, India. Natl Med J India. 2017 Apr; 30(2):69–72. PMID: 28816212
13. Sivaprakasam E, Rajan M, Pasupathy U, Ravichandran L. Clinical Characteristics and Predictors of Severity of Pediatric Scrub Typhus in a Tertiary Level Hospital in South India. Archives of Pediatric Infectious Diseases. 2020; 8: https://doi.org/10.5812/pedinfect.92752
14. Devasagayam E, Dayanand D, Kundu D, Kamath MS, Kirubakaran R, Varghese GM. The burden of scrub typhus in India: A systematic review. PLoS neglected tropical diseases. 2021 Jul 27;15(7):e0009619.

15. Kit insert

4.0. ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

2. Salmonellosis

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology laboratories, Medical centre's Consortium for one health project

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP-2	**Salmonellosis**	**1.0**
	Effective Date: 17-02-2022	

The content in this standard operating procedures are to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1.1. Introduction: Salmonellosis in humans usually takes the form of self-limiting food poisoning (gastroenteritis), but occasionally manifests as a serious systemic infection (enteric fever) that requires prompt antibiotic treatment. It occurs in humans by consumption of food contaminated with animal faeces. In addition, salmonellosis causes substantial losses of livestock. Salmonella occurs in gastrointestinal tracts of mammals, birds, animals and insects. Non-typhoidal salmonellosis is a worldwide disease of humans and animals. Animals are the main reservoir, and the disease is usually foodborne, although it can be spread from person to person. The salmonellae that cause typhoid fever and other enteric fevers spread mainly from person to person via the fecal-oral route and have no significant animal reservoirs. Asymptomatic human carriers ('typhoid Marys') may spread the disease. Thus, transmission of *S. enterica* provides classic example of the One Health

paradigm because reducing human infections will require the reduction of *Salmonella* in animals and limitation of transmission from the environment.

The incubation period for Salmonella gastroenteritis (food poisoning) depends on the dose of bacteria. Symptoms usually begin 6 to 48 hours after ingestion of contaminated food or water and usually take the form of nausea, vomiting, diarrhoea, and abdominal pain. Myalgia and headache are common; however, the cardinal manifestation is diarrhoea. Fever (38°C to 39°C) and chills are also common. At least two-thirds of patients complain of abdominal cramps. The duration of fever and diarrhoea varies but is usually 2 to 7 days.

1.2. Purpose: *Diagnosis of* Salmonellosis in stool samples or rectal swabs, a blood samples using conventional microbiological and molecular (PCR) techniques.

1.3. Case definition: The most common form of salmonellosis is self-limited, uncomplicated gastroenteritis. Pathogenic salmonellae ingested in food survive passage through the gastric acid barrier and invade the mucosa of the small and large intestine and produce toxins. Invasion of epithelial cells stimulates the release of pro-inflammatory cytokines which induce an inflammatory reaction. The acute inflammatory response causes diarrhoea and may lead to ulceration and destruction of the mucosa. The bacteria can disseminate from the intestines to cause systemic disease.

The septicaemic form of *Salmonella* infection can be an intermediate stage of infection in which the patient is not experiencing intestinal symptoms and the bacteria cannot be isolated from faecal specimens. The severity of the infection and whether it remains localized in the intestine or disseminates to the bloodstream may depend on the resistance of the patient and the virulence of the Salmonella isolate.

Enteric fevers are severe systemic forms of salmonellosis. The best-studied enteric fever is typhoid fever, the form caused by *S.* Typhi, but any species of *Salmonella* may cause this type of disease. The symptoms begin after an incubation period of 10 to 14 days. Enteric fevers may be preceded by gastroenteritis, which usually resolves before the onset of systemic disease. The symptoms of enteric fevers are non-specific and include fever, anorexia, headache, myalgias, and constipation. Enteric fevers are severe infections and may be fatal if antibiotics are not promptly administered.

Diagnosis is by cultures of blood, stool, or site specimens.

Laboratory Confirmation

• Isolation of Salmonella (except S. Typhi)* from a clinical specimen.

*S. Typhi is reportable as Typhoid Fever Case Classifications

• Confirmed:

A case that meets the laboratory criteria for diagnosis. When available, Salmonella serotype characterization should be reported.

• Probable:

A case with Salmonella sp. detected, in a clinical specimen, by use of culture independent laboratory methods (non-culture based),

OR

A clinically compatible case that is epidemiologically linked to a case that meets the probable or confirmed laboratory criteria for diagnosis

Note: Both asymptomatic infections and infections at sites other than the gastrointestinal tract, if laboratory confirmed, are considered confirmed cases that should be reported.

Note: A case should not be counted as a new case if laboratory results were reported within 365 days of a previously reported infection in the same individual, unless additional information is available indicating a separate infection, e.g., different serotype

Ref: https://www.dshs.texas.gov/IDCU/investigation/electronic/Salmonellosis.pdf

1.4. Materials & Methods

1.4.1. Sampling: Preferably stool samples or rectal swabs, a blood samples (in case of invasive Salmonellosis). Additionally, food samples will be subjected to isolation of salmonellae

1.4.2. Sampling kit: A clean container (a disposable plate or a cardboard kidney bowl), sampling container with a spoon attached to the lid, sealable plastic bag, sticker for recording personal details.

1.4.3. 1. Collection of a Stool sample

a. When to collect? During the period of active diarrhoea (preferably as soon as possible after the onset of illness).

b. How much to collect? A fresh stool sample from ill persons; Two rectal swabs or swabs of fresh stool can be substituted if necessary.

1.4.3.2. Method for collection

1.4.3.2.1. Rectal swabs: For rectal swabs, moisten 2 swabs in an appropriate transport medium (e.g., Cary-Blair, Stuart, Amies; buffered glycerol-saline). Insert swab 1-1.5 inches into the rectum and gently rotate. Place both swabs into the same tube deep enough that medium covers the cotton tips. Break off the top portion of sticks and discard.

1.4.3.2.2. Whole stool samples:

- Collect some stools (faeces) in the clean container.
- Open the lid of the sampling container. Using the spoon attached to the lid fill 1/3 of the container (containing Cary-Blair medium).
- Close the lid carefully. Attach a sticker to the sampling container.
- Write the sampling date and time on the sticker.
- Put the sampling container in a plastic bag and seal the bag carefully.

1.4.4.1. Storage and transportation of specimens after collection

Refrigerate swabs in transport media at 4°C. When possible, test within 48 hours after collection; otherwise, freeze samples at -70°C. Refrigerate the whole stool, process it within 2 hours after collection. Store the portion of each stool specimen at less than -15°C for antigen or PCR testing.

1.4.4.2. Blood sample: Collect 3 to 5 ml of blood in EDTA coated vials. The blood samples can be used for culturing the pathogen or can be used to extract the DNA to detect the pathogen. The samples can be stored at 4ºC without freezing.

1.4.4.3. Food sample: Take a 25 g analytical unit at random from each 100 g sample unit. When a sample unit consists of more than one container, aseptically mix the contents of each container before taking the 25 g analytical unit. To reduce the analytical workload, the analytical units may be composited. The maximum size of a composite unit is 375 g or 15 analytical units.

1.5. Laboratory detection of Salmonellosis

Salmonellosis can be detected by the cultural isolation of clinical specimens. Infection is diagnosed when a laboratory test detects *Salmonella* bacteria in the stool or bodily fluids. The test could be a culture that isolates the bacteria or a culture-independent diagnostic test (CIDT) that detects the genetic material of the bacteria such as PCR.

1.5.1. Protocol:

The stool samples are pre-enriched in buffered peptone water, enriched in Selenite- F broth (Selenite F broth enhances the selective growth of *Salmonella* organisms), and DNA is extracted from the enriched Selenite F broth overnight stool culture. Alternatively, the enriched samples could be plated on selective plates. The DNA is used as a template to detect *Salmonella* targeting *InvA* gene using gene-specific primers. The samples which are PCR positive are considered positive for Salmonellosis

1.5.2. Protocol for isolation of *Salmonella*:

Though isolation of the *Salmonella* from the specimens is a time-consuming process, it is the gold standard for the detection of Salmonellosis. A matchstick head-size sample of stool is inoculated in 10 ml of buffered peptone water. The inoculated sample is incubated at 37 °C for 18-24 hours. Later, a loopful culture from the pre-enriched samples is enriched in selenite- F broth and aerobically incubated overnight at 37 °C for 18-24 hours. The top layer (1 ml) of an overnight culture is spun at 20,000 g for 5 minutes. A 1 µl loop is used to subculture *Salmonella* from the pellet by spreading on selective agar plates to achieve characteristic colonies.

A. MacConkey agar. Typical colonies appear transparent and colourless, sometimes with a dark center. Colonies of *Salmonella* will clear areas of precipitated bile caused by other organisms sometimes present.

B. Hektoen enteric (HE) agar: Blue-green to blue colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

C. Xylose lysine deoxycholate (XLD) agar: Pink colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies.

D. Bismuth sulphite (BS) agar: Brown, gray, or black colonies; sometimes they have a metallic sheen. The surrounding medium is usually brown at first but may turn black in time with increased incubation, producing the so-called halo effect.

1.5.3. **Bacterial Identification**:

A. TSI agar: 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. Incubate TSI slants at 35°C for 24 ± 2 h.

Salmonella in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI.

B. MR-VP test: Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella*. Incubate 48 ± 2 h at 35°C.

Perform Voges-Proskauer (VP) test at room temperature as follows: Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth an additional 48 h 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 results after 4 h; 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.

Perform methyl red test as follows: To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator. Read results immediately. 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 test.

Simmons citrate test: Inoculate this agar, using needle containing growth from unclassified TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 h at 35°C. Read results as follows:

- Positive- presence of growth, usually accompanied by colour change from green to blue. Most cultures of *Salmonella* are citrate-positive.
- Negative- no growth or very little growth and no colour change.
-

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

Biochemical and serological reactions of *Salmonella*				
#	Test or substrate	Result		*Salmonella* species reaction[(a)]
		Positive	Negative	
1.	Glucose (TSI)	yellow butt	red butt	+
2.	Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3.	H_2S (TSI and LIA)	Blackening	no blackening	+
4.	Urease	purple-red colour	no colour change	−
5.	Lysine decarboxylase broth	purple colour	yellow colour	+
6.	Phenol red dulcitol broth	yellow colour and/or gas	no gas; no colour change	+[(b)]
7.	KCN broth	Growth	no growth	−
8.	Malonate broth	blue colour	no colour change	−[(c)]
9.	Indole test	red colour at surface	yellow colour at surface	−
10.	Polyvalent flagellar test	Agglutination	no agglutination	+
11.	Polyvalent somatic test	Agglutination	no agglutination	+
12.	Phenol red lactose broth	yellow colour and/or gas	no gas; no colour change	−[(c)]
13.	Phenol red sucrose broth	yellow colour and/or gas	no gas; no colour change	−
14.	Voges-Proskauer test	pink-to-red colour	no colour change	−
15.	Methyl red test	diffuse red colour	diffuse yellow colour	+
16.	Simmons citrate	growth; blue colour	no growth; no colour change	v
[a] +: 90% or more positive in 1 or 2 days; −: 90% or more negative in 1 or 2 days; v: variable. [b] Majority of *S. arizonae* cultures are negative. [c] Majority of *S. arizonae* cultures are positive.				

The characteristic colonies will then be confirmed by PCR.

1.5.4. Detection of *Salmonella* spp. by PCR

***Salmonella* genus identification**

Organism	Gene	Primer Sequence	Product Size	Reference
Salmonella	inv A	F: (5´-TCG TGA CTC GCG TAA ATG GCG ATA-3´)	423 bp	Nair et al., 2015
		R:(5´-GCA GGC GCA CGC CAT AAT CAA TAA-3´)		

PCR Master mix:

Sl. No.	Name	Add (µl) per reaction
1.	10x PCR buffer	2.5
2.	MgCl2 (50mM)	1.0
3.	dNTP mix (10mM)	1.0
4.	InvA Forward primer	1.0 µL each
5.	InvA Reverse primer	1.0 µL each
6.	Taq DNA Polymerase (1U/µl)	1.0
7.	DNA template	4.0
8.	D/W	13.5

Thermal cycler Conditions

Temp. (0C)	Time (min/sec)	Cycle (s)
94	5 min	1
94	30 sec	35
56	1 min	
72	1min 30 sec	
72	10 min	1
4	Hold	

The amplified PCR products were resolved by agarose gel electrophoresis, using 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized and documented using UV gel documentation system.

1.5.5. Serotype identification of *Salmonella* isolates by multiplex PCR

The serotype identification namely *Salmonella* Enteritidis, *Salmonella* Typhi, and *Salmonella* Typhimurium for the confirmed *Salmonella* isolates can be carried out using multiplex PCR assay as described earlier (De Freitas *et al.* 2010) with slight modifications. The details of primers, genes targeted and the amplicon size for the serotypes mentioned above are presented in Table-1.

Table 1: Primers for *Salmonella* Serotype (*Salmonella* Enteritidis, *Salmonella* Typhi, *Salmonella* Typhimurium) Identification

Salmonella Enteritidis	Sdf I	Forward	TGT GTT TTA TCT GAT GCA AGA GG	304	De Freitas et al. 2010
		Reverse	TGA ACT ACG TTC GTT CTT CTG G		

Salmonella Typhi	Via B	Forward	CAC GCA CCA TCA TTT CAC CG	738	De Freitas et al. 2010
		Reverse	AAC AGG CTG TAG CGA TTT AGG		
Salmonella Typhimurium	Spy	Forward	TTG TTC ACT TTT TAC CCC TGA A	401	De Freitas et al. 2010
		Reverse	CCC TGA CAG CCG TTA GAT ATT		

PCR Master mix:

Sl. No.	Name	Add (µl) per reaction (25 µl)
1.	10x PCR buffer	2.5
2.	$MgCl_2$ (50mM)	3.0
3.	dNTP mix (10mM)	1.0
4.	Forward primer (Sdf I/ Via B/ Spy)	1.0 µL each
5.	Reverse primer (Sdf I/ Via B/ Spy)	1.0 µL each
6.	Taq DNA Polymerase (1U/µl)	3.0
7.	DNA template	5.0
8.	D/W	3.5

Thermal cycler Conditions:

Temp. (0C)	Time (min/sec)	Cycle (s)
94	5 min	1
94	30 sec	35
57	1 min	
72	1min 30 sec	
72	10 min	1
4	Hold	

The amplified PCR products were resolved by agarose gel electrophoresis, using 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized and documented using UV gel documentation system.

1.5.6. Tests for the detection of Typhoidal Salmonellosis (*Salmonella* Typhi, *S.* Paratyphi)

There are several serological tests for the rapid detection of Typhoidal Salmonellosis which include the Widal test, Typhidot test, TPT test. Widal test is the most commonly employed test for the detection of Typhoidal Salmonellosis.

Widal test:

1. Prepare a serial dilution of the patient serum @ 1:40 to 1:320
2. Add an equal volume of *Salmonella* antigen. This can be done as a Slide method or as a Tube method.
3. When running in the tube, then incubate tubes for 12 hours or overnight.

Interpretation:

1. The highest dilution of serum exhibiting agglutination is noted. If it ends at 1:320, then that is the titre.
2. **The Widal test is positive:**

1. if "O" antigen titre is >1:160 = active infection.
2. If the "H" antigen titre is >1:160, it indicates past infection or in immunized persons.
3. A fourfold increase in the titre (e.g., from 1:40 to 1:160) is diagnostic.

2. ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

3. Q-fever

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology laboratories, Medical centre's Consortium for one health project.

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP-3	**Q-fever**	**1.0**
Effective Date: 17-02-2022		

The content in this standard operating procedures are to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1. 1. Introduction:

Q fever is a bacterial infection caused by the Coxiella burnetii bacteria. It causes flu-like symptoms such as a high temperature, muscle pain and headaches. The infection results from inhalation of a spore-like small cell variant, and from contact with the milk, urine, feces, vaginal mucus, or semen of infected animals. Rarely, the disease is also known as tick borne. The 'Q' in Q fever stands for 'query'. This is because when the infection was first identified, its cause was unknown. The cause is now understood but the name has remained the same.

Q fever, first described in 1937, is a worldwide zoonosis that has long been considered an underreported and underdiagnosed illness because symptoms frequently are nonspecific, making diagnosis challenging. The causative organism, *Coxiella burnetii*, is an intracellular bacterium that tends to infect mononuclear phagocytes but can infect other cell types as well. Infection in humans usually occurs by inhalation of bacteria from air that is contaminated by excreta of infected animals. Other modes of transmission to humans, including tick bites, ingestion of unpasteurized milk or dairy products, and human-to-human transmission, are rare. Laboratory diagnosis relies mainly on serology, and doxycycline is the most effective treatment for acute illness.

Prevalence of Q-fever among animals in India was about 24.5% in cattle and 8.9% in buffaloes. On a global scale, data on the prevalence, distribution and movement are primarily form scattered reports. Serologic evidence of Q-fever has been found in a large number of wild and domestic animals, but infection is almost always subclinical. The primary animal reservoir of C. *burnetii* varies from area to area. *C.burnetii*is carried by many species of tick, and although ticks may serve as a vector between animals, they probably do not transmit the disease to humans. Humans are usually infected when they inhale aerosols generated by infected livestock. Infection occurs most frequently in abbatoirs, sheep research facilities, dairies, and in animal husbandry operations.

1.2. Purpose: Hospital based serosurveillance of Q-fever with IgG capture ELISA in human serum.

1.3. Case Definition

A suspected Q-fever case is a person meeting the definition of Q-fever. The Q-fever clinical case definition is a person of any age at any time of year with acute or insidious onset, of nonspecific febrile illness that might occur in conjunction with pneumonia or hepatitis. The most frequently reported symptoms include fever, fatigue, chills, and myalgia. Clinical laboratory findings may include elevated liver enzyme levels, leukocytosis, and thrombocytopenia. Acute fever usually accompanied by rigors, myalgia, malaise, and a severe retrobulbar headache. Fatigue, night-sweats, dyspnea, confusion, nausea, diarrhea, abdominal pain, vomiting, non-productive cough, and chest pain have also been reported. Severe disease can include acute hepatitis, atypical pneumonia with abnormal radiograph, and meningoencephalitis. Pregnant women are at risk for fetal death and abortion. Clinical laboratory findings may include elevated liver enzyme levels, leukocytosis, and thrombocytopenia. Asymptomatic infections may also occur. Note: Serologic profiles of pregnant women infected with acute Q fever during gestation may progress frequently and rapidly having chronic infection.

Clinical Criteria

Acute fever and one or more of the following: rigors, severe retrobulbar headache, acute hepatitis, pneumonia, or elevated liver enzyme levels.

1.4. Materials and Methods:

1.4.1. Study Design: Cross sectional study

Inclusion Criteria: As per below Case-definition

Case Classification:

Laboratory-Confirmed case: A suspected case with any one of the following markers:

- Serological evidence of a fourfold change in immunoglobulin G (IgG)-specific antibody titer to *C. burnetii* phase II antigen by indirect immunofluorescence assay (IFA) between paired serum samples, (CDC suggests one taken during the first week of illness and a second 3-6 weeks later, antibody titers to phase I antigen may be elevated or rise as well), **OR**

- Detection of *C. burnetii* DNA in a clinical specimen via amplification of a specific target by polymerase chain reaction (PCR) assay, **OR**
- Demonstration of *C. burnetii* in a clinical specimen by immunohistochemical methods (IHC), **OR**
- Isolation of *C. burnetii* from a clinical specimen by culture.

Probable Cases: A clinically compatible case of acute illness (meets clinical evidence criteria for acute Q fever illness) that has laboratory supportive results for past or present acute disease (antibody to Phase II antigen) but is not laboratory confirmed.

Exclusion Criteria: A suspected case in which diagnostic testing is performed and an etiological agent other than Q-fever is identified.

1.4.2. Type of sample: Serum

1.4.2.1 Methodology: All Serum Samples will be subjected to Q-fever IgG or IgM ELISA.

Principle:

Coxiella burnetii ELISA antigen is prepared by growth of standard strains in either embryonated hens' eggs or in cell culture, as described below under IFA. Wells of the microplate are coated with *C. burnetii* whole-cell inactivated antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the number of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically.

1.4.2.2. Primary sample: The test can be performed in serum.

1.4.2.2.1. Materials and reagents:

1. Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with *C. burnetii* antigen;
2. 37°C humidified incubator;

3. 8-and 12-channel pipettes with disposable plastic tips; microplate shaker (optional).
4. Positive and negative control sera; conjugate (ruminant anti-immunoglobulin or protein A/G labelled with peroxidase);
5. Tenfold concentration of diluent (PBS–Tween);
6. Distilled water;
7. Substrate or chromogen (TMB [tetramethylbenzidine],
8. ABTS [2,2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulphonic acid)] for peroxidase);
9. Hydrogen peroxide.

1.4.2.2.2. Materials (Not provided in the kit):

1. Biosafety Cabinet
2. Microplate washer
3. Microplate reader
4. Incubator (37°C), humidified with CO_2
5. Adjustable micropipette with disposable tips (5 µl to 500 µl)
6. High grade glass distilled water
7. Bread –box, tissue paper roll, aluminium foil
8. Glass tubes or Deep well plate for dilution of sample & graduated cylinder
9. Timer & Vortex

Procedure:

1. Dilute the serum samples, including control sera, to the appropriate dilution (1/100 or 1/400 depending on the kit used) and distribute 0.1 ml per well in duplicate. Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.
2. Cover the plate with a lid and incubate at room temperature for 30–90 minutes. Empty out the contents and wash three times in washing solution at room temperature.
3. Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well).
4. Cover each plate and incubate as in step ii. Wash again three times.

5. Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example: TMB in 0.1 M acetic acid and 30% H_2O_2 solution [0.2 µl/ml]; or 0.25 mM ABTS in citrate phosphate buffer, pH 5.0, and 30% H_2O_2 solution [0.1 µl/ml]).
6. Shake the plate; incubate according to the manufacturer recommendations, stop the reaction by adding stopping solution to each well, e.g., 0.05 ml 2 M sulphuric acid for TMB or 10% sodium dodecyl sulphate for ABTS.
7. Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450 nm (TMB). The absorbance values will be used to calculate the results.

Precautions:

1. Read the kit insert thoroughly before carrying out the test

2. Do not use kit reagents after expiry date

3. Do not use reagents from a different batch number

4. Do not freeze any of the reagents

5. On the day of performing a test, do not bring the reagents to room temperature

6. Avoid exposure of kit reagents to direct sunlight or higher temperature than recommended storage temperature

7.Use protective clothing, hand gloves, glasses while performing the assay.

8. Avoid mouth pipetting

9. If any of the reagents show precipitation, mix it by repeated pipetting.

10. Use clean, sterile, low protein binding tips and calibrated micropipettes

11. Use bench/disposable absorbent paper(filter paper) to cover the working table.

12. Avoid Microbial contamination of any of the reagents or cross contamination of different reagents.

13. Do not bring any of the reagents to room temperature. Take the vial from refrigerator, add to the wells and keep it back to the refrigerator.

14. Use the kit for testing Serum only. The kit is not optimized for testing other body fluids or whole blood. Serum with bacterial /fungal contamination or hemolysed may give invalid results.

15. Do not use heat inactivated or frozen and thawed sera sample.

16. Incomplete washing may adversely affect the results of the assay.

17. Pink/Yellow colour of the antigen is due to phenol red. It does not indicate contamination.

18. Poor quality distilled water (or dilution of wash buffer concentrate) can lead to erroneous results.

19.After washing the wells, add the next reagent immediately as per the protocol. Do not allow the wells to dry.

20. Consider all reagents as potential infectious and toxic. Handle them with care

21. Dispose of all left-over reagents as per the Biosafety norms followed in your organization

Kits Storage and Stability:

It is essential that all reagents /material is stored at 2-8^0C.Do not freeze any of the reagents. Do not bring/equilibrate any of the reagents to room temperature even on the day of performing the test. Monoclonal Antibody coated test strips must be protected from moisture. The unused strips should be placed safely in self-sealing pouch with desiccant.

1.5. Interpretation of results:

For commercial kits, interpretations and values are provided with the kit. For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Abpos) and negative (Abneg) control sera, and for each serum, calculate the percentage.

Ab - Ab / Abpos - Abneg x 100

Interpret the results as follows:

Ab <30% negative serum

Ab > 30% positive serum

Prepare a control chart and estimate the measurement uncertainty around the cut-off in order to interpret results close to the cut-off.

1.6. Safety Precautions:

1. Handle all specimens as potentially infectious.
2. Avoid contact with eyes, skin and mucous membrane.
3. Wear disposable gloves and face mask while doing the procedure.

2.0 REFERENCES

1. **Zoonotic Diseases of Public Health Importance (3rd Edition-Zoonosis Division-NCDC.**
2. **Centre Diseases Control**
3. **Q-fever -OIE Terrestrial Manual 2018.**
4.

ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

4. Brucellosis

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology laboratories, Medical centre's Consortium for one health project.

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP- 4	**Brucellosis**	**1.0**
Effective Date: 01-03-2022		

The content in these standard operating procedures is to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1.1. Introduction:

Brucellosis is a zoonotic disease (any disease or infection that is naturally transmissible from vertebrate animals to humans and vice-versa is classified as a zoonotic disease). It is also known as "undulant fever", "Mediterranean fever" or "Malta fever". Brucellosis is a disease of mainly cattle, swine, goats, sheep and dogs.

The infection is transmitted to humans by animals through direct contact with infected materials like afterbirth or indirectly by ingestion of animal products and by inhalation of airborne agents. Consumption of raw milk and cheese made from raw milk (fresh cheese) is the major source of infection in man. It is also an occupational disease for people who work in the livestock sector. It affects people of all age groups and of both sexes.

Brucellosis is an acute or sub-acute febrile illness usually marked by an intermittent or remittent fever accompanied by malaise, anorexia, and prostration, and which, in the absence of specific treatment, may persist for weeks or months. Typically, few objective signs are apparent but enlargement of the liver, spleen and/or lymph nodes may occur, as may signs referable to almost any other organ system. The acute phase may progress to a chronic one with relapse, development of persistent localized infection or a non-specific syndrome resembling the "chronic fatigue syndrome". The disease is always caused by infection with a *Brucella* strain and diagnosis must be supported by laboratory tests which indicate the presence of the organism or a specific immune response to its antigens.

1.2. Purpose: Hospital based serosurveillance of Brucellosis Monoclonal Antibody (MAb) blocking ELISA in human serum.

1.3. Case definition according to WHO

A suspected Brucellosis case: is a person meeting the definition of Brucellosis. The Brucellosis clinical case definition is a person of any age at any time of year with acute or insidious onset, with continued, intermittent or irregular fever of variable duration, profuse sweating particularly at night, fatigue, anorexia, weight loss, headache, arthralgia and generalized aching.

Laboratory-Confirmed case: A suspected case with any one of the following markers: Presence of Monoclonal MAb antibody in serum to a specific antigen. **Probable Cases**: A clinically compatible case that is epidemiologically linked to a confirmed case or that has supportive serology.

Exclusion Criteria: A suspected case in which diagnostic testing is performed and an etiological agent other than brucellosis is identified.

Type of sample: Serum

1.4. Materials and Methods:

1.4.1. **Study Design:** Cross sectional study

Inclusion Criteria: As per below Case-definition

Methodology: All Serum Samples will be subjected to Brucellosis MAb ELISA.

Principle:

Brucella ELISA is developed based on the principle of MAb blocking ELISA, where sLPS is precoated on the polystyrene surface of maxisorb microwell modules, to which the brucella specific antibodies of field sera binds and blocks the sLPS epitope. The unbound serum is removed by washing and peroxidise (HRP) conjugated brucella specific Mab is added. The unbound conjugate is washed and TMB substrate is added. The substrate is hydrolysed by the enzyme and subsequent colour development is indicative of the presence of brucella antibodies in the tested bovine sera.

1.4.2. **Primary sample:** The test can be performed in serum.

Materials provided in the kit:

1. Brucella sLPS coated microwells – (12 x 8 wells). Ready for use. Microwells should be resealed immediately and stored in the presence of desiccant. Stable at 2-8°C for one year.
2. Wash Buffer (10 x)- one bottle: 50ml of 10 x concentrate. Dilute one part wash buffer with 9 parts of distilled water. Diluted buffer may be stored for one week at room temperature.
3. Sample diluent- one bottle, 12ml ready to use. Stable at 2-8° for 6 months.
4. HRP conjugated brucella specific MAbone bottle; 12 ml ready to use horseradish peroxidise conjugated MAb with preservative. Stable at 2-8° for 6 months.
5. Substrate- one bottle, 12ml ready to use. Stable at 2-8° for 6 months.
6. Positive control – one vial, 200 µl bovine serum. Stable at 2-8° for 6 months.
7. Negative control - one vial, 200 µl bovine serum. Stable at 2-8° for 6 months.

8. Quality control – one vial, 200 µl of sera. Stable at 2-8° for 6 months.
9. Stop solution – one bottle, 15 ml ready to use. Stable at room temperature.

Materials (Not provided in the kit) :

1. Biosafety Cabinet
2. Microplate washer
3. Microplate reader
4. Incubator (37ºC), humidified with CO_2
5. Adjustable micropipette with disposable tips (5 µl to 500 µl)
6. High grade glass distilled water
7. Bread –box, tissue paper roll, aluminium foil
8. Glass tubes or Deep well plate for dilution of sample & graduated cylinder
9. Timer & Vortex.

Procedure:

1. All reagents should be equilibrated to room temperature (22-25°C).

2. Remove the required number of microwells from the foil sachet and insert into strip holder Along with the samples, six microwells are required for negative control (N), positive control (P) and quality control (QC) in duplicate. The remaining unused microwells should be sealed tightly and replaced in the foil sachet.

3. Pipette 100 µl of positive, negative, and quality controls to respective control wells.

4. Pipette 90 µl of sample diluents to the sample wells and 10 µl of sera samples to the respective sample wells.

5. Gently mix the contents of the wells, seal the plate and incubate for 1 hour at room temperature (20-25 ° C).

6. Wash 4 times with PBST (300 µl per well).

7. Add 100 µl of conjugate to each well. Seal the plate and incubate for 1 hour at room temperature (20-25 ° C)

8. Wash 4 times with PBST.

9. Add 100 µl of substrate (TMB) to each well. Keep the plate for 10 minutes at room temperature.

10. Add 100 µl of stop solution to each well.

11. Read optical density (OD) of each well at 450 nm within 5 minutes after addition of stop solution.

Precautions:

1. Read the kit insert thoroughly before carrying out the test

2. Do not use kit reagents after expiry date

3. Do not use reagents from a different batch number

4. Do not freeze any of the reagents

5. On the day of performing a test, do not bring the reagents to room temperature

6. Avoid exposure of kit reagents to direct sunlight or higher temperature than recommended storage temperature

7.Use protective clothing, hand gloves, glasses while performing the assay.

8. Avoid mouth pipetting

9. If any of the reagents show precipitation, mix it by repeated pipetting.

10. Use clean, sterile, low protein binding tips and calibrated micropipettes

11. Use bench/disposable absorbent paper (filter paper) to cover the working table

12. Avoid Microbial contamination of any of the reagents or cross contamination of different reagents

13. Do not bring any of the reagents to room temperature. Take the vial from refrigerator, add to the wells and keep it back to the refrigerator.

14. Use the kit for testing Serum only. The kit is not optimized for testing other body fluids or whole blood. Serum with bacterial /fungal contamination or hemolysed may give invalid results.

15. Do not use heat inactivated or frozen and thawed sera sample.

16. Incomplete washing may adversely affect the results of the assay.

17. Pink/Yellow colour of the antigen is due to phenol red. It does not indicate contamination.

18. Poor quality distilled water (or dilution of wash buffer concentrate) can lead to erroneous results.

19.After washing the wells, add the next reagent immediately as per the protocol. Do not allow the wells to dry.

20. Consider all reagents as potential infectious and toxic. Handle them with care.

21. Dispose of all left-over reagents as per the Biosafety norms followed in your organization

Kits Storage and Stability: It is essential that all reagents /material is stored at 2-8^{0}C.Do not freeze any of the reagents. Do not bring/equilibrate any of the reagents to room temperature even on the day of performing the test. Monoclonal Antibody coated test strips must be protected from moisture. The unused strips should be placed safely in self-sealing pouch with desiccant.

QUALITY CONTROL

Validation criteria for assay:

Negative control should be above 1.0 OD.

Positive control should be above 80 PI

Quality control should be between 40 to 70 PI

2.0. Results:

Cut-off analysis

Percentage Inhibition (PI) is calculated by the formula:

PI = 100 – {(Test sample OD/Negative control OD)} X 100.

Cut-off achieved for Indian bovine population

Percentage Inhibition of 40 is achieved as cut-off for Indian cattle population for brucella sero-diagnostics by two graph ROC analysis using reference sera samples (n = 200).

Safety Precautions:

1. Handle all specimens as potentially infectious.
2. Avoid contact with eyes, skin and mucous membrane.
3. Wear disposable gloves and face mask while doing the procedure.

3.0. REFERENCES

Kit inserted provided by TRPVB-TANUVAS

1. **Zoonotic Diseases of Public Health Importance (3rd Edition-Zoonosis Division-NCDC.**
2. **Brucellosis in Human and Animals -OIE-WHO.**

ANNEXURES & FORMS:

ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

Annexure 2 - SOP prepared by and Kit

5. Japanese Encephalitis

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology laboratories, Medical centre's Consortium for one health project.

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP- 5	**NIV JE**	**1.0**
Effective Date: 17-02-2022		

The content in this standard operating procedures are to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1.1. Introduction:

Acute encephalitis syndrome (AES) is a serious public health problem in India. It is characterized as acute-onset of fever and a change in mental status (mental confusion, disorientation, delirium, or coma) and/or new-onset of seizures in a person of any age at

any time of the year. The disease most commonly affects children and young adults and can lead to considerable morbidity and mortality (1).

Japanese encephalitis virus (JEV) is the major cause of AES in India (ranging from 5%-35%). AES due to JEV was clinically diagnosed in India for the first time in 1955 in the southern State of Madras, now Tamil Nadu. During 2018, 10485 AES cases and 632 deaths were reported from 17 states to the National Vector Borne Diseases Control Programme (NVBDCP)*in India, with a case fatality rate around 6 per cent. AES cases were reported mainly from Assam, Bihar, Jharkhand, Karnataka, Manipur, Meghalaya, Tripura, Tamil Nadu, Uttar Pradesh (1)

Japanese Encephalitis (JE) is a mosquito borne zoonotic viral disease. The virus is maintained in animals, birds, pigs, particularly the birds belonging to family Ardeidae (eg. Cattle egrets, pond herons etc) which act as the natural hosts. Pigs & wild birds are reservoirs of infection and are called as amplifier hosts in the transmission cycle, while man and horse are dead end hosts. The virus does not cause any disease among its natural hosts and transmission continues through mosquitoes primarily belonging to vishnui group culex. Vector mosquito is able to transmit JE virus to a healthy person after biting an infected host with an incubation period ranging from 5 to 14 days (2).

The disease affects the Central Nervous System and can cause severe complications, seizures and even death. The Case Fatality Rate (CFR) of this disease is very high and those who survive may suffer from various degrees of neurological sequale. (An estimated 25% of the affected children die, and among those who survive, about 30- 40% suffers from physical & mental impairment). The children suffer the highest attack rate because of lack of cumulative immunity due to natural infections. (2)

Infection with Japanese Encephalitis virus may be asymptomatic, or may cause febrile illness, meningitis, myelitis or encephalitis. Encephalitis is the most commonly recognized presentation of JE and is clinically indistinguishable from other causes of an acute encephalitis syndrome (AES).

JE surveillance therefore, aims to identify patients with AES followed by serologically confirming JE viral infection using standardized laboratory techniques (3). Strong surveillance system is an integral part and a pre-requisite for any disease control programme especially in a disease like JE, which has epidemic potential and high case fatality. (3)

1.2. Purpose: Hospital based serosurveillance of JE IgM ELISA in human serum and CSF samples

1.3. Case definition according to WHO

A suspected JE case: is a person meeting the definition of AES. The AES clinical case definition is a person of any age at any time of year with the acute onset of fever and at least one of the following:

1. a change in mental status (including symptoms such as confusion, disorientation, coma or inability to talk) or
2. new onset of seizures (excluding simple febrile seizures).

(A simple febrile seizure is defined as a seizure that occurs in a child aged 6 months to < 6 years old, whose only finding is fever and a single generalized convulsion lasting less than 15 minutes, and who recovers consciousness within 60 minutes of the seizure.)

Case Classification:

Laboratory-Confirmed case: A suspected case with any one of the following markers:

- Presence of lgM antibody in serum and/ or CSF to a specific virus including JE/Entero Virus or others
- Four-fold difference in lgG antibody titre in paired sera
- Virus isolation from brain tissue
- Antigen detection by immunofluroscence
- Nucleic acid detection by PCR

In the sentinel surveillance network, AES/JE will be diagnosed by lgM Capture ELISA, and virus isolation will be done in National Reference Laboratory.

Probable Cases: Suspected case in close geographic and temporal relationship to a laboratory confirmed case of AES/JE in an outbreak

Exclusion Criteria: A suspected case in which diagnostic testing is performed and an etiological agent other than AES/JE is identified

Type of sample: Serum /CSF.

1.4.Materials and Methods:

1.4.1. **Study Design**: Cross sectional study

Inclusion Criteria:

Sample Size: 290

1.5. Methodology: All Serum/CSF Samples will be subjected to JE **IgM** ELISA **(NIV, Pune)**

Principle:

IgM antibodies in the patient's serum/CSF are captured by anti-human IgM (µ chain specific) coated on to solid surface(wells). In the next step, JE antigen is added which binds to captured human IgM in the sample. Unbound antigen is removed during the washing step. In the subsequent step biotinylated Flavivirus anti JE monoclonal antibodies are added followed by Avidin-HRP. Subsequently, chromogenic substrate (TMB/H_2O_2) is added, the reaction is stopped by 1N H_2SO_4.The intensity of color/optical density is measured at 450nm.The kit is for in vitro use only.

1.6. PROCEDURE:

1.6.1. Primary sample: The test can be performed in serum /CSF.

1.6.2. Materials (Not provided in the kit):

1. Biosafety Cabinet
2. Microplate washer
3. Microplate reader
4. Incubator (37ºC), humidified with CO_2
5. Adjustable micropipette with disposable tips (5 µl to 500 µl)
6. High grade glass distilled water
7. Bread –box, tissue paper roll, aluminium foil
8. Glass tubes or Deep well plate for dilution of sample & graduated cylinder
9. Timer & Vortex

1.7. Materials provided in the kit:

1. Anti-human IgM coated microwells (12x8 wells)- Ready for use.

Twelve strips with eight wells each are coated with polyclonal rabbit anti Human IgM antibodies and post coated to block nonspecific binding of any protein, and also to stabilize the coated antibody. Stable at 2-8^{0}C if protected from moisture.

1. **Sample Diluent for JE IgM- Ready for use.**
   One bottle (60ml) /kit.

   Phosphate Buffered Saline with additives and antibiotics for dilution of test samples (serum/CSF). Do not dilute JE IgM Positive Control or JE IgM Negative Control.

2. **Wash Buffer Concentrate (20x)**
   One bottle (60ml) /kit.

   Phosphate buffered saline with surfactant and antibiotics. If the Wash Buffer Concentrate shows crystallization, warm the bottle at 37^0C until crystallization disappears. Before use, dilute wash buffer concentrates 1:20 (1 part of buffer concentrate + 19 parts of high-grade distilled water). For assay of eight samples (six clinical samples and two controls),100 ml of diluted wash buffer is sufficient.

3. **Japanese Encephalitis Antigen –Ready to use**
   One vial (6ml)/kit JE antigen in stabilizer with additives and antibiotics. The antigen may show pink/yellowish colour due to the phenol red indicator. Unused antigen must be decontaminated before it is discarded.

4. **Anti JE monoclonal antibody (Biotin labelled)-Ready to use.**
   One vial (6ml)/kit Anti JE Monoclonal Antibody-Biotin labelled diluted in stabilizer with additives and antibiotics.

5. **Avidin-HRP-Ready to use.**
   One vial (6ml)/kit

   Avidin-HRP diluted in stabilizer with additives and antibiotics

   Protect the solution from direct exposure to light.

6. **Liquid TMB substrate –Ready to use.**
   One vial (12ml)/kit.

   Tetramethyl benzidine Dihydrochloride. It is light sensitive.

   Protect from light.

7. **Stop solution-Ready to use.**

   One vial (12ml)/kit.

   1 N H_2SO_4 Wear protective gloves mask and eye glasses while handling stop solution.

8. **JE IgM Positive Control-Ready to use**

   One vial (0.8ml)/kit

   Human serum positive for JEV IgM antibodies diluted in stabilizer with additives and antibiotics.

   This is ready to use reagent. Do not dilute.

9. **JE IgM Negative Control –Ready to use**

   One vial (0.8ml)/kit

   Human serum negative for Dengue IgM antibodies diluted in stabilizer with additives. This will help to monitor integrity of the kit. This is ready to use reagent. Do not dilute.

It is essential that all reagents /material is stored at 2-8^{0}C.

2.0. Precautions:

1. Read the kit insert thoroughly before carrying out the test

2. Do not use kit reagents after expiry date

3. Do not use reagents from a different batch number

4. Do not freeze any of the reagents

5. On the day of performing a test, do not bring the reagents to room temperature

6. Avoid exposure of kit reagents to direct sunlight or higher temperature than recommended storage temperature

7.Use protective clothing, hand gloves, glasses while performing the assay.

8. Avoid mouth pipetting

9. If any of the reagents show precipitation, mix it by repeated pipetting.

10. Use clean, sterile, low protein binding tips and calibrated micropipettes

11. Use bench/disposable absorbent paper (filter paper) to cover the working table

12. Avoid Microbial contamination of any of the reagents or cross contamination of different reagents.

13. Do not bring any of the reagents to room temperature. Take the vial from refrigerator, add to the wells and keep it back to the refrigerator.

14. Use the kit for testing Serum/CSF only. The kit is not optimized for testing other body fluids or whole blood. Serum/CSF with bacterial /fungal contamination or hemolysed may give invalid results.

15. Do not use heat inactivated or frozen and thawed sera sample.

16. Incomplete washing may adversely affect the results of the assay.

17. Pink/Yellow colour of the antigen is due to phenol red.It does not indicate contamination.

18. Poor quality distilled water (or dilution of wash buffer concentrate) can lead to erroneous results.

19.After washing the wells, add the next reagent immediately as per the protocol. Do not allow the wells to dry.

20. Consider all reagents as potential infectious and toxic. Handle them with care

21. Dispose of all left-over reagents as per the Biosafety norms followed in your organization.

2.1. Kits Storage and Stability:

It is essential that all reagents /material is stored at 2-8^{0}C.Do not freeze any of the reagents. Do not bring/equilibrate any of the reagents to room temperature even on the day of performing the test. Anti-Human IgM coated test strips must be protected from moisture. The unused strips should be placed safely in self-sealing pouch with desiccant.

2.2. Procedure:

Thoroughly mix all the reagents using the micropipette tips before addition.The test procedure must be followed meticulously. Don’t bring any of the kit reagents to room

temperature before commencing the test. All the reagents are stable at 2-8^{0}C and for in vitro use only.

Preparation of reagents before performing test

(a) Dilute wash buffer concentrate to 1X (mix one part of wash buffer concentrate with 19 parts of high grade glass distilled water. For assay of eight samples (six clinical samples and two controls),100ml of diluted wash buffer is sufficient. The unused diluted Wash Buffer can be stored in a refrigerator for two months or until it shows microbial growth.

(b) Remove the Anti Human IgM coated strip (a strip of 8 wells) as per the number of samples to be tested and fix it on to the strip holder. The unused strips must be sealed immediately with the film and kept in the plastic pouch. The unused strips must be protected from moisture.

(c) At the time of addition take the reagents from the refrigerator, add required quantity of the reagent to the wells and keep the vial back to the refrigerator. **Do not bring any of the reagents to room temperature**. This may lower the shelf life of the kit.

(d) After washing the strips, the next reagents must be added immediately before the well become dry. Dried wells may give erroneous results.

2.3. Protocol

1. Select the samples to be assayed. Write down the protocol on ELISA sheet provided with each kit.

2. Dilute serum 1:100 in tubes or CSF 1:10 in tubes or preferably in deep well plate using Sample Diluent for JE IgM.

3. Remove required number of anti IgM coated strips. Number the test strips as 1,2,3, …

4. Wash the strips 3 times with 1X Wash Buffer. Do not allow the wells to dry.

5. Transfer 50 µl of diluted samples from deep well plate to respective wells as per the protocol on ELISA sheet using multichannel pipette.

6.Add 50 µl of JE IgM Positive control and JE IgM Negative control to respective wells as per the protocol.

7. Cover the plate with aluminium foil to prevent evaporation of samples. Keep the plate in a closed humidified box (A bread box with a soaked cotton/tissue paper) inside the incubator and incubate the plate at 37^0C for 1 hour. Use timer for accurate incubation period.

8. At the end of incubation, wash the plate five times with wash buffer. Tap the plate after last wash on a tissue paper.

9.Add 50 µl of JE antigen to all each well of the plate. Take out the JE antigen vial from the refrigerator, add 50 µl to each well and put the vial back to the refrigerator. **Do not equilibrate with room temperature.**

10. Repeat Step No 7 and 8.

11.Add 50 µl of Anti JE Monoclonal antibody (Biotin labelled) to each well.

12.Repeat Step No 7 and 8.

13.Add 50 µl of Avidin-HRP to each well.

14.Cover the plate with aluminium foil to prevent evaporation of samples. Keep the plate in a humidified box (A bread box with a soaked cotton/tissue paper) and incubate the plate at 37^0C for 30 minutes. Use timer for accurate incubation period.

15.Repeat Step No 8.

16.Add 100 µl of Liquid TMB substrate (TMB/H_2O_2) to each well.

17. Incubate at room temperature **in Dark** for 10 minutes.

18.Stop the reaction exactly after 10 minutes with 100 µl Stop Solution.

19.Measure the absorbance at 450 nm within 10 minutes after termination of reaction.

2.4. QUALITY CONTROL

Each kit contains one vial of “Positive Control: and one vial of “Negative control”. These work as marker of kit performance.

1.If OD of Negative control is more than 0.18 (Or)

2. If OD of the Positive control is less than 5 times the OD of Negative Control.

In both situations, the test should be considered as invalid.

3.0. RESULT:

INTERPRETATION OF RESULTS:

1. If OD value of sample tested is less than OD of Negative control by a factor 3.0 (Sample OD$\leq$Negative Control OD ×3.0), **the sample should be considered as “Negative”.**
2. If OD value of sample tested exceeds OD of Negative control by a factor 5.0 (Sample OD$\geq$Negative Control OD ×5.0,)**the sample should be considered as “Positive**”.
3. If OD value of sample tested exceeds OD of Negative control by a factor 3.0 (Sample OD $\geq$Negative control OD × 3.0), but it is less than OD of Negative control by factor 5.0(Sample OD $\leq$ Negative control OD × 5.0),**the sample should be considered as “Equivocal”.**

RESULT	INTERPRETATION

Negative	Sample does not contain detectable levels of JE IgM and indicates no recent JEV infection. But if the clinical symptoms still suggest possible role of JEV, the sample should be subjected to virus isolation/genome detection or immunofluorescence studies to show presence of JE viral antigen in the sample to rule out that the sample was probably collected during early acute phase of infection before development of detectable levels of JEV IgM antibodies.
Positive	The sample contains detectable levels of JE IgM and is interrupted as **Presumptive JE infection or a Probable case of JE**. Since serological cross reactivity among the flavivirus group is common, role of other flaviviruses like West Nile, and Dengue serotypes circulating in the geographic areas must be ruled out.The results must be confirmed by PRNT or four fold rise in IgG titres of paired sample or follow the guidelines laid down by WHO from time to time.
Equivocal	It indicates that second sample collected 8-10 days after the first sample should be tested for JE IgM or else by an alternate method of testing should be adopted.

3.1. Limitations:

(a) Clinical signs, symptoms and epidemiology of disease should be considered critical for interpretation of the results. Detection of JE cases in the new area should be validated by another confirmatory test preferably by PRNT.

(b) Serological cross reactions are common within the flaviviruses i.e. Dengue Serotypes 1,2,3,4, West nile encephalitis,etc. Possible involvement of other circulating flaviviruses in the geographical region must be excluded before confirmation of Diagnosis.

(c) The performance of the assay has not been optimized for visual determination of OD.

(d) IgM results of the immunocompromised individuals must be interpreted with care.

(e) NIV JE IgM ELISA has not been optimized for

- Seroconversion studies after JE vaccination.
- Screening general population without symptoms of encephalitis.
- Testing samples from blood donors.
- Screening cord blood, neonates or prenatal antibodies.

3.2. Safety Precautions:

1. Handle all specimens as potentially infectious.

2. Avoid contact with eyes, skin and mucous membrane.

3. Wear disposable gloves and face mask while doing the procedure.

4.0. REFERENCES

Kit insert provided by NIV

1.Acute encephalitis syndrome | National Health Portal Of India (nhp.gov.in)-2019

2. Operational Guidelines (nvbdcp.gov.in) -2014

3. JE Book.cdr (nvbdcp.gov.in) -2006

ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

6. Listeriosis

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology laboratories, Medical centre's Consortium for one health project

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP-6	**Listeriosis**	**1.0**
	Effective Date: 17-02-2022	

The content in this standard operating procedures are to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1.1. Introduction

Listeria monocytogenes, the causative agent of listeriosis, has been implicated in increasing foodborne outbreaks worldwide. The disease is manifested in various forms ranging from severe sepsis in immune-compromised individuals, febrile gastroenteritis, still birth, abortions and meningoencephalitis in humans and abortions, stillbirths, mastitis, reproductive disorders, encephalitis in animals.

With the recent discovery of several new species, the genus *Listeria* comprises 26 species: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, L. grayi, L. marthii, L. costaricensis, L. rocourtiae, L. fleischmannii, L. newyorkensis, L. weihenstephanensis, L. floridensis, L. aquatica, L. thailandensis, L. cornellensis, L. riparia, L. booriae, L. goaensis* and *L. grandensis*, *L. valentina*, *L. farberi, L. portnoyi, L. cossartiae, L. rustica*, and *L. immobilis*. Among these species, *L. monocytogenes* is the most frequent pathogen in humans and animals, while *L. ivanovii* causes disease in animals.

The majority of cases in adults and juveniles occur amongst the immunosuppressed, i.e. patients receiving steroid or cytotoxic therapy or with malignant neoplasms. Other 'at risk' groups include patients with AIDS, diabetics, elderly people, kidney dialysis patients, individuals with prosthetic heart valves or replacement joints and individuals with alcoholism or alcoholic liver disease.

L. monocytogenes as a major pathogen lies in its ubiquitous nature and wide host range, which includes 40 mammals, 20 birds, crustaceans, ticks and fishes. *L. monocytogenes* has been isolated from various animals like sheep, goat, cattle, swine, dog, cat and horses besides rodents and birds. However, many wild and domestic animal species such as sheep, goat, buffalo, cattle, pig, poultry, bird and fish are intestinal carriers of listeriae.

The pathogen can breach blood-brain, intestinal and placental barrier and has tropism for all tissues.

1.2.Purpose: The purpose of the present SOP is isolation and identification of Listeria monocytogenes from clinical cases and foods.

1.3.**Case definition**: There are two main types of listeriosis: a non-invasive form and an invasive form.

Non-invasive listeriosis (febrile listerial gastroenteritis): A mild form of the disease affecting mainly otherwise healthy people. Symptoms include **diarrhoea, fever, headache and myalgia (muscle pain).**

I**nvasive listeriosis:** A more severe form of the disease and affects certain high-risk groups of the population. These include pregnant women, patients undergoing treatment for cancer, AIDS and organ transplants, elderly people and infants. This form of disease is characterized by severe symptoms and a high mortality rate (20%–30%). The symptoms include **fever, myalgia (muscle pain), septicemia, meningitis.** The incubation period is usually one to two weeks but can vary between a few days and up to 90 days.

1.4.Materials & Methods

Samples to be collected

Humans: Blood (5 ml), cerebrospinal fluid, vomitus, meconium in case of aborted foetuses, placental bits, deep cervical swab.

Animals: Blood, serum (for serological examination), vaginal secretions, faecal matter, milk from mastitic animals (5 to 10 ml)

Foods: Meat, milk and their products: about 25 grams

The samples may be transported in sterile containers. The samples can be stored in refrigerator if not processed immediately.

1. Which test to be used (preferably one suitable for surveillance), protocol (may be brief, but can be expansive), interpretation of the result (including any cut-off), reporting (including to whom).

The protocol for isolation and identification of Listeria monocytogenes *from clinical cases and foods*

ISOLATION

(Clinical samples, food)

SELECTIVE ENRICHMENT

USDA (University of Vermont Medium I and II) (USDA) or Fraser broth (ISO)

SELECTIVE PLATING

PALCAM AGAR or Chromogenic media

CONFIRMATION

Routinely used methods

PHENOTYPE	**GENOTYPE**
CULTURE	BIOCHEMICALS
Gram Stain	Sugar Fermentation
Motility	Biochemical testing

Hemolysis

Camp Test

PI-PLC Assay

TYPING

Serotyping

RAPD, PCR

Typical Isolation of *L. monocytogenes* by USDA/ISO method

1.4.1. **Enrichment of sample:**

For solid sample such as meat, fish, aborted material, tissue etc. homogenization or maceration is recommended. Take approx. 5g 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 45ml of UVM-I or Half Fraser Broth. Incubate at 37^0C for 24 h. After incubation transfer 0.1 ml of UVM-I or Half Fraser to the UVM-II or Full Fraser broth and further incubate at 37^0C for 24-48h. For sample from humans which are to be resuscitated, a pre-enrichment step is required. 5 ml of each of the blood and urine samples and 5 g of the placental tissue to be inoculated into 50 ml of Pre-enrichment Broth (PEB; Tryptic Soy broth with 0.6% yeast extract for 24 h at 30^OC.

1.4.2. Isolation of *Listeria*

Loopful from enriched UVM-II or full Fraser Broth, streak on selective agar such as PALCAM agar plate. Incubate at 37^0C for 24h-48h. 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 presume as of *Listeria.* The agar plates should be observed daily and peculiar colonies should be sub-cultured as early as possible before storing the agar plate in refrigerator. Pick such colonies and transfer to Brain Heart Infusion Broth (appendix) and preserve at 4^0C.

Confirmation of *Listeria spp.*

Suspected isolates then can be confirmed by ALOA and hemolysis test.

1.4.2.1.ALOA Test:

Isolates can be confirmed by observing Phosphatidyl-inositol Phospholipase C activity by spot inoculating on ALOA agar plate (Himedia or any other) and incubating at 37^0C for 48 h. Isolates showing green coloured on agar plate will be *Listeria* spp. while green colour with halo will be pathogenic spp. (either *L. monocytogenes* or *L. ivanovii*).

1.4.2.2.Hemolysis test:

Pathogenic listerial spp. can be differentiated from non-pathogenic spp. by hemolysis test. Streak the isolates on 5% sheep blood agar, incubate at 37^0C for 24h and observe for hemolysis. Pathogenic spp. of *Listeria* exhibit weak β-hemolysis.

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

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 fig. 1.

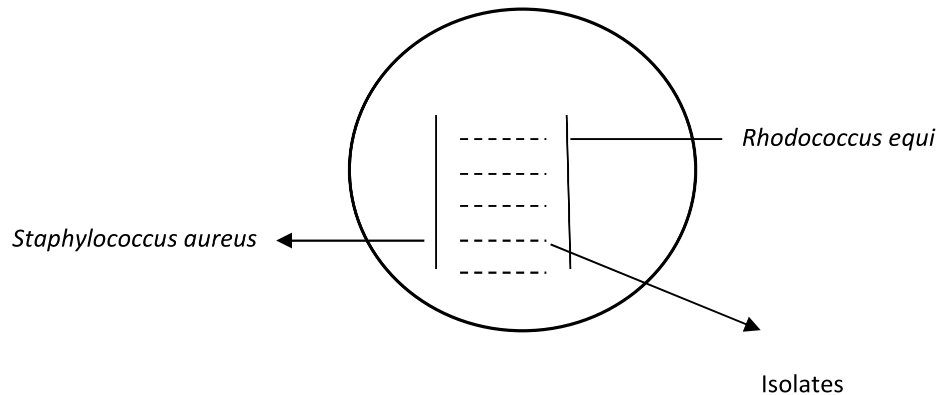


Fig. 1: Streaking for CAMP test on 5% sheep blood agar plate. All dotted lines are horizontal streaking lines of suspected pathogenic strains of *Listeria* spp.

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 strains streaking line. Inoculate these plates and incubated at 37^0C for 24 h. 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. *L. mononcytogenes* strain gives hemolysis synchronously with *S. aureus* and *R. equi,* while *L. ivanovii* strains gives hemolysis with *R. equi* only.

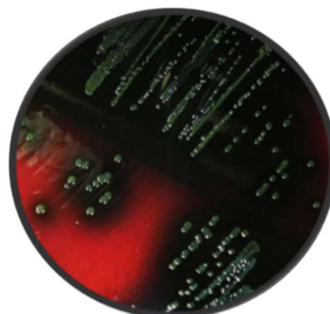
1.4.2.3.Biochemical differentiation:

Perform the Gram staining for typical colonies of presumptive *Listeria*. Presence of Gram-positive coccobacilli can be presumed as *Listeria* spp. Perform further biochemical as in **Table 1** to differentiate the listerial spp.

Table 1

	L. monocytog	L.innocua	L.ivanovii	L.seeligeri	L.welshimeri	L.grayi	L. marthii	L. rocoucrtia
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: Differentiation of *Listeria* spp. (+: postive reaction; -: negative reaction; ?: not known



Listeria colonies on PALCAM agar

ISO 11290 method for the detection of *L. monocytogenes* from foods

The method prescribed by ISO 11290 had an overall sensitivity of 85.6% and a specificity of 97.4%. *L. monocytogenes* can be detected after primary enrichment, although secondary enrichment often yielded further specificity.

The Bureau of Indian standards has adopted the methods for detection and enumeration of *L. monocytogenes* from foods as ISO 11290 Part 1 and Part 2 (1996) equivalent to IS 14988 Parts 1 and 2 (2001) in India.

IS 14988 (Part 1):2001/ ISO 11290-1:1996 Microbiology of Food and Animal Feeding Stuffs Horizontal Method for Detection and Enumeration of *Listeria monocytogenes*: Part 1 Detection Method. Bureau of Indian Standards, New Delhi, India.

IS 14988 (Part 2):2001/ ISO 11290–2:1996 Microbiology of Food and Animal Feeding Stuffs— Horizontal Method for Detection and Enumeration of *Listeria monocytogenes*: Part 2 Enumeration Method. Bureau of Indian Standards, New Delhi, India.

(Note: For solid samples, blend the sample with blender in a sterile bag)

1. Take 25 gm (or 25 ml) of the solid (or liquid) food sample.
2. For primary enrichment, inoculate sample in 225 ml of half-Fraser broth and incubate at 30ºC for 24 h.
3. For secondary enrichment, transfer 0.1 ml of the enriched half-Fraser broth to 10 ml of the Fraser broth at 37ºC for 24 h.
4. Streak a loopful of enriched culture from half-Fraser broth (step 3) and Fraser broth (step 4) on PALCAM selective or Chromogenic media (ALOA) plate and incubate at 30ºC (for Oxford) or 37ºC for (PALCAM).
5. Examine the plate after 24 h of incubation (if necessary, observe after 48 h) for the presence of the characteristic colonies.
6. Confirm the *L. monocytogenes* isolates by means of appropriate morphological, and biochemical tests on five presumptive colonies from a single plate.
   (Note: For preparation of Half-Fraser broth, add half content of Fraser supplement.)

1.4.3. Detection of *Listeria monocytogenes* by PCR

1.4.3.1. <u>Genus and species-specific multiplex PCR</u>

The isolates showing characteristic biochemical properties are further subjected to genus and species-specific multiplex PCR targeting *prs* (Genus specific) and *isp* (Species specific) gene.

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

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

b.

PCR protocol :

10X PCR buffer - 2.5 µL (final concentration of 1X)

10 mM dNTP mix - 2.0 µL (final concentration of 0.8 mM)

50 mM $MgCl_2$ - 2.0 µL (final concentration of 4.0 mM)

10 pM Primer *Prs* (Forward) - 1.0 µL (final concentration of 0.4µM)

10 pM Primer *Prs* (Reverse) - 1.0 µL (final concentration of 0.4µM)

10 pM Primer *Isp* (Forward) - 1.0 µL (final concentration of 0.4µM)

10 pM Primer *Isp* (Reverse) - 1.0 µL (final concentration of 0.4 µM)

Taq DNA polymerase (1U/µL) - 1 Unit

Template - Single colony

Nuclease Free Water - Upto 25 µL

c. Tap the PCR tube (0.2 ml) containing the reaction mixture thoroughly with finger and then flash spin in a micro centrifuge to settle reactants at the bottom.
d. Place the tubes on to the thermal cycler and perform the reaction with a preheated lid.
e. The **cycling conditions** for PCR includes:
f. Store the resultant PCR products at -20ºC until the products are visualized using 1.2% agarose gel electrophoresis under UV trans-illuminator and record digitally by gel documentation system (UVP Gel Seq Software). Materials contaminated with ethidium bromide are disposed according to the local guideline.

Primers (forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
prs; isp	95°C 5 min	95°C 30 sec	53°C 1 min	72°C 2 min	72°C 10 min
		Repeated for 40 Cycles			

1.4.3.2. Serotyping PCR:

A multiplex PCR can be performed for molecular serotyping as per Doumith *et al*. (2004). 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.

Target gene	Primer sequence	Product size (bp)
*lmo*0737	"F" 5'-AGGGCTTCAAGGACTTACCC-3' "R" 5'- ACGATTTCTGCTTGCCATTC-3'	691
*lmo*1118	"F" 5'-AGGGGTCTTAAATCCTGGAA-3' "R" 5'-CGGCTTGTTCGGCATACTTA-3'	906
ORF2819	"F" 5'-AGCAAAATGCCAAAACTCGT-3' "R" 5'- CATCACTAAAGCCTCCCATTG-3'	471
ORF2110	"F" 5'-AGTGGACAATTGATTGGTGAA-3' "R" 5'-CATCCATCCCTTACTTTGGAC-3'	597

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

b. **PCR protocol**:

10x PCR Buffer	- 2.5µl (final concentration of 1X)
50mM MgCl2	- 4.0µl (final concentration of 8.0 mM)
10mM dNTP mix	- 4.0µl (final concentration of 1.6 mM)
10pM lmo 0737 'F'	- 1.0µl (final concentration of 0.4 µM)
10pM lmo 0737 'R'	- 1.0µl (final concentration of 0.4 µM
10pM lmo 1118 'F'	- 1.0µl (final concentration of 0.4 µM)

10pM lmo 1118‘R’	- 1.0µl (final concentration of 0.4 µM)
10pM ORF 2819 ‘F’	- 1.0µl (final concentration of 0.4 µM)
10pM ORF 2819 ‘R’	- 1.0µl (final concentration of 0.4 µM)
10pM ORF 2110‘F’	- 1.0µl (final concentration of 0.4 µM)
10pM ORF 2110‘R’	- 1.0µl (final concentration of 0.4 µM)
Taq Poly (1 units/ µl)	- 2.0 units

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

d. Place the tubes on to the thermal cycler and perform the reaction with a preheated lid.

e. The **cycling conditions** for PCR includes:

Primers (forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
*lmo*0737 *lmo*1118 ORF2819 ORF2110	95°C 5 min	94°C 30 sec	56°C 1 min	72°C 2 min	72°C 10 min
		Repeated for 40 Cycles			

f. Store the resultant PCR products at -20^{0}C until the products are visualized using 1.2% agarose gel electrophoresis under UV trans- illuminator and record digitally by gel documentation system (UVP Gel Seq Software). Materials contaminated with ethidium bromide are disposed according to the local guidelines.

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

iii) <u>Multiplex PCR targeting virulence associated genes</u>:

A multiplex PCR is performed for the detection of virulence associated genes viz., haemolysin called listeriolysin O (*hly*A), phosphatidylinositol-specific phospholipaseC

(*plc*A), Actin filament protein (*act*A), positive regulatory factor – PrfA (*prf*A) and invasion associated internalin gene (*Inl*C).

a. Primer details: The primers for the mPCR targeting virulence associated genes are listed below:

Target gene	Primer sequence	Product size (bp)
plc A	Forward 5'-CTGCTTGAGCGTTCATGTCTCATCCCCC-3' Reverse 5'-CATGGGTTTCACTCTCCTTCTAC-3'	1484
prf A	Forward 5'-CTGTTGGAGCTCTTCTTGGTGAAGCAATCG-3' Reverse 5'-AGCAACCTCGGTACCATATACTAACTC-3'	1060
hly A	Forward 5'-GCAGTTGCAAGCGCTTGGAGTGAA-3' Reverse 5'-GCAACGTATCCTCCAGAGTGATCG-3'	456
act A	Forward 5'-CGCCGCGGAAATTAAAAAAAGA-3' Reverse 5'- ACGAAGGAACCGGGCTGCTAG - 3'	839
InlC	Forward 5'-AATTCCCACAGGACACAACC-3' Reverse 5'-CGGGAATGCAATTTTTCACTA-3'	517

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

b. **PCR protocol** :

10X PCR buffer - 2.5µl (final concentration of 1X)

10 mM dNTP mix - 2.0µl (final concentration of 0.8 mM),

25 mM $MgCl_2$ - 3.0µl (final concentration 3.0 mM)

Five sets of primer

10pM of primer (Forward) - 1.0 µl (final concentration of 0.4 µM)

10pM of primer (Reverse) - 1.0 µl (final concentration of 0.4 µM)

Taq DNA polymerase (1U/µL) - 5.0 Units

DNA template - a single colony of *Listeria*

Sterilized milliQ water - to make up the reaction volume

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

c. Place the tubes on to the thermal cycler and perform the reaction with a preheated lid.

d. The **cycling conditions** for PCR includes:

Primers (forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
*hly*A; *plc*A; *act*A; *prf*A; *Inl*C	95°C 5 min	94°C 15 sec	60°C 30 sec	72°C 1 min 30 sec	72°C 10 min
		Repeated for 35 Cycles			

e. Store the resultant PCR products at -20ºC until the products are visualized using 1.2% agarose gel electrophoresis under UV trans- illuminator and record digitally by gel documentation system (UVP GelSeq Software). Materials contaminated with ethidium bromide are disposed according to the local guidelines.

1.4.3.3. Multiplex PCR for detection of Lineages of *Listeria monocytogenes*:

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).

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′	

a. PCR protocol

10x PCR Buffer	- 2.5µl (final concentration of 1X)
50mM MgCl2	- 5.0µl (final concentration of 8.0 mM)
10mM dNTP mix	- 5.0µl (final concentration of 1.6 mM)
10pM prs ‘F’	- 1.0µl (final concentration of 0.4 µM)
10pM prs ‘R’	- 1.0µl (final concentration of 0.4 µM
10pM isp ‘F’	- 1.0µl (final concentration of 0.4 µM)
10pM isp ‘R’	- 1.0µl (final concentration of 0.4 µM)
10pM L1 ‘F’	- 1.0µl (final concentration of 0.4 µM)
10pM L1 ‘R’	- 1.0µl (final concentration of 0.4 µM)
10pM L2 ‘F’	- 1.0µl (final concentration of 0.4 µM)
10pML2‘R’	- 1.0µl (final concentration of 0.4 µM)
10pM L3 ‘F’	- 1.0µl (final concentration of 0.4 µM)
10pML3‘R’	- 1.0µl (final concentration of 0.4 µM)
Taq Poly (1 units/ µl)	- 5.0 µl

a. Tap the PCR tube (0.2 ml) containing the reaction mixture thoroughly with finger and then flash spin in a micro centrifuge to settle reactants at the bottom.
b. Place the tubes on to the thermal cycler and perform the reaction with a preheated lid.

c. The **cycling conditions** for PCR includes:

Primers (forward and reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final Extension
Prs Isp L1 L2 L3	95°C 5 min	95°C 30 sec	56°C 1 min	72°C 2 min	72°C 10 min
		Repeated for 40 Cycles			

a. Store the resultant PCR products at -20^0C until the products are visualized using 1.2% agarose gel electrophoresis under UV trans- illuminator and record digitally by gel documentation system (UVP Gel Seq Software). Materials contaminated with ethidium bromide are disposed according to the local guidelines.

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

ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

7. Cryptosporidiosis

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology Laboratories, Medical centre's Consortium for one health project

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP-7	**Cryptosporidiosis-**	**1.0**
	Effective Date: 17-02-2022	

The content in this standard operating procedures are to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1.1.Introduction

Cryptosporidiosis is a gastrointestinal illness caused by small protozoan parasite Cryptosporidium. It infects the microvillous region of epithelial cells in the digestive and respiratory tract of vertebrates. It is an obligate intracellular parasite of man and other mammals, birds, reptiles and fish. It requires a host to multiply. The two-layered thick-walled oocyst is the primary source of transmission. A low dose of 10–100 oocysts can transmit the infection. These oocysts can survive the adverse conditions on the environment for months until it is ingested by a new suitable host. Most human cases of cryptosporidiosis are due to two species; *Cryptosporidium hominis*, which mainly infects humans and *Cryptosporidium parvum,* which infects humans as well as domestic animals.

1.2.**Purpose:** *Cryptosporidium parvum* ELISA (targeting monoclonal antibodies) test kit will be used for direct detection of *Cryptosporidium parvum* in faecal samples.

1.3.Case definition:

Confirmed case

Laboratory confirmation of infection with or without symptoms from an appropriate clinical specimen (e.g. **stool**, intestinal fluid or small bowel biopsy):

- demonstration of *Cryptosporidium* oocysts

or

- detection of *Cryptosporidium* DNA

or

- demonstration of *Cryptosporidium* antigen by an approved method (e.g. EIA, immunochromatographic - ICT)

Probable case

Clinical illness in a person who is epidemiologically linked to a confirmed case.

Clinical evidence

Clinical illness is characterized **by diarrhoea (often profuse and watery), diarrhea duration of 72 hours or more, abdominal cramps, anorexia, fever, nausea, general malaise and vomiting**.

1.4. Materials & Methods

Laboratory Criteria for Diagnosis:

Confirmed: Evidence of *Cryptosporidium* organisms or DNA in stool, intestinal fluid, tissue samples, biopsy specimens, or other biological sample by certain laboratory methods with a high positive predictive value (PPV), e.g.,

- Direct fluorescent antibody [DFA] test
- Polymerase chain reaction [PCR]
- Enzyme immunoassay [EIA]
- Light microscopy of stained specimen

Specimen: Stool

Numbers:

1.4.1. **Sample collection and Storage**:

- Label the collection container with the patient's name (or other identifier), date and time of collection.
- Collect 5 to 10 gm of Stool sample from diarrhoea patient using the labelled faecal container.
- Place the container into a sealable safety bag.
- Store at 2°C to 8°C immediately after collection and process within 72 hours. Longer storage is possible at -20°C. Repeated freezing and thawing of samples should be avoided.

1.4.2. Transport:

- Label the specimen container with the patient ID and seal with waterproof tape. Wrap the container in absorbent packing material.
- Place the specimen container in secondary packaging.
- Place the secondary package in the outer packaging (*i.e.,* the shipping container). Label the shipping container "Clinical Specimen" on the outside of the package.

- Include the following information: Patient's name, age, brief clinical history, and travel history; specimen collection date.
- Ship at 2°C to 8°C.

1.4.3. Precaution:

- Wear personal protective equipment such as safety glasses, gloves, laboratory coats.
- If you have cuts or abrasions on the skin of your hands, cover them with adhesive dressing.
- Never transfer sample from one sample container to another.
- Dispose all used material and personal protective equipment appropriately.

Which test to be used (preferably one suitable for surveillance), protocol (may be brief, but can be expansive), interpretation of the result (including any cut-off), reporting (including to whom)

Test: *Cryptosporidium parvum* ELISA test kit is used for direct detection of *Cryptosporidium parvum* in faecal samples

The Serazym *Cryptosporidium parvum* is based on monoclonal antibodies to *Cryptosporidium parvum* specific proteins.

KIT: store in +2 to +8°C till expiry date

Items supplied with kit:

- Microtitration plate coated with monoclonal anti-C, parvum antibodies (mouse): 12 single breakable 8 well strips colour coding black vacuum sealed with desiccant.
- Wash buffer 10-fold: 100 ml concentrate for 1000ml solution white cap.
- Sample diluent: 100 ml ready to use coloured yellow black cap
- Positive control; Cryptosporidium parvum Oocysts, inactivated: 2.0 ml ready to use coloured blue red cap.
- Negative control; Cryptosporidium parvum negative sample: 2.0 ml ready to use coloured blue green cap.
- HRP-conjugate; HRP-labelled monoclonal anti-C parvum antibodies (mouse): 15 ml ready to use coloured green violet cap.
- Substrate; 3,3', 5,5'-Tetramethylbenzidine and hydrogen peroxide: 15 ml ready to use blue cap.
- Stop solution; 0.25M sulphuric acid: 15 ml- ready to use yellow cap.

1.4.4. Materials and Equipment required:

- ELISA microwell plate reader, equipped for the measurement of absorbance at 450/620 nm
- Manual or automatic equipment for rinsing wells
- Pipettes to deliver volumes between 10 and 1000 µl
- Distilled water
- Disposable tubes

1.4.4.1. Reagent preparation:

- Allow all components to reach room temperature prior to use in the assay.
- The microtitration plate is vacuum-sealed in a foil with desiccant. The plate consists of a frame and strips with breakable wells.
- Allow the sealed plate to reach room temperature before opening.
- Unused wells should be stored refrigerated and protected from moisture in the original cover carefully resealed.
- Prepare a sufficient amount of wash solution by diluting the 10-fold concentrated wash buffer 1 + 9 with distilled or deionized water. Example: 10 ml wash buffer concentrate +90 ml distilled or deionized water.

1.4.4.2. Sample preparation:

- Preparation of untreated samples: Warm samples to room temperature and mix well. Pipette 1000 ul of sample diluent into a clean tube. Using a disposable stirring rod transfer about 200 mg (diameter about 4 - 6 mm) of faeces if solid or 200 ul if liquid into the tube and suspend thoroughly. If necessary, sediment floating particles by a centrifugation step with a micro centrifuge for one min at maximum speed.
- Conserved samples, prediluted in transportation media: Mix sample thoroughly and apply 100 ul without any further dilution in the Serazym *Cryptosporidium parvum*.

1.4.5. Assay procedure:

1. Dilute samples with sample diluent 1:6, e.g., 200 mg or 200 ul faeces + 1.0 ml sample diluent.
2. Avoid any time shift during dispensing of reagents and samples. Make sure the soak time of the wash buffer in the wells is at least 5 seconds per wash cycle and that residual fluid is completely drained in every single wash cycle. Avoid light exposure of the TMB substrate solution.
3. Warm all reagents to room temperature (RT) before use. Mix gently without causing foam.
4. Dispense 100 µl standards/controls and diluted samples into their respective wells.
5. Cover the plate and incubate for 60 min at room temperature.
6. Decant, then wash each well 5x with 300 ul wash solution and tap dry onto absorbent paper.
7. Dispense 3 drops (or100 ul) HRP conjugate in each well.
8. Cover the plate and incubate for 30 min at room temperature.
9. Decant, then wash each well 5 x with 300 µl wash solution and tap dry onto absorbent paper.
10. Dispense 3 drops (or 100 ul) substrate per well.
11. Incubate for 10 mins at room temperature protected from light.
12. Dispense 3 drops (or 100 ul) stop solution per well, mix gently.
13. Read OD at 450nm/ 620nm with a microplate reader within 30 mins after addition of the stop solution.

2.0. Result interpretation:

Qualitative evaluation:

Cut-off determination: OD negative control + 0.10

Samples with OD values equal with or higher than the cut-off is considered positive, Samples with OD values below the cut-off is considered negative for *Cryptosporidium parvum* antigen.

Reference Values:

Positive	≥ Cut-off
Negative	< Cut-off

It is recommended that each laboratory establishes its own normal and pathological reference ranges as usually done for other diagnostic parameters, too. Therefore, the mentioned reference values provide a guide only to values which might be expected.

Test validity: The test run is valid, if:

• The mean OD of the negative control is ≤ 0.20 (manual performance), ≤ 0.30 (automatic performance)

• The mean OD of the positive control is ≥ 0.80

If the above-mentioned quality criteria are not met, repeat the test and make sure that the test procedures are followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier.

Specificity and Sensitivity:

Specificity: 99.4% to 100%

Sensitivity: 96.9% to 100%

3.0. ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

8. Crimean-Congo hemorrhagic fever (CCHF)

MICROBIOLOGY LABORATORIES

MEDICAL CENTRES CONSORTIUM FOR ONE HEALTH PROJECT

DEPARTMENT OF MICROBIOLOGY

This document contains proprietary information. It is provided to Microbiology Laboratories, Medical centre's Consortium for one health project

SOP No.	TITLE	ISSUE No.
GH/MICRO/SER/SOP-8	**CCHF**	**1.0**
Effective Date: 17-02-2022		

The content in this standard operating procedures are to be followed only by Clinical Microbiology Laboratory staff, for the Medical Centre's consortium for one health project.

1.1. Introduction: Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne zoonotic viral disease that can be severe in humans but does not produce clinical signs in domestic and wild ruminants (cattle, sheep, and goats), insectivores, small lagomorphs, and rodents. Humans are very susceptible to CCHF, which, after a 3–7 day incubation period, causes fever, myalgia, headaches, and gastrointestinal symptoms.1 Though CCHF is endemic in many parts of Africa, Asia, Eastern Europe, and the Middle East, it does not currently exist in the United States.

1.2. Purpose: Serological diagnosis of Crimean Congo Hemorrhagic fever virus in clinical samples.

1.3. Case Definition:

✓ **Suspected case:** A patient with abrupt onset of high fever >38.5°C and one of the following symptoms: severe headache, myalgia, nausea, vomiting, and/or diarrhea **AND/OR** History of insect (tick) bite within 14 days before the onset of symptoms; or History of contact with tissues, blood, or other biological fluids from a possibly infected animal (e.g., abattoir workers, livestock owners, veterinarians) within 14 days before the onset of symptoms; or History of exposure to a suspect, probable, or laboratory-confirmed CCHF case, within 14-days before the onset of symptoms (contacts of the patient including health care workers).

✓ **Probable case:** A probable CCHF case is defined as a suspected CCHF case fulfilling, in addition, the following criteria: Thrombocytopenia < 50,000/cmm

AND

Two of the following hemorrhagic manifestations: hematoma at an injection site, petechiae, purpuric rash, rhinorrhagia, hematemesis, hemoptysis, gastrointestinal hemorrhage, gingival hemorrhage, or any other hemorrhagic manifestation in the absence of any known precipitating factor for hemorrhagic manifestation.

1.4. Materials & Methods

Sample type:

- Blood
- Serum

- Plasma

4. **Transportation, Aliquoting and Storage**

- It is recommended that specimens be kept refrigerated at 2-8ºC and tested within 48 hours. If there is a delay of more than 48 hours before testing, samples may be kept frozen at -20°C for up to 7 days. For storage longer than 7 days, specimens should be frozen at -80°C. Repeated freezing and thawing of specimens should be avoided.
- Before proceeding with the test, 3 labeled tubes are prepared for aliquoting of the sample. The following aliquots are prepared:
  1stAliquot: To be stored at –80ºC for long term preservation.
  2ndAliquot: To be stored at –80ºC for QA/QC or if any repeat testing is required.
  3rdAliquot: To be used for performing the diagnostic test requested.
- Frozen specimen must be thawed and mixed homogenously before the test.
- Specimen must not be repeatedly thawed and frozen.

5. **Turnaround Time:** Around 4 hrs

7 **Requirements for processing:**

➢ **Equipment:**

- Micro centrifuge
- Micro pipettes (2-20 µl Single channel, 20-200 µl single channel, 100-1000µl)
- Refrigerator
- ELISA Reader
- Timer
- Biosafety cabinet

➢ **Reagents**

- ELISA kit
- Blood collection kit

➢ **Consumables**

- PPE (Personal protective equipment) kit (Disposable- gloves, cap, lab coat, shoe cover, N95 mask and eye goggle)
- Aerosol Barrier tips: 0.5- 10 µl, 10-100 µl, 20-200 µl

- Marker pen

9. **Calibration:** Calibration/Efficiency testing of the pipettes, refrigerators, Bio-Safety cabinet and centrifuge should be done annually.

1.5.**Procedural steps**

PPE DONNING

a) Before entering patient room/ward, assemble all the PPE, sample collection and packaging materials.

b) Wear first layer of protective clothing (scrubs) after removing your outermost clothing and perform hand hygiene. Note: Donning and donning off of PPE must be done in a specified area different from the patients' ward/area. Put on rubber boots one at a time (Note: always put on a boot size bigger than your normal shoe size for easy removal).

c) Put on the coverall by opening the zip and inserting both legs into the pairs of trousers of the coverall (one leg at a time), make a thumb hole and zip it up to the neck and hood up. Note: Put on the plastic apron/heavy duty apron and tie the two projections of the apron behind your back in a rift-knot-like form. Use heavy duty apron, gloves and coverall if the patient is bleeding profusely

d) Put on the N95 mask in a manner that fits well on your face.

e) Put on the goggles

f) Put on first pair of gloves (inner gloves) and ensure cuffs are tucked under the sleeves of the coverall.

g) Put on the second pair of gloves and ensure cuffs are tucked over sleeves of the coverall.

h) Seal the outer gloves with adhesive tape if is raining. Note: Ask your buddy to check and confirm whether you are properly donned or not. Besides, you must check yourself on a wall mirror to further confirm that you are properly donned.

1.6.**Sample Collection**: Two laboratory personnel must be involved in the sample collection and packaging process. One should be responsible for sample collection whilst the other personnel proceed to the designated area for packaging.

a) After putting on the appropriate PPE, label the sample collection tube with the patient's details including the date of collection (labeling should be done before entering the ward/patient area).
b) Walk into the patient room with your blood sample collection kits.
c) Inform patient of the purpose and method for collecting the blood sample.

d) Tie a disposable tourniquet around the patient's arm to visualize the veins.

e) Disinfect the selected puncture site with an alcohol pad and let it air-dry (approximately 15-20 seconds).

f) If using a vacuum extraction system, fasten the needle in the Vacutainer sleeve and put it in place (in kits' tray). Alternatively, when using a needle and syringe, fasten the needle into the hub of the syringe and put it on kits' tray.

g) Position the needle at a 45 degrees angle to the arm and insert into the selected vein with the beveled-edge facing upwards.

h) Collect 4-5ml of blood.

i) When using a Vacuum extraction method, push the Vacutainer tube into the needle to collect the blood.

j) When using a needle and syringe, loosen the tourniquet after collecting the blood and place a small pad of dry cotton wool on the punctured site as you withdraw the needle out of the vein. Ask the patient to bend the arm or help bend the arm with the cotton wool in place to stop the bleeding.

a) Dispense the blood by piercing the needle right through the top part of the rubber stopper of the Vacutainer.

b) When all the blood is collected into the Vacutainer, pull off the needle and syringe and appropriately dispose off into the sharps container.

c) If a vacuum extraction system is used, unplug the needle and dispose into the sharps container. DO NOT RECAP THE NEEDLE!

d) Make sure that the punctured site is dry, if not place a small bandage on the punctured site to stop the bleeding. Remember to discard the cotton wool applied on the punctured site into a biohazard bag.

e) Bath the tube containing the blood sample in 0.5% active chlorine solution and place on a rack.

f) Hand over the specimen to the other laboratory personnel (buddy) for packaging.

g) Proceed to the designated donning off area and remove PPE in the following sequence as described below: Note: Your companion has to help you with the disinfection process using a spray bottle containing 0.5% active chlorine solution.

1.7. DONNING OFF PPE

a) Spray the front and back of the suit with 0.5% active chlorine solution disinfectant

b) Disinfect the outer pair of gloves by spraying with 0.5% active chlorine solution.

c) Disinfect the boots by asking your buddy/partner to pour 0.5% active chlorine solution on both boots into an empty container.

d) Disinfect the apron by spraying it with 0.5% active chlorine solution. 5. Remove the apron by untying from the back and bend a little bit forward. Then remove the apron over the head from the back.

e) Turn contaminated outside toward the inside. 2. Fold or roll into a bundle and discard into the disposable biohazard bag or reusable waste container if the apron is a reusable type (heavy duty).

f) Remove the coverall by unzipping it and remove it in an inside out manner.

g) Remove the goggles by pulling it away from your face and move it upwards to completely remove it.

h) Remove the outer pair of gloves by grasping the outside edge near the wrist and then peel away from hand, turning the glove inside-out. Then hold in opposite gloved hand (i.e the other hand).

i) Disinfect the inner pair of gloves by spraying with the 0.5% active chlorine. 7. Remove the mask by pulling it away from your face and move it upward to completely remove it.

j) Remove the rubber boots by using one foot to step on the backside of the other.

k) Disinfect the gloved hands by spraying with the 0.5% active chlorine

l) Remove the inner pair of gloves

m) Soak hands in 0.05% active chlorine and soap and clean water. Note: All reusable PPE are to be soaked into a container containing 0.5% active chlorine solution during the donning off process.

1.8. **Specimen packaging**

a) Ensure that all tubes are tightly closed to avoid any leakage of the sample.

b) Wrap every tube in an absorbent material like dry cotton wool and place it in a watertight, leak-proof screw-cap container.

c) Wrap the sample tube (EDTA) with dry cotton wool or other suitable absorbent material and place it in the water-tight, leak-proof container (secondary container) provided.

d) Collect four frozen ice packs from the freezer (-20 degrees Celsius or colder) and place them on the sides of the UN specified cold box (UN 2814) ensuring that the secondary container is in the middle of the ice packs.

e) Place the case investigation forms in a leak-proof, zip-lock plastic bag to prevent them from becoming contaminated or destroyed by the wet ice packs and put them inside the cold box on top of the secondary packaging.

f) Seal the cold container and disinfect the outer packaging with 0.5% active chlorine solution before transportation.

g) Samples should be transported at 4-10 degrees Celsius within 24 hours after collection.

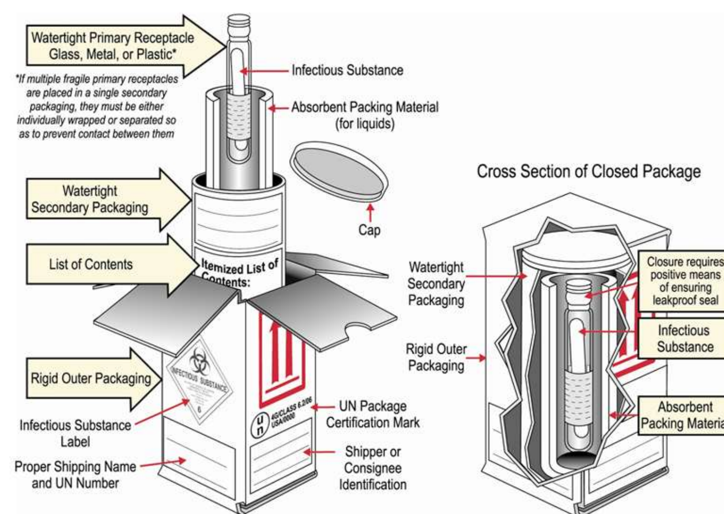


Figure 1: Triple Packaging of clinical samples

1.9. ELISA Protocol

1.9.1. Sample preparation

a) **Serum preparation** After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2,000-3,000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifuged again.

b) **Plasma preparation** Collect the whole blood into tubes with anticoagulant (EDTA or citrate). After incubated at room temperature for 10-20 minutes, tubes are centrifuged for 20 min at 2,000-3,000rpm. Collect the supernatant carefully as plasma samples. If precipitates appear during reservation, the sample should be centrifuged again.

Procedure

Following ELISA kit will be used for the testing

Commercial ELISA and IFT/IFA									
Developer	**System**	**Regulatory status**	**Sample Type**	**Target**	**LOD**	**Sensitivity /PPA**	**Specificity /NPA**	**Specimens tested**	**Reference assay**
Alpha Diagnostic International / ADI (US)	**Crimean-Congo hemorrhagic fever virus (CCHFV) IgG, IgM ELISA Kits**	RUO	human serum or plasma of vaccinated, immunized and/or infected hosts	purified recombinant CCHFV nucleoprotein (NP, 482-aa)	no info	no clinical samples	no info	no info	no info
EUROIMMUN (GER) (acquired by Perkin Elmer 2017)	**IIFT and IFA BIOCHIPs** (ELISA in development)	CE-IVD	Serum, plasma, culture media or any other biological fluid	CCHF-IgG, CCHF-IgM two recombinant proteins CCHFV-GPC and CCHFV-N transfected cells	IgG: 1:100 IgM: 1:10	IgG: 89.5% IgM: 97.2%	IgG: 100% IgM: 97.5%	IgG: 206 positives; 88 neg IgM: 184 positives, 204 neg	no info
Vector-Best (RUS)	**VectoCrimea-CHF-IgG VectoCrimea-CHF-IgM VectoCrimea-CHF-antigen**	no info	no info	no info	no info	no info	no info	no info	no info
Abbexa (UK)	**Human CCHF-IgG ELISA Kit**	RUO	serum, plasma, tissue, other biological fluids	96 well plate has been pre-coated with an antigen specific to CCHF-IgM.	no info	no info	no info	no info	no info
Creative Diagnostics (USA)	**CCHF-IgM ELISA Kit**	RUO	serum, plasma, culture media or any biological fluid	CCHFV nucleoprotein	no info	no info	no info	no info	no info
Bernhard Nocht Institute for Tropical Medicine (GER)	**BLACKBOX CCHFV IgG, IgM ELISA Kits**	RUO	human serum	HRP-labelled CCHFV recombinant antigen	Calibrated index value	no info	no info	no info	no info

The ELISA protocol will be performed according to manufacturer's.

2.0. References

1. WHO blood sample collection protocol, 2014- "How to safely collect blood samples from persons suspected to be infected with highly infectious blood-borne pathogens (e.g. Ebola)
2. WHO documents, How to safely collect blood samples by phlebotomy from patients suspected to be infected with CrimeanCongo Haemorrhagic fever (CCHF) virus? https://www.who.int/emergencies/diseases/crimean-congo-haemorrhagic-fever/collection-of-blood-samples.pdf
3. WHO documents, How to safely ship human blood samples from Crimean-Congo Haemorrhagic Fever (CCHF) cases within a country by road, rail and sea? https://www.who.int/emergencies/diseases/crimean-congo-haemorrhagic-fever/shipment-of-blood-samples.pdf?ua=1.

3.0. ANNEXURES & FORMS:

Annexure I – Clinical Case Proforma

Annexure 1: Clinical Case Performa

One Health Consortium Project

Case Performa (☐Hospital/☐Community), Case ID_____

Name of the Centre: ________________________________, Date: ___/______/20___

A. Case Identification:

Name of the Hospital (For IPD/OPD): ____________________________

Patient's Name: ______________________________ Sex: ☐ F/ ☐ M Age: Years months

Religion:__________________________Caste: ☐ Gen / ☐ SC/ ☐ ST/ ☐ OBC

Parent's name: ____

Res. Address:____

PIN:

Landmark:____________________ Village/ Mohalla: _______________________

Block/Urban area: _________________ District:______________________Setting: ☐ Rural/☐ Urban

State:____________________________ Mob.

No.:

GIS co-ordinates: (Latitude) ___________ (Longitude)___________(Community survey only)

Hospital Registration no (where applicable):-___________________________________

Has there been reported outbreak of similar cases in last 3Months from the same area? ☐ Y/☐ N/☐ UK, if yes, specify: ________________________

Contact with animals with zoonotic disease like Bovine-Tb/others: ☐ Y/☐ N/☐ DK

B. Occupational/ other Exposures (reference period preceding 3 months)

a. Occupation at date of onset of illness	b. Any contact with animals within 3 months prior to onset (Check all that apply)
☐ 1.Agriculture Farming	☐ 1.Cattle
☐ 2.Dairy	☐ 2.Goats
☐ 3.Livestock rearing	☐ 3.Cats
☐ 4.Veterinarian	☐ 4.Sheep
☐ 5.Slaughterhouse worker	☐ 5.Pigeon
☐ 6.Live in household with person occupationally related to above	☐ 6.Poultry
☐ 7. Contact with wild animals	☐ 7.Rodents/guinea pig
☐ 8.Other (please specify)- _______________	☐ 8.Rabbit
	☐ 9.Pig
	☐ 10. Dog
	☐ 11.Others (please specify) ______

One Health Consortium Project

Case Performa (□Hospital/□Community), Case ID_____

c. Any exposure to delivery/ abortion of animals-	**d.** Exposure to raw/ unpasteurized milk?	e.1.Travel during last 90 days before the onset of illness:	**e.**Other family member with similar illness in last 3 months
□ 1. Yes □ 2. No □ 3. UK If Yes, which animal:_________	□ 1. Yes □ 2. No □ 3. UK If Yes, which animal: _____________	□Yes/□No 2.If yes, a. Place/s of visit____________ b. Tentative date of visit______ c. Duration of visit__________	□ 1. Yes □ 2. No □ 3. UK

C. **Clinical features**	
f. **□Symptomatic/ □Asymptomatic (community survey)** **g.** **Date of Onset of presenting Complaint** ___ ___ ______ **(dd/mm/yyyy)**	**h. Clinical Symptoms in symptomatic (check all that is applicable)** *(Evidence of clinically compatible illness is necessary. See attached case definition, and case categorization summaries.)* 1.Fever: □ Y/□ N, if Yes, Date of Onset of Fever ___________(dd/mm/yyyy) 2. Myalgia: □ Y/□ N 3. Retro bulbar pain: □ Y/□ N 4. Night sweats: □ Y/□ N 5. Weight loss: □ Y/□ N 6. Loss of appetite: □ Y/□ N 7. Chills: □ Y/□ N 8. Cough>2W: □ Y/□ N 9. Pain in Chest: □ Y/□ N 10. Hemoptysis: □ Y/□ N 11. Dyspnea: □ Y/□ N 12. Vomiting: □ Y/□ N 13. Pneumonia: □ Y/□ N 14. Headache: □ Y/□ N 15. Rash: □ Y/□ N 16. Eschar: □ Y/□ N if Yes, site of eschar____ 17. Splenomegaly □ Y/□ N 18. Hepatomegaly: □ Y/□ N 19. Seizure/convulsion developed*:□ Y/□ N; if Yes, Date of Onset _____(dd/mm/yyyy) 20. Change in Mental Status*:□ Y/□ N ; 21. Neck rigidity: □ Y/□ N 22. Paralysis*: □ Y/□ N if Yes, Site of paralysis*: □RA/□LA/□RL/□LL/□neck/□bulbar/□respiratory muscles/□trunk/□facial/□others:____ 23. Diarrhea: □ Y/□ N if Yes, duration of Diarrhea______(days) 24. Abdominal cramps: □ Y/□ N

	25. Vomiting: Y/ N 26. Back pain: Y/ N 27.Joint pain: Y/ N 28. Red eyes: Y/ N 29.Flushed face: Y/ N 30. Red throat: Y/ N 31. Petechiae (red spots) on the palate: Y/ N 32. Sore throat: Y/ N 33. Dizziness: Y/ N 34. Drowsiness: Y/ N 35. Other (please specify)_________________________________

Any pre-existing medical conditions? (Multiple response)	Was patient hospitalized because of this illness?	Did patient die from complications of this illness? *
1.Immunocompromised___ 2.Valvular HD/Vasc Graft___ 3.Diabetes____ 4.Pregnancy______ 5.Oral steroid________ 6.CKD_____________ 7.Other________________________________	Y/ N/ DK,	Y/ N/ DK, If yes, date _________ (dd/mm/yyyy) [Keep copy of death certificate if possible]

D. Laboratory investigations (Specifically for One Health Consortium)						
i. Collection of specimen type- 4. Blood_____ 2. CSF____ 3. Sputum_____ 4. Stool______ 5. Others (please specify)______						
j. Investigation for	**Microscopy** (Positive/Negative/ Not Done)	**Serology** (Positive/Negative/ Not Done)			**PCR** (Positive/Negative/ Not Done)	**Culture** (Positive/Negative/ Not Done)
		IgM	**IgG**	**IFA**		
Tuberculosis (Bovine TB)						
Brucellosis						
Japanese encephalitis						
Scrub typhus						
Q fever						

One Health Consortium Project

Case Performa (Hospital/Community), Case ID_____

Cysticercosis				
Salmonellosis (NTS)				
Cryptosporidiosis				
CCHF				
Nipah				
Listeriosis				

E. Vaccination Record

1. BCG Vaccination ____1. Yes 2. No 3. Unk if Yes, Date/ Year of Vaccination __________(dd/mm/yyyy)
2. JE Vaccination______1. Yes 2. No 3. Unk if Yes, Date/Year of Vaccination __________(dd/mm/yyyy)

Diagnosis: (as per record / lab investigation)

1.1 Probable diagnosis: ______________________

2.1 Confirmed diagnosis: ______________________

Outcome (as per available records):

Discharged/ LAMA/Referred out/ Death / not admitted (Rx at OPD)

Signature of data collector

Annexure 2: Details of the Kits & SOP

Disease	Sample	Test	Kits to used	SOP prepared by
Tuberculosis	Sputum	Microscopy (ZN/Aura mine staining if samples are few) PCR & Isolation if needed (only for in contact persons when animals are found positive)	?	Dr M Mawlong & Dr Ravisekhar, AIIMS Jodhpur
Brucellosis	Serum	IgG ELISA	IgG ELISA Novatech Kit	Dr Vikram Saini, AIIMS Delhi
JE	Serum, CSF	Active cases – CSF – IgM Serum IgG (for household)	IgM ELISA NIV Pune Kit IgG ELISA Inbios Int. Kit	Dr K Nagamani, GMC Hyderabad
CCHF	Serum	IgG ELISA	ID Screen ELISA Kit	Dr Ravisekhar, AIIMS Jodhpur
Q fever	Serum	IgG ELISA	IgG ELISA Novatech Kit	Dr Pushkala Subramanian, TN Dr MGR MU, Chennai
Scrub typhus	Serum	IgM ELISA	IgM ELISA Inbios Int. Kit	Dr K Nagamani GMC Hyderabad
Cysticercosis	Serum	IgG ELISA	IgG ELISA Novatech Kit	Dr Siraj Khan, ICMR-RMRC Dibrugarh

Crypto-sporidiosis	Stool	Ag ELISA PCR	Ag ELISA Kit Abbexa	Dr Siraj Khan, ICMR-RMRC Dibrugarh
Salmonellosis (NTS)	Stool	Culture on XLD & PCR	PCR details by Dr Barbuddhe	Dr Barbuddhe, ICAR-NRC MEAT, Hyderabad
Listeriosis	Placental bits, meconium, Deep cervical swab, stool, CSF	Culture	Culture details by Dr Barbuddhe	Dr Barbuddhe, ICAR-NRC MEAT, Hyderabad
Nipah	Blood, serum, CSF	RT PCR	Dr Barman to request Dr Simon, Pirbright	Dr Barman/ Dr Arnab

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

STANDARD OPERATING PROCEDURE MANUAL