Formalin-ether Concentration Method

PRINCIPLE

Fecal concentration is a routine part of the ova and parasite examination and allows the detection of small numbers of organisms that may be missed by using a direct wet smear. Sedimentation methods use centrifugation to concentrate the protozoa, helminth ova and larva in the bottom of the tube. Ether is used as an extractor of debris and fat from the feces.

SPECIMEN

- Stool preserved in SAF
- Preserved duodenal aspirates
- External QC aspirates
- Aspirates from abscesses

MATERIALS

Reagents

Normal saline (0.85%)(Commercial product, PML)

Diethyl ether (Commercial product, PML)

10% neutral buffered formalin (pH 7.0) (Commercial product, PML)

IF MADE IN HOUSE:

Formaldehyde (Commercial product)	1200mls
Na_2HPO_4	10.7gr
NaH_2PO_4	0.23gr
Distilled water	10.8 L
Triton X-100 (commercial product)	12mls

1. mix thoroughly before dispensing. Smaller quantities can be prepared.
2. titrate pH to 7 using concentrated HCl or NaOH.

Normal Saline Wash Solution (commercial reagent, PML):

IF MADE IN HOUSE:

NaCl	34gr

distilled water	4000mL
Triton X-100 (commercial product, BDH)	4mL

Equipment:
Fume hood
safety centrifuge
Microscope with ocular micrometer and set for Kohler illumination
Funnel filter – disposable (PML)
Applicator sticks
Centrifuge tubes and caps (PML)
Pasteur pipette
Sharps discard
Cotton tip applicator
Glass microscope slides
Cover slips (22 x 40 mm)

QUALITY CONTROL

1. Check all reagents each time they are used and ensure that formalin and saline appear clear without any visible contamination.
2. Ensure that all reagents and chemicals have not expired.
3. The microscope should be calibrated (within the last 12 months)
4. All QC results should be appropriately recorded and "out-of-control" results should be referred to laboratory director for action.
5. Whenever possible, one technologist will read the concentrate and a different one will read the smear. Discordant results will be recorded and resolved with the lab director before reporting.
6. Whenever possible multiple samples from the same individual should be read by a different technologist.

Special Safety Notes

Ether is highly flammable and should only be used in a fume hood and should be disposed of in the appropriate hazardous waste container (see appendix and WHMIS manual). Formalin is hazardous and all processing should be performed in a fume hood. Stool samples should be treated as biohazards and universal precautions are always indicated (see appendix).

PROCEDURE

ALL STEPS SHOULD BE PERFORMED IN THE FUME HOOD.

FOR SAMPLES IN SAF

1. Invert the preserved specimen several times and ensure sample is uniformly resuspended and well mixed with the fixative. Examine the specimen macroscopically for worms, tapeworm segments and note any unusual features such as minimal amount or mucous specimens.

2. Strain approximately 3-4 mLs of well resuspended SAF fixed stool through a commercial funnel filter into a 15mL disposable plastic centrifuge tube. It may be necessary to use more than 3-4 mLs of a diarrheic stool. **DO NOT filter small samples, aspirates or those with a lot of mucous - SEE SECTION ON PROBLEM SPECIMENS BELOW.**

3. Using a fine steam of normal saline Wash Solution from a wash bottle, wash as much of the specimen as possible through the filter device until the volume in the centrifuge tube is 15mls.

4. Before discarding filter device, carefully examine it for worms or tapeworm segments. Save and place any identified specimens into SAF for identification by a technologist or referral.

5. Centrifuge at 2000 rpm (500x *g*) for 10 minutes. CHECK that the amount of sediment is ~1.0mLs. The amount of sediment can be adjusted at this stage. If there is excess, mix the sediment and remove the excess. If there is too little sediment, add more strained specimen before proceeding.

6. Decant the supernatant. Add 10mLs of normal saline Wash Solution, mix the sediment well with an applicator stick and centrifuge at 2000 rpm for 10 minutes.

7. Repeat wash step 6. The supernatant should be fairly clear after the second wash, if not it may be repeated one more time.

8. Decant supernatant and drain tube well onto paper towel. The final sediment should be between 0.5-1.0mLs.

9. **Make slides for permanent stain smears. See Permanent smear section.**

10. Add 8mLs of 10% neutral buffered formalin to the remaining sediment. Mix well with an applicator stick and allow specimen to stand for at least 10 minutes.

11. Add 3mLs of diethyl ether, stopper well and shake vigorously for 60 seconds. DO NOT VORTEX. After a 15-30 sec. delay, carefully open the stopper with the tube pointing away from your face, to let off pressure. RE-CAP tube.

12. Centrifuge at 2000 rpm for 10 minutes. Four layers should result: a small amount of sediment (containing the parasites) in the bottom of the tube; a layer of formalin; a plug of fecal debris on top of the formalin layer; and a layer of ether at the top.

13. Carefully open the stopper away from your face (avoid aerosols). Free the plug of debris by "ringing" the plug with an applicator stick; decant the supernatant fluid and detritus (BUT NOT THE SEDIMENT) into a discard container. DO NOT discard directly into sink or drain.

14. After proper decanting, 1 to 2 drops of fluid remaining on the side of the tube may run into the sediment. Mix this fluid with the sediment. The final sediment volume should be ~0.25 to 0.5mLs. Tubes should be left open in the fume hood for ~ 1hour to let residual ether evaporate. Re-cap the tubes until ready to examine.

EXAMINING SPECIMENS

15. Using a plastic transfer pipette, mix the remaining sediment and transfer 1 drop of the resuspended sediment onto a clean slide and cover with a 22 x 40 mm cover slip. If the sediment is too thick to pipette, 1 or 2 drops of 10% formalin can be added to the sediment. DO NOT ADD TOO MUCH or the sample will be diluted and the concentration will be ineffective. Sterile saline can be used in place of formalin but it must be changed at least daily to avoid contamination of sediment.

16. Systematically scan the entire cover slip with overlapping fields under low power (total magnification x 100) especially examining for helminth ova. Suspicious organisms may be reviewed with the 40x objective. At least one third of the cover slip should be examined under high dry power. Total examination should require at least 10 minutes, more if the technologist has less experience.

MUCOUS OR MINIMAL AMOUNT SPECIMENS

1. If the specimen contains a lot of mucous and/or only a small amount of fecal material (such as that derived in an aspirate or from a neonate), DO NOT FILTER but

centrifuge the entire sample in 1 or more centrifuge tubes at 2000 rpm (500x *g*) for 10 minutes.

2. Wash sample with saline Wash Solution as usual but carefully remove supernatant each time with a transfer pipette rather than by pouring. After the final wash, remove supernatant carefully with a transfer pipette being especially careful NOT TO DISRUPT THE SEDIMENT.

3. Prepare 2 slides from washed sediment - as above. DO NOT PROCEED WITH formalin-ether concentration. Re-cap tube and retain. The technologist will prepare a direct smear from the remaining sediment.

4. If washed sediment is mainly mucous, place 1 drop of Mayer's albumen on 2 slides. Remove an equal volume of sediment with an applicator stick, mix with albumen on one of the slides. Invert the other slide onto the first slide and squash specimen between the two slides. Carefully draw slides apart horizontally and allow to dry thoroughly before staining. Both slides should be stained and read. Re-cap tube and retain. The technologist will prepare a direct smear from the remaining sediment.

PROCEDURE NOTES

1. Too much (> 0.5mL) or too little (<0.25mL) sediment will result in an ineffective concentration.
2. Centrifuge time should be calculated after centrifuge reaches full speed.
3. Vernier measurements should be taken if an unusual parasite is identified or if a confirmation is required.

Problem solving

1. Too little sediment:
   - Possible Problem: - less than 0.5mL of sediment before adding formalin
     - too much ether used
   - Corrective action: - adjust amount of sediment used before adding formalin
     - adjust amount of ether added

2. Too much sediment:
   - Possible Problem: - more than 1.0mL of sediment before adding formalin
     - too little ether used

Corrective action: - adjust amount of sediment used before adding formalin
- adjust amount of ether added

3. No sediment:

Possible Problem: - centrifugation speed too low
- tube "shaken" before "ringing"
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Corrective action: - check centrifuge speed and time
- remove tubes from centrifuge without shaking

REPORTING

1. Protozoan trophozoites and/or cysts, and helminth eggs and larva may all be seen and identified. Protozoan trophozoites and cysts may or may not be identified to species level and are bes confirmed by examination of the permanent stained smear. Oocysts of *Cryptosporidium parvum, Isospora belli,* and *Cyclospora* sp. Can be seen but can also be identified on modified acid fast or sequential stains (see Section of Stains for Coccidia). Identification of microsporidia require special techniques (see Section on Stains for Microsporidia).
2. Use *"Bench Aids in the Diagnosis of Intestinal Parasites"* or attached tables to aid in a species identification of the observed parasites.
3. Helminth eggs and larva including *Ascaris lumbricoides* and hookworm larva may be identified and reported.
4. Report presence of Charcot-Leyden crystals (which are breakdown products of the eosinophils and may be increased in certain parasitic infections) but do not quantitate.
5. Report pus cells (as "Pus cells seen") and erythrocytes (as "Red blood cells seen") if > 3/oil immersion field, but do not quantitate. Do not report yeast.
6. Do not quantitate parasites.
7. Any atypical parasites or those with unusual morphology should be marked with Vernier coordinates and reviewed with the lab director before reporting.

1. LIMITATIONS OF THE PROCEDURE

1. Results obtained by concentrated specimens should usually be confirmed by a permanent stained smear. Confirmation is particularly important in the case of *Entamoeba histolytica* vs *Entamoeba coli.*

2. Coccidial oocysts may concentrate better with flotation methods. If PVA-preserved specimens are used, *Giardia lamblia*, hook worm, trichuris and strongyloides larva may not be well concentrated or easily identified.

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