A COMPARISON OF THE ZINC SULFATE AND THE FORMALIN-ETHER(406th MGL) TECHNIC

L. S. Ritchie,* C. Pan,** and G. W. Hunter, III***
Medical Zoology Department, 406th Medical General Laboratory, APO 500

Many technics have been devised to detect helminth ova and/or protozoan cysts. These may be classified according to features of manipulation and physical attributes as follows: direct smear (wet saline mounts, with or without stains, and permanently stained mounts); sedimentation, followed by direct smear; flotation procedures, with or without centrifugation; and ether sedimentation with centrifugation. Another classification based on the range of effectiveness might be as follows: effectiveness limited to helminths; effectiveness limited to protozoa; effective for both helminth eggs and protozoan cysts; effective for specific helminth eggs; and adaptations for making egg counts.

At least in the minds of some, direct saline smears and permanent stains for protozoa are relegated to the identification of protozoan trophozoites and for confirmation of findings by concentration methods. A number of the concentration technics have been developed in the last three decades, for which the above classifications are inclusive.

The zinc sulfate flotation technic as developed by Faust et al[1] and Tobie et al[2] has been widely used and considered highly effective. It was the first stool concentration method that acceptably fulfilled the dual role of recovering both eggs and cysts. Recently, a formalin-ether sedimentation procedure[3] has been developed that is also highly effective for concentrating eggs and cysts. This method, a simple modification of Telemann's,[4] has been extensively used at the 406th Medical General Laboratory since 1946, and has become identified as the 406th MGL technic.[5] Two other ether sedimentation procedures have been reported for the recovery of cysts as well as eggs, one in 1928 by DeRivas,[6] and the other in 1952 by Laarman.[7]

A direct comparison has been made of the formalin-ether and the zinc sulfate technics by means of parallel examinations on 161 "single stool" specimens from a highly parasitized population.

METHODS

In order to gain proficiency with the zinc sulfate technic, two persons, highly capable in the detection and identification of both helminth eggs and protozoan cysts, prepared and examined a series of specimens by this method before the comparison was started. Critical attention was given to details of preparing the specimens: zinc sulfate with a specific gravity of 1.18 was used and final recovery of eggs and cysts was by means of the wire loop. For any one group of specimens, the microscopics for both methods were done by the same technician.

The procedures for the formalin-ether (406th MGL) method are as follows:

Comminution of the Stool: Partial comminution of the entire stool with an appropriate amount of tap water can be accomplished in the stool box. A suitable amount of water will make it possible to recover 10-12 ml. of strained emulsion, which when centrifuged, will yield 0.5-1 ml. of fecal sediment.

Straining: Two layers of Curity gauze are superimposed over 50 mm. funnels (a rack supporting 12 is appropriate) so that the emulsion can be conveniently collected in 15 ml. centrifuge tubes.

Washing: The emulsion is centrifuged and decanted.

Formalization: The remaining fecal sediment is thoroughly mixed with 10 ml. of 7.5% formalin and allowed to stand 10-30 minutes. Fixation of formalization eliminates distortion of protozoan cysts. Formalin is decanted following centrifugation and replaced with tap water.

Addition of Ether: About 3 ml. of ether are added to the formalized specimen, the tube is stoppered by thumb, vigorously shaken, and then centrifuged at a relatively low speed (at 2 on the angle-arm table centrifuge) for about two minutes. Ether, superficial debris and formalin are completely decanted, using an applicator to free the superficial debris from the centrifuge tube.

Coverslip Preparation: The sediment remaining in the tube is mixed thoroughly with the fluid that drains back from the tube wall and is then poured onto a glass slide. An applicator may be used to drag the few drops to the lip of the tube and is especially useful in controlling the amount of sediment that escapes onto the slide; an excess should be avoided. A small drop of 2% iodine solution is placed near the drop of sediment and mixed with it by using the edge of a coverslip. Finally the edge of the coverslip is pushed into the drop allowing the fluid to run under the coverslip, and, at the same time, pushing the coarse debris aside. The step is critical in obtaining a suitable microscopic preparation.

RESULTS AND DISCUSSION

The results of the comparison of the two methods are summarized in Tables I and II. With combined find-

---

* Professional Adviser to the Medical Zoology Department, 406th Medical General Laboratory, APO 500.
** Presently at Harvard School of Public Health, Boston, Mass.
*** Colonel, MSC, US Army; formerly Chief of Department; presently Chief, Section of Parasitology and Entomology, Fourth Army Area Medical Laboratory, Fort Sam Houston, Texas.

ings of both technics as 100%, the percentage recovered by each was used as the basis of comparison. Minimal advantages for the formalin-ether technic were noted for the recovery of Ascaris lumbricoides, Trichuris trichiura and hookworm eggs. In the case of Trichostrongylus orientalis and Schistosoma japonicum, its efficiency exceeded considerably that of the zinc sulfate. It should be noted that the formalin-ether method is mediocre for S. japonicum as compared with the AMS III method of Hunter et al.[2] The zinc sulfate appeared superior for the few cases of Enterobius vermicularis, but the diagnosis of this parasite is dependent upon an anal-swab recovery of these eggs.

For the helminths collectively, 92.4% of the total cases were diagnosed by the formalin-ether as compared to 80.1% by the zinc sulfate. The more effective diagnosis of Ascaris by the former technic was quite definitely associated with the unfertile eggs. Larger numbers of the fertile eggs were usually obtained by the zinc sulfate, but this advantage was offset by its relatively ineffectiveness where only unfertile eggs occurred. Presumably these are heavier than the fertile ones and consequently are more effectively handled by a sedimentation procedure.

TABLE I

A COMPARISON OF THE FORMALIN-ETHER (406TH MGL) AND ZINC SULFATE TECHNICS

	Total Findings	Formalin-ether		Zinc Sulfate	
		Nbr	%	Nbr	%
Nbr persons exam'd	161	161		161	
Nbr parasitized	161	160		158	
Nbr with Helminths	161	159		158	
Nbr with Protozoa	109	107		87	
Helminths:					
A. lumbricoides	142	141	99.3	132	93.0
T. trichiura	149	143	96.0	128	85.9
Hookworm	101	93	92.1	87	86.1
Trichostrongylus sp.	45	33	73.3	18	40.0
S. japonicum	27	24	88.9	5	18.5
E. vermicularis	9	3	33.3	9	100.0
TOTAL	473	437	92.4	379	80.1
Protozoa:					
E. histolytica	47	45*	95.7	21**	44.7
E. coli	84	81*	96.4	71***	84.5
E. nana	54	51*	94.4	32**	59.3
I. bütschlii	4	4*	100.0	4***	100.0
G. lamblia	16	14	87.5	8	50.0
C. mesnili	2	1*	50.0	0	-
TOTAL	207	196	94.7	136	65.7

*Essentially no distortion of cysts.
**Distortion excessive, making identification of the small race of E. histolytica particularly difficult.
***Some distortion, but identification not greatly hampered.

Observations on the relative efficiency of the technics for the detection and identification of protozoan cysts indicated even a greater advantage for the formalin-ether procedure. Of 47 cases of Endamoeba histolytica, 45 (95.7%) were detected by it in contrast to 21 (44.7%) by the zinc sulfate. This marked difference was clearly associated with the small race of E. histolytica (see Table II). It has been shown by actual measurements that about 50% of the infections in the village from which the specimens were collected were of this type, e.g., measuring less than 10 microns, commonly 7 microns. Of 21 cases so designated, without actual measurements, only 3 were detected with zinc sulfate, in contrast to 20 by formalin-ether. The degree of difference was much less for infections where the cysts measured 10 microns or over, the percentages of comparison being 94.1% and 64.7%.

For 84 infections of E. coli, 96.4% were detected by the formalin-ether and 84.5% by the zinc sulfate. Correspondingly for Endolimax nana the figures were 94.4% and 59.3%, and for 16 cases of Giardia lamblia they were 87.5% and 50.0%.

In the case of E. histolytica and E. nana the amount of cyst distortion was the critical factor. Whereas the zinc sulfate causes considerable, it is negligible with the formalin-ether procedure; with the latter even precysts were undistorted. The matter of distortion allows for a personal factor to enter into the comparison, since the technician must evaluate what constitutes a diagnosable cyst. The nature of the distortion produced by zinc sulfate is distinctive, and there is tendency, finally, to identify on the basis of the distortion.

TABLE II

RELATIVE EFFICIENCIES OF TECHNICS FOR SMALL AND LARGE RACES OF E. HISTOLYTICA

	Total Cases	Formalin-ether		Zinc-sulfate	
		Nbr	%	Nbr	%
Large Race	17	16	94.1	11	64.7
Small Race	21	20	95.2	3	14.3

An added advantage of the 406th MGL method is the fact that specimens may be preserved in formalin and kept for a period of time before concentration. This is convenient when the source of specimens is at a distance from the laboratory, or the rate of collection exceeds that at which the microscopics can be done. Furthermore the concentrated specimens do not require immediate examination, but may be held over from one day to the next, or even longer, if the centrifuge tubes are corked and placed in a refrigerator. The technic is known to be effective on helminth eggs other than those occurring in the comparison, including those of various trematodes and cestodes. Nematode larvae are also concentrated.

SUMMARY

The formalin-ether (406th MGL) sedimentation technic for detecting helminth eggs and protozoan cysts has been shown by direct comparison of 161 stools to be consistently more effective than the zinc sulfate flotation method. Relative ineffectiveness of the letter for E. histolytica was due to excessive distortion of the cysts of the small race, which constituted about half the infections handled.

REFERENCES

1. FAUST, E. C.; D'ANTONI, J. S.; ODOM, V.; MILLER,

M. J.; PERES, C.; SAWITZ, W.; THOMEN, L. F.; TOBIE, J.E., and WALKER, H.: A Critical Study of Clinial Laboratory Technics for the Diagnosis of Protozoan Cysts and Helminth Eggs in Feces: Am. J. Trop. Med. 18:169-183. 1938.
2. TOBIE, J.E.; REARDON, L. V.; BOZICEVICH, J.; SHIH, Bao-Chih; MANTEL N., and THOMAS E. H.: The Efficiency of the Zinc Sulfate Technic in the Detection of Intestinal Protozoa by Successive Stool Examinations: Am. J. Trop. Med. 31(5): 552-560. 1951.
3. RITCHIE, L. S.: An Ether Sedimentation Technic for Routine Stool Exminations: Bull. U.S. Army Med. Dept. 8(4):326. 1948.
4. TELEMANN, W.: Eine methode zur erleichterung der auffindung von parasiteneieren in den faeces: Deutch. Med. Wchnsche. 34:1510-1511. 1908.
5. TIGERTT, W. D.; HUNTER, G. W. III, and RITCHIE, L. S.: Parasitological Studies in the Far East I. Methods and Review of Japanese Literature.: Jap. J. of Med. Sci. and Biol. 5(5):357-385. 1952.
6. DeRIVAS, D.: An Efficient and Rapid Method of Concentration for the Detection of Ova and Cysts of Intestinal Parasites: Am. J. Trop. Med. 8(1):63-72. 1928.
7. LAARMAN, J. J.: New Methods of Concentrating Amoebic Cysts in Faeces: Doc. Med. Geograph. et Trop. Amsterdam. 4(1): 9-11. 1952.
8. HUNTER, G.W. III; HODGES, E. P.; JAHNES, W. G.; DIAMOND, L.S, and INGALLS, J.W., Jr.: Studies on Schistosomiasis. II. Summary of Further Studies on Methods of Recovering Eggs of S. Japonicum from Stools.: Bull. U. S. Army Med. Dept. 8(2): 128-131. 1948.

EXPERIMENTAL TRANSMISSION OF JAPANESE "B" ENCEPHALITIS VIRUS FROM BIRD TO BIRD BY MOSQUITOES (PRELIMINARY REPORT)

Lieutenant M. Z. Rosenberg, MC; Lieutenant J. E. Scanlon, MSC;
R. Cedeno; and Captain E. L. Buescher, MC,
406th Medical General Laboratory, APO 500

THE literature concerning experimental infection of mosquitoes with Japanese "B" encephalitis virus and the ability of infected mosquitoes to transmit infection under laboratory conditions, has been reviewed by Hammon, et al.[1] Transmission to suckling mice by mosquitoes fed on artificially mixed blood-virus suspensions has been accomplished by Japanese and American investigators with at least 17 different mosquito species. Of the few experiments designed to infect mosquitoes by feeding them on infected vertebrate hosts, all but one employed the suckling mouse as the source of virus. One investigator[2] reported a single successful transmission of Japanese "B" encephalitis virus from chicken to suckling mouse with Culex tritaeniorhynchus. Search of available publications discloses no reports of attempts to transmit virus from bird to bird with any species of mosquito. Since suggestive evidence of bird to bird transmission of JBE virus in nature has been recently obtained,[3] it seemed advisable to establish the possibility of bird-mosquito-bird infection cycle in the laboratory. The following report describes two successful bird-mosquito-bird transmissions, a convenient technique for infecting mosquitoes under circumstances closely approaching those that may occur in nature, and the transmission potentials of infected mosquitoes in the laboratory.

METHODS

Culex tritaeniorhynchus has been the only mosquito species in which naturally occurring infection with Japanese "B" encephalitis virus has been consistently demonstrated. For this reason, it would be the species of choice for any experimental study. However, the problems of maintaining this mosquito in a laboratory environment have thus far proved insurmountable. Because Culex pipiens var pallens is readily adapted to laboratory colonization, this mosquito was used exclusively in the following studies.

Previous experience with experimental JBE infections in birds[3] suggested the chick as an ideal vertebrate host for virus in mosquito transmission experiments. Eighty to 85% of a given group of chicks, less than three weeks of age, could be expected to have titratable viremia on the third and fourth day after the inoculation of as little as 5 mouse LD_{50} of virus. Thus, because circulating virus in the chick could be readily quantitated at the time of mosquito feeding, the quantity of mosquito ingested virus could be calculated if the volume of the mosquito blood meal were known. Chicks exposed to the bite of infected mosquitoes could be bled daily for the detection of circulating virus for confirmatory demonstration of specific antibody response.

Several presumable susceptible chicks were inoculated with approximately 100 LD_{50} of virus. On the night of the third day following inoculation, the inoculated chicks were caged separately, each with 100 hungry female C. pipiens. Blood obtained from chicks before exposure to mosquitoes was titrated in mice for virus content. After an overnight exposure to mosquitoes, blood from the same chicks was similarly titrated in mice. The average infectivity of blood calculated from these two titrations was considered an estimate of the virus content of the blood of each chick at the time of mosquito feeding. Unengorged mosquitoes were discarded and the remainder were grouped according to source of blood meal and maintained at an average temperature of 80°F. and a relative humidity of 70%. After the presence of viremia was established in the chick host, the mosquitoes obtaining an infected blood meal were pooled and the remainder discarded. Sample lots of mosquitoes were titrated in lots of ten for virus content periodically. Other mosquitoes from the same pools were used for attempts at transmission of infection to susceptible chicks 7, 14, and 21 days after the initial feeding.

RESULTS

The yield of mosquitoes that engorged on infected chicks was never greater than 50% and was usually