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NEW BIOASSAY SYSTEM FOR EVALUATING PER CENT SURVIVAL OF *ENTAMOEBA HISTOLYTICA* CYSTS*

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ABSTRACT: Excysting *Entamoeba histolytica* trophozoites shed a translucent cyst wall which can easily be distinguished from the intact cyst under high-dry magnification of a microscope. A new cyst bioassay system is based on the assumption that an empty cyst represents an ameba which was alive and excysted, in contrast to a full cyst which represents a dead ameba or one unable to excyst. Live amebae can be stimulated to excyst by incubating them at 37 C in a monophasic nutrient medium. The percentage of live cysts in a population can then be calculated from a count of the full and empty forms in a drop of the excystation culture which is transferred to a slide, covered with a cover slip, and microscopically examined. The unique aspects of the bioassay system are that it makes it possible for cyst survival to be expressed as a percentage of the total number of cysts in a sample, and for the results from any test to be corrected for control cysts. This new bioassay system has been utilized successfully in studies designed to evaluate the ability of *E. histolytica* cysts from a variety of sources to survive exposure to halogen disinfectants and storage at various temperatures and relative humidities.

When *Entamoeba histolytica* cysts are placed in a modified TP-S-1 medium and incubated at 37 C for 10 hr, live amebae excyst through a rupture in the cyst wall. Dead amebae remain entombed within the cyst. Clearly evident in a microscopic view of an aliquant of the excystation culture after the incubation period are empty cyst walls—active trophozoites which have excysted—and full cysts which contain dead amebae. Figure 1 illustrates the ease with which the empty cyst wall can be differentiated from the intact cyst. The per cent of live forms in a population of cysts can be calculated from the number of full (dead) and empty (live) forms counted. Since the new cyst bioassay system is easy to perform and enables cyst survival to be expressed as a per cent, it constitutes an improvement over the variety of systems, based on culturing and staining procedures, which previously have been used to express the results from cyst survival studies.

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MATERIALS AND METHODS

Concentrating and cleaning cysts

Entamoeba histolytica cysts were separated from most of the debris accompanying them in cultures and in human and simian feces by using a modification of the zinc sulfate flotation method developed by Snyder and Meleney (1941). The material containing cysts was placed in centrifuge tubes and vigorously mixed with water prior to centrifugation at 3,000 rpm for 3 min. Washing–centrifugation–resuspension sequences were repeated 4 times. The sediment was resuspended in a zinc sulfate solution (sp gr 1.18). An overlay of 1 ml of water was gently added to the surface of the zinc sulfate solution. After a 2-min centrifugation, cysts were found in the top 5 ml of solution. This supernatant was transferred to a clean 15-ml centrifuge tube, and 3 more washing sequences were performed to remove traces of the zinc sulfate solution. The cyst suspension was passed through cheesecloth and then through a sterile NBS 325, 44-μ sieve to filter out any large chunks of debris which were still present. An additional 15 ml of water was passed through the sieve to wash its pores free of adhering cysts. In the sieve effluent were relatively clean cysts. Approximately 25% of the cysts were lost during the cleaning procedure.

Preparing excystation culture

Washed cysts were then concentrated down to 0.4 ml and mixed with 2.6 ml of the excystation medium in a 15-ml conical-bottom centrifuge tube. The medium which promoted maximum excystation was a modification of liquid TP-S-1 monophasic, which was developed by Diamond (1968) to maintain axenic cultures of *E. histolytica*. Vitamins (mixture 107) were omitted from this medium and antibiotics were added. TP broth for the excystation medium was prepared by combining

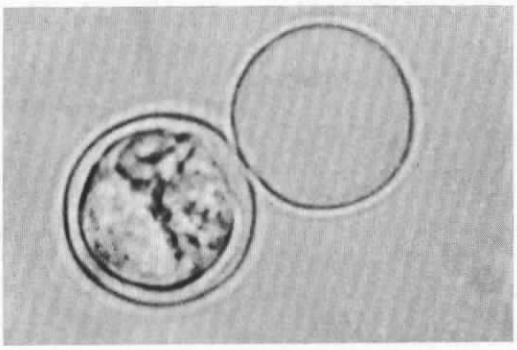

FIGURE 1. Left: A full *Entamoeba histolytica* cyst (culture strain 200-NIH) containing a dead ameba. Right: An empty cyst from which the ameba has excysted. Cysts were incubated in modified TP-S-1 medium for 11 hr at 37 C. Diameter of cysts: 9 μ.

the following: 1.0 g trypticase (Baltimore Biological Laboratories); 2.0 g Panmede liver digest (Paines and Byrne); 0.50 g glucose; 0.10 g L-cysteine monohydrochloride; 0.02 g ascorbic acid; 0.50 g sodium chloride; 0.06 g potassium phosphate, monobasic; 0.10 g potassium phosphate, dibasic, anhydrous; 90 ml water, deionized distilled. pH was adjusted to 7.0 with 1 N sodium hydroxide and the medium was suction-filtered through Whatman No. 1 filter paper and autoclaved for 10 min. Excystation medium contained 1 part horse serum to 9 parts TP broth. Antibiotics were also added (1,500 units/ml of penicillin and 1,000 units/ml of streptomycin).*

Evaluating cyst survival

When the excystation culture was incubated at 37 C, viable amebae began to excyst after the 1st hr. By the 10th hr, when the maximum number of amebae had excysted, the culture was centrifuged (3,000 rpm for 3 min) and the contents were pipetted out except for the final 0.07 ml which contained the concentrated cysts.

A drop of the concentrated cyst suspension was transferred to a glass slide. The drop was covered with a vaseline-edged cover slip and examined under high-dry magnification of a microscope. A random count was made of the visible full and empty cysts. From this count the percentage of empty cysts was calculated with Formula 1:

$$\% \text{ of empty cysts} = \frac{\text{number of empty cysts}}{\text{number of full and empty cysts}} \times 100.$$

Formula 2 was used to calculate cyst survival, corrected for control cysts:

$$\% \text{ of viable amebae excysting} = \frac{\% \text{ of empty cysts in test}}{\% \text{ of empty cysts in control}} \times 100.$$

RESULTS

The new cyst bioassay system makes it possible to obtain the caliber of experimental results shown in Table I. The table summarizes a study demonstrating how the pH value of an iodine solution influences its cysticidal activity. Protocol for this experiment is presented in previous publications (Stringer, 1970; Stringer and Krusé, 1971). As seen in the table a solution of iodine with a pH value of 6.5 is a cysticide far superior to an iodine solution of the same concentration with a pH value of 8. At pH 6.5, no cysts survived after a 10-min exposure to a concentration of iodine sufficient to give a 10-min residual of 8.5 ppm. At the higher pH value (pH 8.0), 34.1% of the test cysts, or 57.6% of the viable control cysts,

* TP-S-1 monophasic medium is now available in the premixed form from General Biochemicals, Laboratory Park, Chagrin Falls, Ohio (Order No. 69251). Horse serum and antibiotics must be added to this premixed medium.

TABLE I. *Per cent survival of in vitro* E. histolytica *(200-NIH) cysts after 10 min exposure to three concentrations of iodine at pH 6.5 and pH 8.0.*

10-min iodine residual (ppm)	pH 6.5		pH 8.0	
	In test	Control* corrected	In test	Control* corrected
1.5 ppm	22.3%	38.7%[a]	44.3%	74.8%
1.5	18.2	30.7	39.2	66.2
3.9	0.37	0.62	43.3	73.1
3.9	1.3	2.2	42.9	72.5
8.5	0	0	34.1	57.6
8.5	0	0	—[b]	—[b]

* 59.2% of control cysts excysted.

[a] $\frac{22.3\%}{59.2\%} \times 100 = 38.7\%$.

[b] Test not done.

excysted after exposure to the same concentration of iodine. The weaker solution of iodine (1.5 ppm) killed fewer cysts. Control-corrected excystation values from duplicate tests in which cysts were exposed to a 1.5-ppm iodine solution at pH 6.5 were 38.7 and 30.7%. These survival values are lower than those which occurred at pH 8, 74.8 and 66.2%.

Although only one example of a successful use of the new bioassay system for *E. histolytica* cysts has been demonstrated in this report, it has found wide application in studies designed to evaluate the ability of amebic cysts to survive exposure to a variety of halogen disinfectants and storage at various temperatures and relative humidities.

DISCUSSION

Ability to differentiate between full and empty cysts could be mastered by a novice after 5 to 10 hr of practice. To facilitate cyst counts it was essential that the number of cysts present be sufficiently large. It seldom took longer than 15 min to count 500 full and empty cysts when 100,000 cysts had been inoculated into the excystation culture. Only whole spherical cysts and cyst walls were included in the count since occasionally cysts were torn apart during the concentration procedure and pieces of the ruptured cysts were present. Since there are some morphological similarities between empty ameba cysts and yeast spores, it was essential that the technician be able to recognize the difference between these cells.

Sensitivity of results obtained with the new cyst bioassay system was dependent on the total number of cysts counted in a random sample and on the per cent of control amebae excysting. Twenty to 70% of *E. histolytica* (strain 200-NIH) cysts from modified egg-slant cultures (Diamond, 1968), and *E. histolytica* cysts from infected humans, normally excysted in freshly prepared medium. This same excystation medium was less effective in promoting excystation of *E. histolytica* cysts from naturally infected monkeys since fewer than 30% of cysts from simian hosts normally excysted. On rare occasions no amebae excysted.

The critical assumption of the new cyst bioassay system is that an empty cyst signifies an ameba which was alive and excysted, whereas a full cyst signifies an ameba which was unable to excyst because it was dead. Undoubtedly, there is the possibility that some amebae were alive but did not excyst and conversely some empty cysts were occasionally seen from which dead amebae had been forced by pressure from a cover slip or by mechanical shock caused by the cleaning and concentrating routine. Two observations were made that lessened the likelihood that an empty cyst represented anything other than a cyst which had been alive and had been stimulated to excyst by incubation at 37 C in the excystation medium: (1) no empty cysts were ever found in a population of cysts prior to being incubated in the monophasic excystation medium, and (2) no empty cysts could be found in excystation cultures after the cysts were exposed to lethal doses of chlorine and iodine. The other assumption, that a full cyst found in an excystation culture after incubation was a dead cyst, was supported by the observation that prior to excysting the trophozoite goes through characteristic pseudopodial movements which the author has documented with time-lapse microcinematography. This characteristic pseudopodial movement of the live encysted trophozoite was never seen in a cyst which remained full after being incubated in the excystation medium for more than 10 hr.

This new amebic cyst bioassay system can be compared to a number of the systems which previously have been developed for the purpose of detecting amebic cyst survival. Kuenen and Schwellengrebel (1913) were the first investigators to evaluate *E. histolytica* cyst viability through the use of a 1/1,000 dilution of eosin, which they believed stained only dead cysts. Until more sophisticated cultural techniques were developed, this system was considered reliable by Wenyon and O'Connor (1917), Stone (1937), Mills et al. (1925), Spector et al. (1934), and other workers. Chang (1944a) pointed out the lack of specificity of this system by demonstrating that as many as 70% of dead cysts were not stained by eosin. Mills et al. (1925) attempted to improve on the strict eosin method by using an iodine–eosin mixed stain. Dead cysts which failed to take the red eosin stain were stained green by iodine. Root (1921) found that neutral red stained a slightly higher percentage of dead cysts than did eosin. He used neutral red to

evaluate the viability of protozoan cysts, including those of *E. histolytica* and *E. coli* in the gut of flies. Chang and Fair (1941) used a drop of orthotolidine in a 5-ml cyst suspension. They then observed the washed cysts under a microscope and computed the per cent of cysts stained yellow. They presumed that only cysts penetrated by chlorine were yellow. Chang (1944a) and Bercovitz (1924) stained the cysts with iron–hematoxylin and observed morphological changes which might be associated with death. Bercovitz actually used these changes as criteria for mortality of cysts after exposure to various substances. Yoshida (1920), Boeck (1921), and Kessel (1925) concluded that plasmolysis in cysts was a better criterion for mortality, since some cysts showed extensive internal plasmolysis and were obviously not viable, yet they were not stained by eosin. Mills et al. (1925) and Garcia (1935), working with chlorine disinfection of *E. histolytica* cysts, used plasmolysis as a criterion for cyst mortality. Stone (1937) used microscopic observation and eosin staining in addition to cultural methods for evaluating cyst viability in his studies. He noted, as had Penfold et al. in 1916, that cysts which showed distinct nuclei after treatment with chlorine were dead. The fact that cysts with distinct nuclei stained only very lightly with eosin suggests that the nuclear morphology criterion was more indicative of cyst mortality than was the eosin-staining criterion.

Cyst viability was assayed most frequently by inoculating the cysts into a medium suitable for growing *E. histolytica*. Essential bacteria were added frequently, and the culture was incubated at 37 C before being examined for the presence of trophozoites. This method was limited in that it told only whether or not 100% of the cysts were killed. It did not indicate the percentage of surviving cysts unless a most-probable number technique (described below) was used. Investigators have introduced variations into this macroculture procedure. The varieties of media which have been used include liver infusion agar medium (Chang and Fair, 1941), Boeck–Drbohlav medium (Stone, 1937), Balamuth medium (Beaver and Deschamps, 1949), Ringer's egg serum medium (Kessel et al., 1944), Locke's egg serum medium (Brady et al., 1943), and blood medium (Penfold et al., 1916). Becker et al. (1946) indicated that a negative culture at 24 hr should be subcultured to insure the detection of a positive culture. He transplanted the sediment from a culture to two fresh tubes of medium. After 48 hr he examined the sediment; and, if it proved to be negative for trophozoites, transfers were made and these were examined 48 to 72 hr later. Wright and Wright (1932) and Beaver and Deschamps (1949) were proponents of subculturing. Chang (1944b) and Stone (1937), in contrast, found that subculturing did not improve the sensitivity of the system for detecting viable cysts. Stone examined a culture for as many as 10 days and Chang for as many as 7 days before conceding that it was negative. The sensitivity of the culture method depended on the number and viability of the cysts inoculated into the culture. Chang and Fair (1941) demonstrated that as few as five cysts could produce a positive culture. Brady et al. (1943) indicated that he could still obtain a positive culture if 99% of the cysts were dead. Chang (1944b) pointed out that when only a few viable cysts were present, a less nutritious medium, such as Boeck–Drbohlav's, would be less likely to give a positive culture than a rich one, such as the liver infusion agar medium. Chang and Kabler (1956) used a most-probable number procedure for calculating cyst survival. Five samples of a known number of cysts were inoculated into separate tubes containing modified liver infusion agar. The cultures were labeled positive if trophozoites were found in them after an incubation period. The most-probable number of viable cysts was then calculated from a formula. Chang stated in a personal communication that the MPN method was time-consuming and tedious, but it was reliable within the statistically permitted range of error.

Penfold et al. (1916) placed 1 part washed cysts in 5 parts nutrient broth and 2 or 3 parts liquor pancreaticus. A drop of the mixture was placed on a slide and covered with a ringed cover slip to prevent evaporation. This microculture was incubated at 37 C for 5 to 7 hr and then microscopically examined for the presence of trophozoites and empty cysts. They concluded that viable cysts had been present when trophozoites and empty cysts were seen. Rees et al. (1949) placed bacteria-free cysts in a micropipette with an excystation

medium. The micropipette was then sealed and incubated for 8 to 10 hr at 37 C. Following this incubation, it was placed in water and microscopically examined for the presence of the cysts and trophozoites. These investigators claimed that since excysted trophozoites would not reproduce in their system, one trophozoite represented one excysted trophozoite.

The unique aspects of the new amebic cyst bioassay system described in this report are that it offers a simple and reliable method for evaluating the percentage of living cysts in a population of amebic cysts, and it makes it possible to correct cyst survival for control cysts.

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