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**CSF Samples**

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**1 Aim**

 This SOP describes processes involved in examining cerebrospinal fluid (CSF) for the detection of meningitis and encephalitis caused by bacteria and *Cryptococcus neoformans*.

**2 Responsibility, Personnel and Competence Assessment**

 The Laboratory Management Team is responsible for ensuring the implementation and maintenance of this procedure.

 This procedure may be performed by fully trained laboratory staff (technicians and assistants), who are deemed to be competent. Competence is assessed using the ‘CSF samples’ competency assessment form (see section 13)

**3 Principles**

**3.1 Introduction**

Cerebrospinal fluid (CSF) is fluid that surrounds the brain and spinal cord. CSF is taken when meningitis, encephalitis or other neurological infections are suspected. Meningitis is inflammation of the meninges. This may be acute, chronic, infective or non-infective. Many infective agents have been shown to cause meningitis including viruses, bacteria, fungi and parasites. Cell counts, CSF staining and culture can all aid its diagnosis.

**Appearance:** The appearance of the CSF can give clues to the aetiology of disease. It is normally a clear colourless fluid with a consistency similar to water. If cloudy this can indicate increased white blood cells (WBC), organisms and/or protein. If pink this indicates blood. Red blood cells (RBC) can be present due to an intra-cerebral or sub-arachnoid haemorrhage (bleeding into the brain) or during a traumatic lumbar puncture (contamination of CSF from peripheral blood when it is being taken). If two samples are taken and both contain similar RBC counts then this indicates previous haemorrhage, however, if the numbers are higher in the first sample this suggests a traumatic lumbar puncture. The presence and timing of xanthochromia (yellow, orange or pink colour) can be helpful. If haemorrhage has occurred, 2-4 hours post haemorrhage the RBCs lyse and release oxyhaemoglobin giving a pink/orange colour and after

24 hours haemoglobin is metabolized to bilirubin giving a yellow colour. Therefore, if the CSF

has been processed rapidly in the laboratory and the centrifuged CSF supernant is yellow in colour, this indicates pathological bleeding.

**Cell counts:** Normal CSF contains zero to very few cells and the presence, number and type of cells can indicate whether bacterial, viral, parasitic or non-infectious aetiologies are likely. The composition of CSF found in different conditions is given in appendix 1 and different WBC types in figure 1 below.

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|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Neutrophil polymorph | Eosinophil | Basophil | Monocyte | Lymphocyte |
| Medium size\* (10-14µm);highly variable dense (dark-staining) nucleus with >2 lobes connected by narrow nuclear strands | Medium size (10-14µm);less dense, bi- lobed nucleus connected by a narrow band of nuclear material | Small (8-14 µm);bi-lobed nucleus | Largest cell (12-18µm;less dense, large, horse-shoe shaped nucleus | Smallest cell (8-12µm); dense circular nucleus taking up most of the WBC |

Figure 1. Differentiation of blood cells (illustration by Caoimhe Nic Fhogartaigh, adapted from ‘Tissues of

the Human Body’ (McGraw Hill): <http://www.mhhe.com/biosci/ap/histology_mh/wbc1.html>)

\*Red blood cell (size 7-8 µm) can be used as comparison to estimate size.

Abnormalities associated with bacterial meningitis include:

1. Higher white cell count (especially if mostly polymorphs)

2. High intracranial pressure when taking the CSF

3. Reduced glucose concentration (<60% blood glucose, CSF:serum ratio <0.6)

4. Elevated protein concentration

Increases in different WBCs can be seen with the following:

|  |  |
| --- | --- |
| **Increase in** | **Found in** |
| Lymphocytes | Viral meningitisTB meningitisFungal meningitisParasitic meningitis | Treated bacterial meningitisNon-infectious causes |
| Monocytes | Viral meningitisFungal meningitisParasitic meningitis | Chronic bacterial meningitisTreated bacterial meningitisNon-infectious causes |
| Neutrophils | Bacterial meningitisVery early viral meningitisTB meningitis | Brain abscessNon-infectious causes |
| Eosinophils | Parasitic infection |  |

*\*Note:* If there is blood in the CSF due to traumatic tap the WBC will need to be adjusted.

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**3.2 Organisms not to miss**

Common causes of meningitis are given in the table below.

|  |  |
| --- | --- |
| **Patient Age/Status** | **Organism** |
| Neonates and young infants(< 2 months) | Group B Streptococci*E. coli* and other coliforms*Listeria monocytogenes* |
| Infants & Children(> 2 months) | *Haemophilus influenzae**Neisseria meningitidis**Streptococcus pneumoniae* |
| > 60 years | *Listeria monocytogenes* |
| Immunocompromised | *Cryptococcus neoformans* |
| Others | *Mycobacterium tuberculosis**Streptococcus suis* |

If Cryptococcal meningitis is suspected an Indian ink preparation should be performed. Indian ink cannot penetrate the *Cryptococcus* capsule, so if present you will see yeast cells surrounded by a clear halo.

**4 Specimen collection and Transport**

CSF must be collected aseptically into sterile labeled universal containers. It should be delivered to the laboratory **immediately** after collection and transported to the laboratory at room temperature.

**5 Specimen processing in the laboratory**

When a CSF arrives in a laboratory it **MUST** be treated as an **URGENT** sample and examined

**IMMEDIATELY.** This is because:

1. Rapid analysis of CSF can guide appropriate treatment.

2. Cells degrade quickly and therefore delayed cell counts may be inaccurate.

3. Organisms in CSF can be difficult to grow and die easily and therefore delayed plating of the sample can lead to false negative cultures.

*Although not always possible ideally cell counts should be performed within 10 minutes of a sample being taken, and specimen cultures set-up within 2 hours of being taken. If a delay in setting up cultures is unavoidable the CSF should be kept at room temperature or can be incubated at 35±2°C. (Note: If there is an unavoidable delay in performing the cell count, and the cell count result is felt to be more critical than the culture result, it is preferable to store the CSF at 4±2°C).*

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**6 Equipment and Consumables**

 Counting chamber

 Centrifuge

 Incubators including CO2, Aerobic and 30°C.

 Gilson P20 and tips (or equivalent)

 Glass slides

 Pastettes

 1.5 ml tubes

 Toluidine blue

 Gram staining reagents

 Additional staining reagents as required e.g. Indian ink, Giemsa stain, Auramine and Ziehl- Neelsen.

 Appropriate media including Blood agar (BA), Chocolate agar (CA), MacConkey (MAC) and

Sabouraud agar (SAB).

**7 Methods**

**7.1 Appearance**

 In good lighting conditions describe the appearance of the CSF and record this on the specimen request form. Descriptions include:

o Clear

o Cloudy

o Blood stained (pink in colour)

o Purulent (looks like pus)

o Clot present

o ‘Spider web’ clot present (this is rare but suggestive of *M. tuberculosis*)

 Estimate the volume and record this on the request form.

 *Note: A normal CSF is clear, bright and colourless*

**7.2 Cell count**

 Perform a cell count on CSF samples using a counting chamber, unless the sample is clotted.

 Always use CSF that has **NOT** been centrifuged.

 If multiple CSF samples have been taken from one patient, perform the cell count on the last sample taken.

1. In a BSC mix the CSF.

2. Pipette 10 μl of the CSF into a 1.5 ml tube using a pipette e.g. Gilson P20.

3. With a clean tip add 10 µl of 0.1% toluidine blue reagent and mix well (appendix 2; this stains the white nuclei blue and also dilutes the sample 1:2).

4. Make sure the counting chamber and cover glass are completely clean. They can be cleaned

with a soft tissue using microscope lens cleaning solution e.g. 30% ethanol: 70% ether.

5. Assemble the counting chamber.

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6. Pipette the stained CSF into the counting chamber using a fine pastette or pipette e.g.

Gilson P20. *(note: make sure the CSF* ***does not*** *overflow into the channels on each side of the chamber. If the CSF overflows clean the chamber and the cover glass and repeat filling the*

*chamber).*

7. Wait for 2 minutes for the cells in the CSF to settle.

8. Count the number of WBCs and RBCs microscopically as described below. *(note: WBCs will have stained nuclei and RBCs will be unstained smaller cells without nuclei).*

**7.2.1 White blood cell count**

o Count the WBCs (cells with a nucleus) microscopically using the **x10 objective**.

o Focus on the WBCs and rulings with the condenser down and the diaphragm closed

sufficiently to give good contrast.

o Count the white cells in **5** of the large squares (W1, W2, W3, W4 & W5) as shown below.



o Multiply the number of WBCs counted by 2 (for samples diluted 1:2 with toluidine blue) and convert to number of WBCs per litre of CSF by multiplying by 106. For example if you counted 64 white cells in 5 squares, then 64 X 2 = 128 WBC /μl CSF, which is 128 X

106 WBC/L.

o If no WBCs are seen, report the count as: < 5 cells X 106 WBC/L.

o If there are too many cells to be counted, repeat the whole procedure with a 1:10 dilution of the CSF (2 μl CSF + 18 μl toluidine blue) using a clean chamber. Then multiply the WBC counted by 10 and convert to WBC per litre of CSF. For example if you counted 64 white cells in 5 squares, then 64 X 10 = 640 WBC /μl CSF, which is 640 X

106 WBC/L.

o Estimate the percentage of each WBC type. Polymorphonuclear neutrophils (PMNs)

have lobed nuclei and lymphocytes have a single round nucleus.

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**7.2.2 Red blood cell count**

o Count the RBCs microscopically using the **x40 objective**.

o Focus on the RBCs with the condenser half down and the diaphragm half closed to give

good contrast.

o Count the RBCs in 5 of the large squares of the counting chamber (W1, W2, W3, W4 & W5) as shown above. *Note: RBC will appear smaller than WBC and unstained as they do not have a nucleus.*

o Report the number of RBCs per litre of CSF following the same calculations used for the WBCs i.e. for a sample diluted 1:2 with toluidine blue, multiply the count by 2 and then convert from RBCs /μl to RBCs /L. For example If 105 RBCs counted then report as 210 x 106 RBC/L).

**7.3 CSF concentration**

 If there is sufficient CSF (≥ 1ml) concentrate it by centrifugation.

 Place the CSF sample in a sealed bucket in a centrifuge, ensuring that the centrifuge is balanced.

 For routine samples centrifuge for a minimum 1,200 g for 5-10 min or increase to 3,000 g for 15-

20 min if *M. tuberculosis* is suspected.

 Remove the sealed buckets and open in a BSC and take out the CSF sample (take care not to disturb the pellet).

 Note the colour of the supernatant (yellow colouration is xanthochromia resulting from the degradation of RBC, increased protein or bilirubin)

 With a sterile pastette carefully remove some of the supernatant and discard, leaving behind the pellet and approximately ½ ml (=500 μl) of supernatant.

 Resuspend the pellet with the remaining supernatant using the pastette.

**7.4 Gram stain**

 In a BSC, place 1 drop of the concentrated CSF onto a clean, labeled glass slide and allow to dry.

Then add another drop on top *(note: alternatively if a cytospin is available this should be used).*

 If the sample is clotted break up the clot as much as possible.

 Perform a Gram stain following the method for clinical samples following the appropriate SOP.

 Examine the Gram film using x40 and x100 for at least 10 minutes or until bacteria are seen.

 Record the presence of RBCs, WBCs, bacteria and yeast using the criteria below.

|  |  |
| --- | --- |
| **Number counted****(x100 objective)** | **Record as:** |
| 0 | None |
| <5 | Few |
| 5-25 | Moderate |
| >25 | Numerous |

 If organisms are seen record if they are Gram negative or Gram positive and their shape, bearing in mind possible pathogens:

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|  |  |
| --- | --- |
| **Gram result** | **Possibility of:** |
| Gram negative diplococci | *N. meningitidis* (may look like coffee beans) |
| Gram positive diplococci orshort chains | *S. pneumoniae* |
| Gram negative rods | *H. influenzae* (may be pleomorphic including rods and coccobacilli) |
| Yeast cells | *C. neoformans* (some may be budding) |

**7.5 Indian ink stain**

 If cryptococcal meningitis is suspected perform and Indian ink preparation.

 In a BSC place one drop of the concentrated CSF on a labeled clean glass slide.

 Add one drop of Indian ink.

 Mix well.

 Place a coverslip over the top.

 Examine microscopically using x10 and x40 objectives.

 Record if yeast cells with a capsule are present (yeast-like organisms surrounded by a clear zone indicate *Cryptococcus neoformans*)*.*

**7.6 Additional stains**

 If *M. tuberculosis* is suspected perform an Auramine and/or Ziehl-Neelsen stain following the appropriate SOP.

 If high WBC counts are obtained Giemsa stain can be used to aid cell differentiation.

 *Note: If only a small volume of CSF is obtained then the Gram stain can be over-stained with*

*Giemsa and Ziehl-Neelsen.*

**7.7 Culture**

**Day 1:**

 In a BSC place one drop of the concentrated CSF onto the appropriate media (see below).

 Allow the drop to dry.

 Streak the plates and incubate as indicated.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Media** | **Which****samples** | **Incubation****temperature** | **Incubator** | **Length of****incubation** |
| Blood agar (BA) | All | 35±2°C | CO2 | Min 48 hrs |
| Chocolate agar (CA) | All | 35±2°C | CO2 | Min 48 hrs |
| MacConkey (Mac, optional)\* | Infant<12 monthsOr Gram neg seen on film | 35±2°C | Aerobic | Min 48 hrs |
| Sabouraud agar(SAB)\* | India Ink+Or yeasts seenOr known immunocompromise | 30±2°C | Aerobic | 2-5 days |

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**Day 2, Day 3 and follow-up:**

 Examine the plates for growth.

 Record if the growth is 1+ (growth in pool only), 2+ (growth in pool and 1st streak), 3+ (growth in pool and 2nd streak) or 4+ (growth in pool and 3rd streak).

 Perform a Gram stain on all colony types.

 Using the appropriate SOPs fully identify and perform sensitivities on all different isolates.

***Note: Ensure any work that may generate aerosols are carried out in the BSC e.g. making a suspension of a potential Neisseria* sp*.***

**7.8 Interpretation & Reporting**

 Inform the medical staff or a senior member of the laboratory **IMMEDIATELY** of any positive microscopy and culture results. These **MUST** be dealt with as a matter of **URGENCY**.

 Report the number of WBCs and RBCs per litre, including the percentages for PMN and lymphocytes.

 Report the Gram stain results and results of any other stains if performed.

 Report the culture results as indicated below.

|  |  |  |
| --- | --- | --- |
| **Culture result** | **Report** | **Comments** |
| No growth on plates | No growth at 48 hrs |  Do not add a comment |
| Organism/s on plate/s | Growth of ….Include sensitivities |  Report the identification and the sensitivities of**ALL** isolates. **NEVER** use the comment “no significant growth” **NEVER** use the comment “mixed growth ofdoubtful significance” |

**8 Quality assurance**

 All media and reagents used must have passed their QC checks, must be stored correctly and must be in-date.

 The positive and negative controls of any tests used must be valid for results to be accepted.

**9 Limitations**

 A negative CSF microscopy and culture result does not rule out the possibility of bacterial meningitis.

**10 References**

 Standard Operating Procedures in-use at SMRU, LOMWRU and COMRU.

 Investigation of Cerebrospinal Fluid. UK Standards for Microbiology Investigations. B27. Issue no: 5.1. Issue date: 10.07.12.

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**11 Synopsis/Bench aid**

**CSF sample**

**Describe appearance**

**Perform RBC & WBC**

**counts**

**Centrifuge to concentrate**

**Perform Gram stain and other stains as required**

**Set-up cultures**

**Identify and perform sensitivities on isolates**

**Report cell counts, Gram results and culture results**

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**Appendix 1: CSF values**

**A: Normal CSF values for different age groups**

Adapted from B Fong & JM VanBendegom. Chapter 96: Lumbar Puncture

([http://www.uth.tmc.edu/schools/med/imed/residency/chiefscorner/Documents/procedures/Lumbar\_Puncture.pdf)](http://www.uth.tmc.edu/schools/med/imed/residency/chiefscorner/Documents/procedures/Lumbar_Puncture.pdf)

|  |
| --- |
| **Condition Appearance WBC Protein Glucose CSF/blood (mm3 or (SI units =g/l) (SI units =mmol/l) glucose ratio x106cells/l)** |
| **Preterm infant** Clear 9 (0-32) 0.65-1.5 (mean 1.15) g/l 1.3-3.5 (mean 2.8) mmol/l 55-105%**(normal)** 57% PMNs [65-150 (mean 115) mg/dl] [24-63 (mean 50) mg/dl] |
| **Term infant** Clear 8 (0-22) 0.2-1.7 (mean 0.9) g/l 1.9-6.6 (mean 2.9) mmol/l 44-128%**(normal)** 61% PMNs [20-170 (mean 90) mg/dl] [34-119 (mean 52) mg/dl] |
| **Child** Clear 0-7 0.05-0.4 g/l 2.2-4.4 mmol/l 50%**(normal)** 0% PMNs [5-40 mg/dl] [40-80 mg/dl] |
| **Adult** Clear 0-5 0.15-0.45 g/l 2.8-4.4 mmol/l 60-70%**(normal)** 0% PMNs [15-45 mg/dl] [50-80 mg/dl] |

**B: CSF values for different etiologies of meningitis**

Adapted from The Royal Children’s Hospital Melbourne guidelines

([http://www.rch.org.au/clinicalguide/guideline\_index/CSF\_Interpretation/)](http://www.rch.org.au/clinicalguide/guideline_index/CSF_Interpretation/%29)

**Appearance Neutrophils**

**(x 106 /L)**

**Lymphocytes**

**(x 106 /L)**

**Protein**

**(g/L)**

**Glucose (mmol/L) (CSF:blood ratio)**

**Bacterial meningitis** Clear, cloudy or purulent

100-10,000

(but may be normal)

Usually <20 Raised >1.0 Low <0.4

(but may be normal)

**Viral meningitis** Clear, may have faint opalescence

**TB meningitis** Clear, opalescent or ground glass

Usually <100 10-1000

(raised, but may be normal)

Usually <100 50-1000

(raised but may be normal)

0.4-1

(slightly raised or normal)

1-5

(Raised, but may be normal)

Usually normal, can be low e.g. herpes or mumps

<0.3

(but may be normal)

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**Appendix 2: Preparation of Toluidine blue reagent**

Material needed: Toluidine blue (0.1 g)

Normal saline solution (saline)(100 ml) A clean 100 ml cylinder

A clean 500 ml beaker

A clean brown bottle

*To make 100 ml:*

1. Weigh out 0.1 g of toluidine blue in a plastic tray.

2. Rinse the clean cylinder and the beaker saline and drain well.

3. Measure 100 ml of saline with a cylinder.

4. Pour about half of the saline into the beaker.

5. Transfer the toluidine blue into the beaker.

6. Use the remaining saline in the cylinder to wash off the remaining toluidine powder into the beaker.

7. Mix well and transfer in the brown bottle.

8. Label the bottle as below:

**TOLUIDINE BLUE**

***Date made*:** dd/mm/yyyy

***Expiry date:*** dd/mm/yyyy (expires after 6 months)

**STORE AT 2 – 80C (FRIDGE)**

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**12 Risk Assessment**

**Please also read the risk assessment of ALL SOPs used in conjunction with this SOP.**

|  |
| --- |
| **COSHH risk assessment - COSHH Assessment Form** |
| **Description of procedure**This procedure involves CSF | **Substances used**- Toluidine blue- Indian ink- Gram stain reagents- Giemsa reagents- CSF |
| **Quantities of chemicals used**- ≤ 100 ml | **Frequency of SOP use**-Daily |
| **Hazards identified**Autoclaved liquidInfection risk from pathogenic bacteriaAerosols (centrifugation step) | **Could a less hazardous substance be****used instead?**No |
| **What measures have you taken to control risk?**Training in good laboratory practicesAppropriate PPE provided (lab coat, gloves, eye protection)Use of Biosafety cabinet for high risk samples/procedures including handling CSFdeposits and making smears.Use of sealed centrifuge buckets which are opened in a BSC. Plate racks are used and high piles of culture plates are avoided. Suspensions of potential *Neisseria* spp. are made in BSC. |
| **Checks on control measures**Observation and supervision by senior staff |
| **Is health surveillance required?**No | **Training requirements:**GLP |
| **Emergency procedures**:**Report all incidents to Safety****Adviser**-Eye contact: Wash immediately withEye Wash or water.-Skin contract: Wash immediately.-Spills: Clean immediately. For biological spills use an antibacterialdetergent e.g 1% virkon. For chemical spills containing guanidine, clean first with water and do not use bleach (guanidine can from highly reactive compounds when combined withbleach). | **Waste disposal procedures**:-Sharps: Discard into appropriate rigid containers for incineration.-Infectious waste: Discard into autoclave bags or 1% Virkon solution prior to autoclaving and subsequent incineration.-Chemical waste: Disposed of according to manufacturer’s instructions. Chemical waste containing guanidine is not mixed with bleach. |

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**13 Competency Assessment form**

|  |  |
| --- | --- |
| Competency Assessment Form:**CSF Samples** | Employee/Position: |
| Supervisor/Position: |
| **Assessment methods:**O = ObservationV = VerbalW = Written | **Level of competency:**1 = Not competent2 = Competent if supervised3 = Competent and can perform independently4 = Competent and can perform independently and is able to assess the competency of others |
| **Activity** | **Date** | **Method** | **Level** | **Employee****signature** | **Employer****signature** |
| **O** | **V** | **W** |
| 1. Has read and understood the SOP |  |  |  |  |  |  |  |
| 2. Has read and understood the risk assessment and allaspects of health and safety related to this SOP |  |  |  |  |  |  |  |
| 3. Is competent in describing and recording CSFmacroscopic appearance. |  |  |  |  |  |  |  |
| 4. Is competent in carrying out CSF cell counts using achamber, including differentiating between WBC andRBC. |  |  |  |  |  |  |  |
| 5. Is competent in performing Gram stain on CSF samplesand interpreting the results. |  |  |  |  |  |  |  |
| 6. Knows when and how to concentrate a CSF sample. |  |  |  |  |  |  |  |
| 7. Is competent in setting up culture for CSF samples. |  |  |  |  |  |  |  |
| 8. Is competent in interpreting and knowing how to follow-up CSF cultures. |  |  |  |  |  |  |  |
| 9. Is competent in reporting CSF results on the laboratorysystem. |  |  |  |  |  |  |  |
| **CAF Comments:** |