LABORATORY DIAGNOSIS OF AMEBIASIS*

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THE laboratory diagnosis (that is, the recovery and identification) of *Entamoeba histolytica* is a topic normally reported in publications concerned with the diagnosis of intestinal parasitic infections in general. There has been an increased awareness of the importance of proper diagnostic techniques in parasitology.

These techniques were once merely discussed in appendices of books on tropical medicine or parasitology but are now treated as a distinct and separate subject. Among the books published in recent years are those by Burrows,[1] and by Markell and Voge,[2] there is also a recent Public Health Service (PHS) bulletin by Melvin and Brooke,[3] and an excellent color atlas by Spencer and Monroe.[4]

Few books or monographs have specifically dealt with the laboratory diagnosis of amebiasis, those of Anderson et al.,[5] Faust,[6] Brooke,[7] and a PHS[8] manual are the principal ones of the past 10 years. In contrast, short articles on the laboratory diagnosis of amebiasis have been published by a variety of authors in a number of journals for many years, attesting to the continued importance of the subject[9-20] and the necessity for periodic review. Since it is nearly impossible to consider all facets of laboratory diagnosis in one report, only certain aspects of the subject will be highlighted here.

To the layman, the term "amebiasis" may mean very little. On the other hand, the statement "amebic dysentery" immediately calls to mind for most people exactly what amebiasis is in its classic, well-known form. Indeed, if all infections with *E. histolytica* caused amebic dysentery, laboratory diagnosis would perhaps be much simpler than it is today. When a microscopist is given a bloody, watery, or mucoid stool

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he can usually make a direct wet mount in physiological saline and find the organisms, which move in a unidirectional manner and are often filled with ingested red blood cells. Under such conditions there are few problems in identification.

In many cases, a clinical diagnostician can combine good microscopy of the stool with the ability to aspirate or scrape material from ulcers visualized through a sigmoidoscope to make a direct wet mount of these scrapings, and to determine the presence of motile organisms. With skill the diagnostician can fix and stain these organisms in their natural state so that the classical *E. histolytica* with ingested red blood cells is preserved. However, even at this stage of laboratory diagnosis, the ameba must possess a variety of criteria before it is identified as a classic organism. Some researchers have postulated that the term *E. histolytica* should be applied only to those organisms in which ingested red blood cells are seen; in this way microscopic confirmation that the organism is ingesting red blood cells as it invades the tissue would be available. Some misguided workers have developed stricter criteria, and they have postulated that at least two ingested red blood cells should be seen before the organism is called *E. histolytica!*

Although it is true that in these classic cases of amebic dysentery, examination of the bloody stool or aspiration of ulcers of the colon make diagnosis simple, the fact remains that not all infections are classic presentations, and etiologic diagnosis of amebiasis can be difficult and sometimes a frustrating endeavor.

The eventual identification of *E. histolytica* in a stool specimen depends upon organisms being seen under the microscope, generally at high magnification, subjectively fulfilling certain "objective" criteria. These criteria are unique for amebiasis only as much as classic parasitologic diagnosis is unique. Material obtained on a finger cot or during a rectal swab examination is not for the parasitology microscopist. For enteric bacteriology, a tiny bit of feces or rectal swab inoculated into selected media may provide the diagnostician with all the objective information he needs. However, in amebiasis it has long been recommended that some information about the character of the stool specimen be available to the laboratory. Therefore it is generally recommended that a substantial portion of the entire stool be sent to the laboratory so that the microscopist can select the part of the specimen with which to work. In some cases of amebiasis the stool is of the

TABLE I. SUBSTANCES THAT INTERFERE WITH PARASITOLOGIC EXAMINATION OF FECES

Antidiarrheal preparations Bismuth, kaolin	*Antacids, Laxatives* Oils, magnesium hydroxide
Radiographic procedures Barium sulfate	*Enemas* Water, soap solution, irritants, hypertonic salt solutions
Biologically active drugs Sulfonamides, antibiotic agents, antiprotozoal drugs, anthelmintic agents	

Adapted from Juniper, 1969[22]

classic diarrhetic or mucoid, bloody type; in other cases formed stools may occur without any blood or evidence that the integrity of the intestinal wall has been impaired. The parasitology laboratory must often decide which type of examination should be performed; this depends on the character of the stool rather than on the patient's symptoms.

A factor in the diagnosis of amebiasis which has become more important in recent years is the knowledge that the examining laboratory should receive a specimen of stool in which there is a reasonable chance that organisms are present. In these days of ready access to antibiotics and other preparations, it is often possible that the patient may have taken "interfering substances" at variable periods before the specimen is submitted to the laboratory. Because of extensive travel to various parts of the world, especially to those considered endemic for amebiasis, it has been recommended that the physician, in obtaining a history from the patient, include Maegraith's question, "*unde venis?*"[21] ("Where have you been?").

It is perhaps also appropriate, considering the possibility of interfering substances in amebiasis, that the physician ask: "*Quibus medicamentes uteris?*" (" What medications or drugs are you now taking?")

Table I, from a recent article by Juniper,[23] lists some of the substances which make it difficut to find organisms in a fecal specimen. The substances reduce the organisms to very low numbers or temporarily eliminate them, so that diagnostic procedures become literally a waste of time. The physician's knowledge of whether or not such substances have been ingested by the patient is invaluable. I know of instances in which numerous stool specimens have been examined

over long periods of time while the patients have been taking broad-spectrum antibiotics.

Given the possibility that interfering substances have not been administered to the patient, there are certain guidelines that a microscopist can follow grossly in judging whether or not a particular stool specimen has the characteristics of amebiasis rather than, for example, those of bacillary dysentery. The macroscopical differences between stools in amebiasis and bacillary dysentery have been well outlined recently by Stamm.[23]

Such guidelines for gross distinctions should perhaps be used only by experienced observers. The tendency of the inexperienced might be to make a specific diagnosis of the stool macroscopically, that is, by its solid or liquid state. whether or not it was streaked with blood, whether fecal elements were present, and whether the odor was offensive or alkaline. In amebiasis such "long distance diagnosis" without resort to microscopy is not to be encouraged.

The direct wet mount of a stool, in physiological saline, has always been the standby for initial examination. The procedure for making a direct wet mount is simple. A bit of stool is placed on a slide and emulsified in the saline; a coverslip is added, and a search is made of the entire preparation for trophic or cystic forms. The consistency of the preparation should be such that newsprint can easily be read through it.

In those instances in which the stools are watery, loose, or soft, and rapid passage through the colon has perhaps taken place, there is a tendency for trophic forms of the organism to predominate. A bit of Nair's solution[24] added to the wet mount aids in delineating the morphology of the trophic amebas. In a more solid specimen, if it is produced in chronic amebiasis during such times as the intervals between attacks of diarrhea or dysentery, the stools contain more cystic forms. In such cases the usual procedure is to make a wet mount in physiological saline and also a preparation containing a bit of Dobell's iodine, which helps delineate the glycogen mass, nuclear elements, and the chromatoid bodies, if these are present. For many years some workers have added 0.5% eosin to the physiological saline solution. In this preparation the living cysts, which do not take up the eosin, stand out as refractile bodies and can be detected easily. After this comes the difficult part in diagnosis. Organisms that are seen must be

distinguished by species, that is, they must be classified as *Entamoeba histolytica, E. hartmanni, E. coli, Endolimax nana, Iodamoeba butschlii*, or perhaps as a mixture of many species, as sometimes occurs.[25, 29]

The number of stools which should be examined before infection with *E. histolytica* is ruled out has been the subject of several reports.[11, 26] The rule accepted by many workers is that not less than three normally passed specimens obtained over a period of 7 to 10 days should be examined to determine whether organisms are present. This is the standard "O & P times 3" which appears on so many laboratory forms. The percentage of infections which are detected, based on the number of stools examined, has been reported on by Svensson[26] and by Stamm.[11]

Quoting several sources, Stamm stated that the consensus was that about 30% of the positives present would be found by a single examination. Estimates are that 75 to 95% of the infections are found after examination of the third specimen. How many specimens a laboratory should examine after the third one depends as much on the physician as on the laboratory. Continued stool examinations may occasionally be fruitful. However, there is always the danger that the pressure to find *E. histolytica* may persuade a microscopist that what he thought were artifacts were actually the elusive sarcodine. Strange as it may seem, one occasionally hears the statement: "The patient has amebiasis; therefore, there should be *E. histolytica* in his stool specimen."

Cathartics such as buffered phosphosoda are used to obtain "purged" specimens and are sometimes recommended[7, 9, 13, 15, 22] to increase the yield of organisms in stools. The value of using three normally passed stools as well as purged specimens was noted by Sawitz.[27] Reports by Yarinsky and Sternberg[28] list good reasons for examining always both the first and second specimens taken after a purge.

A stool of recent passage, an experienced microscopist, and time enough for examination are three conditions that rarely coexist. Indeed, in many laboratories, parasitological examinations of feces sometimes occupy a relatively small period of the time of a technologist who is obliged to function also as a bacteriologist, serologist, or hematologist. Separate parasitological laboratories exist in relatively few institutions, such as large hospitals, state health departments, or federal establishments. If an examination of a fresh stool can be accomplished by a

skilled microscopist within a period of one to two hours it is one of the best and most valuable types of examination and is not to be discredited.

The situation in which the corner of a laboratory contains stool cartons collected throughout the day and awaiting examination is not unusual in some institutions. Many years ago it was thought that the amebas coming out in the stool specimen from a warm body should continue to be kept as warm as possible. It was then customary to place stools in a 37° C. incubator if there was to be a long delay in examination. However, it is now recommended that a stool be kept at room temperature or even at 4° C. in a refrigerator both for aesthetic reasons and also in order to retard the action of bacteria which may destroy trophic forms. Experience has shown, however, that a stool specimen may be passed hours before it is examined for parasites. To obviate the problems that arise during the interval between passage and examination, and because of the difficulty in finding sparse organisms, a number of techniques, ancillary to the direct wet mount examination, have been developed. Solutions are now used to preserve the specimen so that it can be examined at leisure, and procedures are used for concentrating the organisms in a small amount of material.

It is interesting that many of the standard procedures in use today for the diagnosis of amebiasis were developed 15 to 30 years ago. In some fecal specimens amebas may be so few that many normal specimens or even purged stools must be examined before the culprit is found. For years it was thought that if the organisms could be concentrated in a small amount of material it would be advantageous. The time spent searching through slide after slide would be eliminated. A technique which concentrates trophic forms of ameba in loose, soft, or watery specimens has not yet been developed.

One of the first laboratory techniques developed for concentrating parasitic organisms in stools was the zinc-sulfate procedure of Faust and his co-workers.[30] Floating the protozoan cysts, helminth eggs, and other portions of the feces in a solution of 33% zinc sulfate, at a specific gravity of about 1.18 to 1.2, enabled the microscopist to "corral" many organisms in a small amount of fluid. This is a very useful procedure. Trophozoites, if not destroyed, are generally distorted, sometimes beyond recognition. The zinc-sulfate concentrating technique, still widely used, was developed in 1938.

In 1948 Ritchie[31] introduced the formalin ether (FE) concentration technique, which has been modified slightly by some workers.[32] It is also a procedure used in general parasitologic diagnosis, that is, for the recovery of helminth larvae and eggs as well as protozoan cysts. Ritchie's FE procedure uses a very common ingredient of the laboratory—the pathologist's "*aqua eterna*," formaldehyde. The stool specimen is placed in a tube containing formalin, a small amount of ether is added, and then the tube is centrifuged. The individual protozoan cysts, helminth larvae, and eggs are separated from most of the fecal debris and can be detected in the sediment.

The FE technique of Ritchie is widely used in a variety of laboratories. It is especially useful because it can be performed with no loss of morphologic integrity in specimens which have been previously collected and preserved in 5 to 10% formalin. However, the FE technique does not concentrate the trophic forms to any degree and is not well suited for the concentration of cysts of *Giardia lamblia.*

The Army's contribution to parasitologic diagnosis in the late 1940's was Ritchie's FE technique; this was followed quickly by the Navy's contribution in 1953, when Sapero and Lawless[33] introduced the merthiolate-iodine formalin (MIF) procedure. This was another method of treating fecal specimens that assured the preservation of any organisms present; also identification could be performed at leisure. The merthiolate and iodine provided a polychromatic staining of the protozoan nuclei, chromatoid bars, glycogen masses, and cell membranes. Both the MIF and FE procedures can be used for specimens collected far away, both in distance and time, from a central diagnostic laboratory.

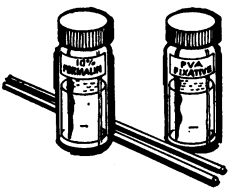
A highly significant contribution to diagnostic parasitology was made by the Public Health Service when the polyvinyl alcohol (PVA) fixative technique was developed by Brooke and Goldman.[34] With the PVA fixative, it was possible to collect stool specimens and to preserve the fragile trophic forms so that their integrity was maintained and distinct morphological characters (nuclear beading, cytoplasm, cell membrane) were preserved for subsequent staining and critical observation.

The PVA fixative only preserves the organisms, which must be stained before the morphology can be critically evaluated. Few routine diagnostic laboratories today use the long Heidenhain iron-hematoxylin

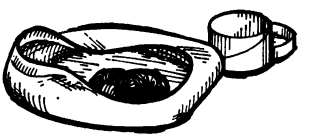
USE OF PVA-FIXATIVE TECHNIQUE FOR SUBMITTING STOOL SPECIMENS TO BE EXAMINED FOR PARASITES

ADAPTED FROM BROOKE AND GOLDMAN, 1949

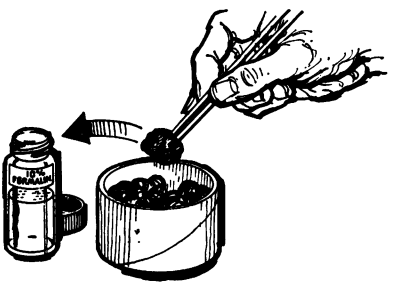
NOTE: BOTH SOFT AND FORMED SPECIMENS SHOULD BE SUBMITTED BY THIS METHOD. SPECIMENS MUST BE FRESH WHEN PLACED IN VIALS.



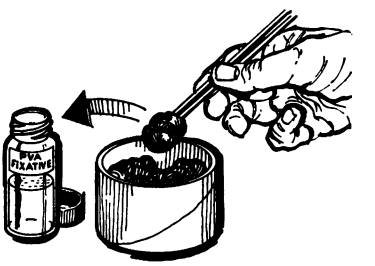
1 The kit consists of two glass vials (one with 10% formalin, and one with PVA).



2 The stool should be passed into a dry container. Urine should not be passed into the same container.

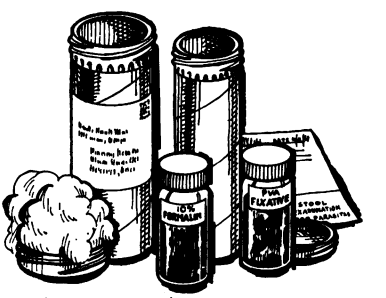


3 Using applicator sticks, place a quantity of the stool into the 10% formalin (ratio of 5 parts formalin to 1 stool).



4 Place a similar quantity into the vial containing the PVA fixative.

5 Thoroughly break up specimen in the 10% formalin and PVA fixative. Shake vigorously.



6 Place the two vials so as to protect against breakage. Enclose appropriate identification and mail or deliver to laboratory.

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techniques since it is time-consuming and subject to errors in the processes of destaining and mordanting. However, when critical examination of protozoan morphology is needed, it is still useful and largely unsurpassed.

For routine diagnostic purposes, the trichrome stain of Wheatley,[35] developed in 1951, is useful since it affords critical staining and presents a pleasant polychromatic picture. The short iron-hematoxylin procedure of Tompkins and Miller[36] is favored by some workers; like the Wheatley trichrome, it can be used with stool specimens that have been preserved by means of PVA. Many workers advocate the formalin-PVA preservation method seen in the accompanying plate for the complete examination of a stool for all parasites, including amebas. This method can be used for routine office, clinic, or hospital work as well as during surveys or on those occasions in which several hours or even days may elapse between collection and examination.

Although diagnostic techniques for amebiasis in particular and parasites in general were developed many years ago there are a number of useful newer procedures which need only be evaluated by various workers over a period of time for more widespread acceptance. Burrows[37] recently reported a much needed improvement in the method of preparing PVA-fixative, and he introduced in 1967[38] an additional fixative, the "PAF," for preservation of diagnostic stages of protozoa and helminths. Arensburger and Markell[39] developed a useful combination direct-concentrate procedure in 1962, and Silva in 1969[40] reported the efficacy of a "larvoocyst" apparatus which utilizes zinc sulfate flotation to collect larvae, eggs, and protozoan cysts.

In 1966[41] Mitchell reported that the penetration of mordant and hematoxylin was improved by the addition of dimethyl sulfoxide to the solutions. In the same year Alger[42] described a modification of the trichrome stain which was simple, precise, and rapid, and could be used by inexperienced workers. A few years ago, in our own laboratory, we investigated[43] the chlorazol-black combination fixative-stain developed by Kohn in 1960,[44] and we found that it was very useful, especially for small laboratories or clinics which do not examine large numbers of stools and do not have the various solutions, stains, and equipment necessary for diagnosis.

The use of cultures for the detection of *E. histolytica* in stool specimens has a long history. The LER medium of Boeck and Drboh-

lav[45] developed in 1925 is still used today, as are the liver extract medium of Cleveland and Collier,[46] the egg infusion of Balamuth,[47] and the alcohol-egg extract of Nelson.[48] Generally the culturing of stools for intestinal amebas is carried out in laboratories in which parasitologic diagnosis is an ongoing activity, not a sideline. Some workers have minimized the use of cultures for routine diagnosis. None of the culture techniques allow the growth and multiplication of *E. histolytica* alone; therefore if cultures are positive and organisms are seen, the microscopist is still faced with the problem of differentiating *E. histoytica* from other amebas in the media.

The experience of some diagnosticians, however, indicates that the use of cultures, in addition to the other diagnostic methods in amebiasis, can be valuable. The recent positive results of McQuay,[49] who used his charcoal medium[50] for cultivating the stools of furloughed missionaries in Chicago, and the positive results of Robinson,[18] who used his newly developed culture medium in Greenwich, England, speak well for the use of cultures as a part of the armamentarium of techniques for the diagnosis of amebiasis.

Although diagnosing amebic infections of the intestine probably constitutes most of the work of the routine laboratory, some strains of *E. histolytica* are capable of penetrating and thriving in tissues other than the wall of the colon. The gynecologist, for example, may encounter diagnostic problems in amebiasis; these were recently pointed out by Munguia *et al.*[51] in their detection of *E. histolytica* in Papanicolaou smears.

The etiologic diagnosis of extraintestinal amebiasis is difficult. Recovery of organisms from tissues such as the liver, the primary focus of the amebas outside the intestine, is not very successful in many diagnostic laboratories. The average laboratory is generally not called upon to search for or identify amebas in tissues from liver biopsy or so called amebic hepatitis.

Fluid obtained from a liver abscess by open drainage or closed aspiration is generally the material which the laboratory receives for examination. Such fluid may be of the "typical" anchovy-paste color and consistency, and a positive diagnosis of amebiasis is often concluded on the recovery of such typical fluid without demonstration of the organism. However, as pointed out by Wilmot,[52] fluid from an amebic liver abscess may be white, cream-colored, greenish, or yellowish. Foul-smell-

ing, greenish or yellowish fluid indicative of bacterial infection does not rule out the fact that the fluid may have originally been sterile. Maddison et al.[53] pointed out that the sterility of the abscess fluid may be proportional to the number of times aspiration has been attemped.

Just as the laboratory can seldom find amebas in stool specimens if the patient is taking interfering substances, the chances are likewise small that isolation and identification of organisms will be successful if the pus from a liver abscess cannot be expected to contain organisms. This is usually the case when the first portions of the fluid drained or aspirated from an abscess are sent to the laboratory. Some authors[12, 54] have pointed out that amebas are found at the periphery of the abscess and are more abundant in the last part of the aspirate or drainage recovered. Such fluid shows the typical red color. With the removal of the static fluid pressure, the wall of the abscess shrinks or collapses, expressing amebas and blood from the tissue.

Lello[54] several years ago outlined a procedure employing streptodornase and streptokinase to free the amebas from the thick coagulum of pus which is often obtained in drainage from liver abscesses. The resultant fluid can be either examined as a wet mount preparation, fixed to slides, placed in PVA for staining, or inoculated into the standard culture media already mentioned. Culture media inoculated with fluid from a liver abscess must also be inoculated or "seeded" with bacteria, particularly if the aspirate fluid is sterile, since amebas seem to thrive better *in vitro* with bacteria. Inoculating abscess fluid into a highly specialized medium such as the axenic type developed by Diamond[55] may be useful but is not recommended as a routine procedure. Mixed bacterial flora or monoconcomitants such as *Escherichia coli* or *Clostridium welchii*[56] are often used with the initiation and maintenance of cultures of amebas.

The number of successful isolations of *E. histolytica* from extraintestinal sites are very few compared with isolations from intestinal infections. Clinical impression, history, and response to chemotherapy are often the only choices open to the physician because of difficulties in obtaining an etiologic diagnosis. Serologic study has been resorted to as an aid in cases in which the organism is difficult to find.

Many years ago serologic techniques were used in the diagnosis of amebiasis, both for intestinal and extraintestinal disease. Difficulties in obtaining standardized antigens, the problem of serologically false nega-

TABLE II. RESULTS OF RECENT SEROLOGIC TESTS FOR AMEBIASIS

Serologic test	Amebic Liver abscess No. sera	Amebic Liver abscess % Positive	Symptomatic intestinal amebiasis No. sera	Symptomatic intestinal amebiasis % Positive	Author	Year
IHA*	35	100%	133	98%	Kessel et al.	1965
IHA	121	96%	83	82%	Milgram et al.	1966
IHA			63	85%	Healy	1968
IHA	31	87%	168	81%	Krupp	1970
IHA	16	75%	6	100%	Prakash et al.	1970
IHA	16	87%	20	85%	Halpern et al.	1967
IHA	48	100%	41	90%	Thompson et al.	1968
IHA	47	92%			Savant and Chaicumpa	1969
CF†	20	100%	92	90%	Kessel et al.	1965
CF	55	100%			Kasliwal et al.	1966
CF	31	84%	30	63%	Thompson et al.	1968
IFA‡	18	100%	10	80%	Coudert et al.	1968
IFA	61	95%	40	75%	Jeanes	1969
IFA	33	91%	33	75%	Boonpucknavig and Nairn	1967
IFA	42	100%	23	91%	Ambroise-Thomas et al.	1969
SAFA§	15	100%	12	100%	Gore and Sadun	1968
IFA	22	90%	32	84%	Goldman	1966
Gel diffusion	528	94%			Powell et al.	1965
Gel diffusion			400	92%	Powell et al.	1967
Gel diffusion	33	93%	32	66%	Boonpucknavig and Nairn	1967
Gel diffusion	12	92%	22	95%	Halpern et al.	1967
Gel diffusion	49	80%	41	54%	Thompson et al.	1968
Tube precipitin	150	97%	150	89%	Powell	1968
Immunoelectrophoresis	93	97%	6	67%	Savant and Chaicumpa	1969
Bentonite flocculation	90	93%	50	86%	Tupasi and Healy	1970
Latex agglutination	100	98%	100	96%	Morris et al.	1970
Bentonite phagocytosis	17	100%	24	96%	Halpern et al.	1967

*Indirect hemagglutination test
†Complement fixation test
‡Indirect fluorescent antibody test
§Soluble antigen fluorescent antibody test

tive and false positive reactions, and the problem of ameba-bacteria antigenic complexes limited the use of serology in diagnosis. In the past decade, however, there has been a renewed interest in the judicious use of serologic techniques, particularly for amebic liver abscess.

This interest has been stimulated to some extent by the advances in the field of serology in general, partly by the development of more sophisticated techniques for making purer antigens and partly by the knowledge that in extraintestinal amebiasis, classic isolation techniques are difficult and often unrewarding.

The results obtained by several groups of workers over the past decade who employed serologic techniques for the detection of amebic infections may be seen in Table II. The list is not exhaustive, since many workers published preliminary papers prior to the reference cited. Virtually every type of serologic procedure has been employed for both symptomatic intestinal and extraintestinal amebiasis. Various workers have obtained different results. The difference in positivity rates reflects not only differences in the populations studied but also individual variations in the criteria used to determine positive and negative serologic results. The number of references in Table II, however, indicates that a sizeable body of literature[57-76] now exists, attesting to the use of serologic techniques. The list continues to grow. For the perplexing problem of suspected amebic liver abscess, in particular, serology would seem to be very promising as a useful diagnostic tool.

In summarizing this review I am encouraged by the progress which has been made. Time-tested, reliable techniques are still in use. Periodically the literature is nourished by the publication of a new technique or a modification of an older method; the new data help to sharpen the tests for this protozoan parasite, so important in medicine and public health. Serology has a place in the laboratory diagnosis of amebiasis. The limits of serologic diagnosis in intestinal amebiasis have been pointed out by some,[66] and doubtless there will be other critical evaluations.

A prospective appraisal of the diagnosis of amebiasis is not within the purview of this presentation. Nearly 20 years ago a group of 93 qualified workers examined the subject in some depth.[10] Results did not show universal agreement on the criteria for diagnosis. Perhaps it is time for another analysis.

If I were asked to forecast the future of laboratory diagnosis, I should say that the outlook is good. The increasing role of serology was noted above. For classic etiologic diagnosis, the passage of the Clinical Laboratories Improvement Act of 1967[77] has stimulated the use of referee laboratories, reference or check specimens, plus the inclusion of quality-control methods in the laboratory diagnosis of all parasitic diseases. Private and governmental institutions are now using evaluation specimens to upgrade and check diagnostic proficiency. Preliminary analysis and results of laboratory proficiency indicate that such evaluations are needed, but the future does look bright.

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