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Enhancement of Circumoval Precipitin Reactivity by Sonication in the Paraformaldehyde-Fixed Eggs of *Schistosoma mansoni* and *S. japonicum*

HARUO KAMIYA[1], KEIKO YAMASHITA[1], SEIKI ISHIGOOKA[1], KENTARO YOSHIMURA[1] AND BAYANI L. BLAS[2]

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Circumoval precipitin (COP) test is now considered to be the serodiagnostic method of choice in schistosomiasis in local, endemic areas, since the test can be easily performed with only minimal equipments. Furthermore, sensitivity and specificity of the COP test are much better than other methods, for instance, ELISA and redioimmunoassay (Hillyer *et al.*, 1979). Although lyophilized eggs have been extensively employed in most laboratories, for the routine COP assay, the preparation of those eggs is not necessarily easy in the local, endemic areas, where there is a great need for the COP test (Kamiya *et al.*, 1980). In view of this, the application of air-dried (Kamiya, 1983) or formalin-fixed eggs (Kamiya and Blas, 1984) to COP test, instead of lyophilized eggs, would provide an alternative method of approach to this problem. On the basis of our previous results, the present study was attempted to examine the usefulness of paraformaldehyde-fixed and then sonicated eggs in COP assay.

Materials and Methods

Egg preparation: Mice used throughout the study were 4 to 5 weeks old males (dd Y strain) and fed pellet food and water *ad libitum*. *Schistosoma japonicum* (Philippine strain) and *S. mansoni* (Puerto Rican strain) eggs were harvested from the livers of mice at 7 and 8 weeks postinfection, respectively. Eggs were collected by a sieving method (Dresden and Payne, 1981), fixed in 2% paraformaldehyde (PFA) in phosphate buffered solution (0.1 M, pH 7.4) for 24 hours and preserved in 0.2% PFA in a refrigerator (4°C) until use. Prior to use, those eggs were washed with several changes of cold phosphate buffered saline soultion (PBS; pH 7.2) for 2 days and treated in PBS using a sonicator (Tomy Co., Tokyo, Model UR-200P).

Serum samples: Twenty serum samples from schistosomiasis japonica patients in Leyte, Philippines, all proven by stool examination and/or COP test using lyophilized eggs, were employed. Fifty human sera from normal Japanese volunteers were used as controls. Six serum pools (Pool nos. 1 to 6) were obtained from mice 10 to 13 weeks after infection with *S. mansoni*; serum pool nos. 1 to 4 were collected from 10 infected mice each and the remaining two pools from 5 mice each. For each infected mouse group used for obtaining the serum pools above, the mean number of paired flukes recovered was determined. Ten serum samples were also obtained from age and sex matched mice and used as controls.

COP test: The test was carried out as described elsewhere (Yokogawa *et al.*, 1967;

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[1] *Department of Parasitology, Akita University School of Medicine, Akita 010, Japan*; [2] *Schistosomiasis Control and Research Center, Palo, Leyte 7118, Philippines.*

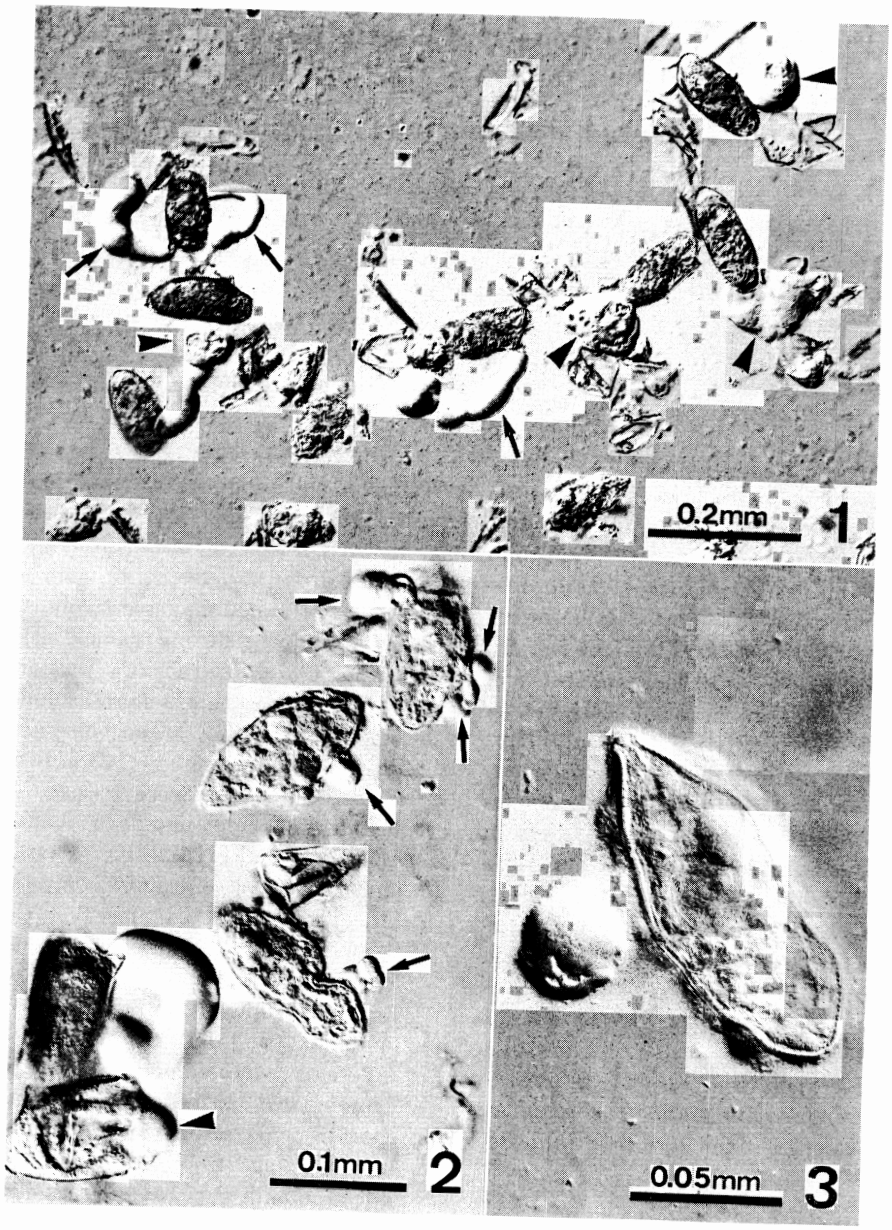

Fig. 1 Long (arrows) and large bleb-like COP reaction products (arrowheads) on sonicated *Schistosoma mansoni* eggs at the portions of eggshell cleavages. Note many empty eggshells as well. Differential interference microphotograph (DIM).
Fig. 2 COP reaction products at the portion of eggshell cleavage (arrowhead) and bleb-like products (arrows) on free miracidia released from eggs of *S. mansoni*. DIM.
Fig. 3 High magnification of a large precipitin product on a free micacidium of *S. mansoni*. DIM.

Table 1 Circumoval precipitin (COP) reaction of paraformaldehyde (PFA)-fixed *Schistosoma mansoni* eggs with or without sonication treatment

Serum pool no. from mouse groups*	Mean no. of paired flukes recovered	% COP positive eggs†	
		Not sonicated	Sonicated‡
1	3.8	1.0	41.4
2	12.9	2.4	44.7
3	23.0	1.8	43.1
4	33.7	1.3	52.0
5	42.4	1.6	49.2
6	65.2	1.8	51.4
Sera of 10 normal ddY mice		0	0

* Infected ddY mice serum.
† Eggs were preserved in 0.2 % PFA for 3 months in a refrigerator (4°C).
‡ Sonicated with 115 watts for 1.5 min.

Table 2 COP reaction of paraformaldehyde-fixed *S. japonicum* eggs, with or without sonication treatment, against sera from schistosomiasis japonica patients

Eggs used in the assay	No. of patient sera	Mean percentage of COP positive egg ± SD (range)
Lyophilized*	20	20.5± 8.8(8.0-40.9)
Sonicated-I†	20	6.2± 3.2(1.4-11.6)§
Sonicated-II‡	20	22.2±10.2(7.0-39.6)

* Lyophilized eggs were harvested from the livers of infected mice and preserved in a refrigerator (4°C) for 4 years.
† Eggs sonicated with 115 watts for 3 min.
‡ Eggs sonidated with 115 watts for 1.5 min.
§ This value significantly differed from those of the lyophilized and sonicated-II groups at the p value less than 0.001, as assessed by Student's "t" test.

Yogore *et al.*, 1968), and mean % of COP positive eggs was calculated from the data of duplicate tests.

Results

Schistosomiasis mansoni

Eggs preserved in 0.2 % PFA for 3 months were sonicated with 115 watts for 1.5 min. Following this treatment, some eggs were found ruptured and eggshells alone, without miracidia, were frequently recognized. COP assays revealed the production of long precipitates and/or big bleb-like precipitates on the sonicated eggs (Figs. 1, 2). COP products were often seen at the portion of eggshell cleavage. Interestingly, COP product was also found on the surface of miracidium free from eggshell (Figs. 2, 3). In contrast to the extremely low reactivities of non-sonicated eggs, sonicated ones exhibited higher reactivities (Table 1). It is of interest to note, however, that there is no direct relationship between the intensity of infection, as assessed by fluke yields, and % of COP positive eggs (Table 1). No positive COP reactions could be found in any of 10 normal mouse sera. Additionally, it was also confirmed that sonicated eggs previously preserved in 5 % for-

malin solution for much longer period, e. g., 8 months, showed similar COP reactivity to that observed in those eggs fixed in PFA (data not shown). It is also worth mentioning that the preservation of assay preparations in a refrigerator (4°C) for one month following their routine incubation at 37°C for 48 hours markedly increased the percentage of the COP positive eggs (data not shown).

Schistosomiasis japonica

Morphological characteristics of COP reaction products on sonicated *S. japonicum* eggs were identical with those on corresponding *S. mansoni* eggs. *S. japonicum* eggs, sonicated with 115 watts for 1.5 min, yielded high COP reactivity which is almost equivalent to that of lyophilized eggs (Table 2). Eggs sonicated for 3 min, however, exhibited significantly lower COP reactivity than those sonicated for 1.5 min ; this is probably due to the fact that a considerable number of eggs were found ruptured in the former group. These data suggest that the magnitude of sonication markedly affects the COP reactivity of PFA-fixed eggs. To the contrary, PFA-fixed eggs without sonication showed extremely low COP reactivity (% of positive eggs=around 1 % or less). No COP reaction was, of course, detected in any of 50 normal human sera.

Discussion

Our previous studies (Kamiya, 1981 ; Kamiya *et al.*, 1981, 1982) have indicated that formalin-fixed egg-sections could be employed as antigens to indirect fluorescent antibody test, indirect immunoperoxidase test and intraoval precipitin reactions. These results have strongly suggested the presence of heat stable intraoval antigen (s), possibly associated with the above-mentioned immunological reactions. Although these results suggest us to use such formalin-fixed eggs in COP assay for the diagnosis of human schistosomiasis japonica, those eggs have been shown to exhibit unexpectedly low reactivity (Kamiya and Blas, 1984), as Yogore *et al.* (1968) reported previously. Our recent study (Kamiya and Blas, 1984) has indicated that less COP reactivity of the formalin-fixed eggs was probably attributable to the nature of eggshell structure ; the leakage of antigenic substances, localized between vitelline membrane and miracidium, could have been blocked in formalin-fixed eggs by some unknown mechanism, since it is well known that antigenic substances responsible for COP reaction leak through the micro-pores of the eggshell (Race *et al.*, 1971 ; Sakumoto *et al.*, 1972 ; Ford *et al.*, 1980 ; Demaree and Hillyer, 1981). The function of micro-pores might have been, therefore, damaged due to formalin or PFA fixation of eggshell, since the eggshell has been shown to be composed of the protein (Byram and Senft, 1979). The current study clearly indicates that sonicating processes for making artificial eggshell cleavages distinctly enhance the COP reactivity of PFA-fixed *S. mansoni* and *S. japonicum* eggs (Tables 1 and 2). Frequent occurrence of COP reaction products at the portion of eggshell cleavages as well as at the surface of free miracidium (Figs. 1-3) also support this notion.

It is also likely that the present COP test with sonicated eggs has only qualitative significance in the diagnosis of schistosomiasis, since no direct relationship could be noted between the intensity of infection and percentage of COP positive eggs, as shown in murine schistosomiasis mansoni (Table 1). Despite the presence of numerous advantages, COP test has been shown to have an essential defect in this aspect of evaluation, although many attempts have been made to quantitate the assay (Bruijning, 1964 ; Tanaka, 1976 ; Yogore *et al.*, 1978 ; Hillyer *et al.*, 1979 ; Ruiz Tiben *et al.*, 1979).

Usefulness of PFA-fixed and sonicated eggs in COP assay would provide a partial solution for egg supply, instead of fresh eggs or lyophilized eggs, for the diagnosis of schistosomiasis in local, endemic areas. These eggs would also contribute to the standardization of COP test procedure as attempted by Garcia *et al.* (1981), since egg antigen(s) associated with the test might be adversely affected by purification or lyophilization processes.

Summary

Paraformaldehyde (PFA)-fixed and sonicated eggs of *Schistosoma mansoni* and *S. japonicum* were assessed for their usefulness in COP assay for the diagnosis of schistosomiasis. Although PFA-fixed eggs exhibited extremely low COP reactivity, their sonicated eggs showed high COP reactivity almost equivalent to that of lyophilized eggs. Sonication affects the nature of eggshell and provide more or less shell-cleavage. Through these artificial cleavages, antigenic substance(s) involved in COP reaction would leak out from the eggs. Possible usefulness of PFA-fixed eggs in COP assay in local endemic areas was discussed.

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超音波処理虫卵を用いた住血吸虫卵周囲沈降反応

神谷晴夫[1] 山下恵子[1] 石郷岡清基[1] 吉村堅太郎[1] BAYANI L. BLAS[2]
([1]秋田大学医学部寄生虫学教室[2] *Schistosomiasis Control and Research Center, Palo, Leyte 7118, Philippines*)

住血吸虫症の流行地の現場において，その手技の簡便さに加え，鋭敏性と特異性の高さから，卵周囲沈降反応 (Circumoval Precipitin Test ; COP) の診断的価値は大きい．しかしながら，通常，診断に用いられる凍結乾燥虫卵は，流行地において十分に供給されているとはいえない．この観点から，日本住血吸虫およびマンソン住血吸虫のパラホルムアルデヒド (PFA) 固定虫卵の COP への応用を検討した．無処理の固定虫卵の反応性は極めて低かったが，超音波処理によって，両種の虫卵の COP 反応性は，凍結乾燥虫卵を用いた場合と同程度に，著しく回復した．また，卵周囲沈降物の形成は超音波処理虫卵の卵殻破損部位にしばしば認められた．したがって，この反応性の回復は，超音波処理により人工的に作出された破損部より，卵内の，卵黄膜 (vitelline membrane) とミラシジウムの間に存在する，COP に関与する抗原が流出することに起因することが示唆された．この事実は，卵殻より遊離したミラシジウムの体表に同様の沈降物が形成されることによつても明らかであった．さらに，今回の COP に関与する抗原の反応性は PFA 固定液中で長期間保持され，安定性の高い抗原であることが示唆された．このような固定虫卵を卵周囲沈降反応へ応用することは，流行地での虫卵抗原の供給に寄与するものと考えられる．