

ATTEMPTS TO STANDARDIZE THE CIRCUMOVAL PRECIPITIN TEST (COPT) FOR SCHITOSOMIASIS JAPONICA

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INTRODUCTION

The circumoval precipitin (COP) test (COPT) demonstrates the formation of precipitates associated with schistosome ova after incubation in patient's sera (Oliver-Gonzales, 1954). It has been proven to be a highly sensitive and specific immunodiagnostic technique in schistosomiasis japonica (Garcia *et al.*, 1968; Yogore *et al.*, 1968; Tanaka *et al.*, 1975; Tanaka, 1976) and is currently regarded as the method of choice for this infection in the Philippines. In our experience (Garcia *et al.*, 1968), the test was positive in 94.3% of cases with parasitologic evidence of infection; that of Yogore *et al.*, (1968) was 94.6% while Tanaka (1976) reported the test to be positive in 97.3% of *S. japonicum* egg passers. The very high specificity is evidenced by the fact that we obtained only 0.5% positive reactions among non-exposed individuals while Yogore *et al.*, (1968) and Tanaka *et al.*, (1975) did not report any positives among individuals from non-endemic areas. Present evidence indicates that there is little relevant to the diagnosis of schistosomiasis which is added by the use of more complicated techniques such as radioimmunoassays (Tapales *et al.*, 1981; Hillyer *et al.*, 1979) or enzyme-linked immunosorbent assays (Hillyer *et al.*, 1979) using isolated egg antigen mixtures and tagged anti-human immunoglobulins.

In addition to its high sensitivity and specificity, the COPT possesses a number of

features which make it a very practical test. It is very easy to perform and requires only minimal equipment. In addition, lyophilized eggs used as a source of antigens for precipitin detection in sera are very stable and readily available (Smithers, 1960; Rivera da Sala *et al.*, 1962). The visual readout which is a characteristic of this test provides for ready interpretation and evaluation of the data. The practice of collecting blood from finger pricks on filter paper, or in heparinized capillary pipettes, has made this test particularly suitable for mass surveys and field work (Cabrera *et al.*, 1968; Yogore *et al.*, 1980).

In spite of the above observations, the test is often criticized as being "not standardized". It is a fact that eggs used as a source of antigens are recovered from animals with different ages of infection, that no constant quantity of eggs is used in the assay, and that there is a lack of agreement amongst workers on the significance of small bleb-like precipitates. Other variables such as period of incubation of eggs with sera and temperature of incubation have not been examined systematically. The study reported in this paper is an attempt to standardize various aspects of the COPT by determining at what age of infection should donor animals be sacrificed for recovery of eggs, the effect of maturity and/or viability of eggs on COPT, the effect of different quanta of eggs used as reactants for the test on the size of precipitates and proportion of reacting eggs, the determination of loss of sensitivity when the

reactants are incubated at room temperature, rate of formation of precipitates during a 48-hour incubation period, and whether bleb-like precipitates (as distinct from segmented precipitates) are the function of the test serum or the eggs used (? degree of maturity and viability).

MATERIALS AND METHODS

Schistosoma japonicum eggs

Rabbits depending on their sizes, were given 300 to 500 cercariae of *S. japonicum* originally obtained from Mindoro or Leyte and produced from *Oncomelania hupensis quadrasi* snails maintained in aerated "aquaterraria". Rabbits were killed at 5-day intervals from the 40th to 75th day of infection for recovery of eggs. This regime was used on the assumption that at some point of time (i.e. duration of infection) there would be an optimal proportion of mature eggs in the liver. Eggs were obtained by digestion of macerated livers in pepsin and trypsin (Smithers, 1960) and lyophilized (Rivera da Sala *et al.*, 1962). They were stored in a dessicator at room temperature, until used in COPT.

Sera

Sera were obtained from patients presenting at the Department of Parasitology, Institute of Public Health and were stored at -20°C until used. Fecal egg counts were performed using the modified Kato-Katz technique (Katz *et al.*, 1972). When eggs were not demonstrable in the Kato-Katz smear, merthiolate-iodine formalin concentration (MIFC) (Blagg *et al.*, 1955) of the feces was done.

Circumoval precipitin (COP) test (COPT)

Two drops of sera were added to lyophilized *S. japonicum* eggs on a glass slide, covered with a cover slip and sealed using paraffin. Slides were incubated at 37°C or at room

temperature (about 28°C) usually for 24 to 48 hour, as indicated in the Results section. The presence of blebs or segmented precipitates was determined using a bench microscope and sizes of precipitates measure in some experiments using a graduated eye piece.

Statistical analyses

Data were expressed as the 95% confidence interval of the mean (arithmetic mean \pm 1.96 x standard error). Statistical analysis of the data was performed using a Student's t test and a P value of 0.05 chosen as the limit of statistical significance when comparing differences between groups.

RESULTS

Influence of age of infection in donor rabbits on COPT

Infected rabbits used as donors of livers for egg isolation were killed at 5-day intervals between 40 and 75 days of infection. The 8 batches of lyophilized eggs were incubated with 2 drops of sera at 37°C for 24 and 48 hours using 50 different sera from patients with parasitological evidences of schistosomiasis japonica. It can be seen in Fig. 1 that eggs harvested at 55 or 60 days of infection were superior to all other batches of eggs in the COPT. Eggs taken earlier or later than two time points resulted in a lower proportion of positive reactions (i.e. precipitates) associated with the eggs. Results using a 24 hour COPT and a 48 hours COPT were very similar for each egg batch.

In the same experiment, the ratio of segmented precipitates to blebs was determined at 24 and 48 hours using the 8 batches of eggs and the 50 human sera. Results are presented in Fig. 2. With lyophilized eggs from 40 to 60 day infected rabbits livers, segmented precipitates predominated over

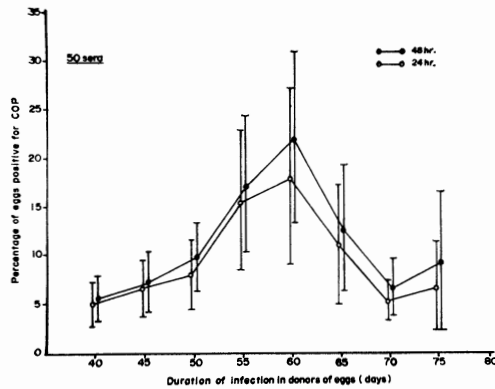


Fig. 1—Effect of duration of infection in rabbits (day 40 to 75) used as donors of eggs for use in the COPT as measured by the percentage of eggs which are positive for a COP reaction. Each of the 8 batches of eggs was incubated for 24 (open symbols) or 48 hour (closed symbols) at 37°C with 2 drops of sera from 50 patients with schistosomiasis japonica (i.e. positive for eggs in feces and COPT positive). Arithmetic means and 95% confidence interval are indicated, approximately 500 eggs being used for each determination.

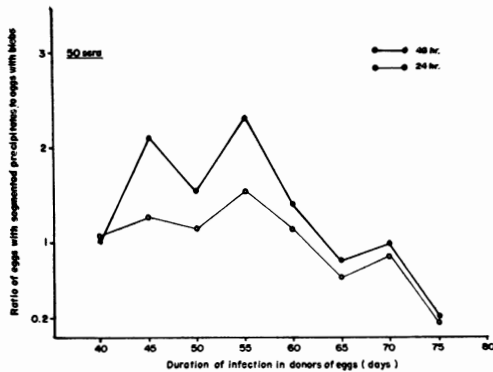


Fig. 2—Effect of duration of infection in rabbits (day 40 to 75) used as donors of eggs for use in the COPT as measured by the ratio of eggs with segmented precipitates to eggs with blebs. Each of the 8 batches of eggs was incubated for 24 (open symbols) or 48 hours (closed symbols) at 37°C with 2 drops of sera from 50 patients with schistosomiasis japonica (see Fig. 1).

blebs especially in the 48 hour COPT. However, the frequency of segmented precipitates was lower than that of blebs using eggs from rabbits infected for greater than 60 days and particularly so at the 75 day point. The results in Figs. 1 and 2 raise the possibility

that immaturity is a cause of a low percentage of positive eggs in the COPT and that dead and dying eggs also result in a low percentage of positive eggs as well as a high percentage of blebs (rather than segmented precipitates). At late time points of infection, these dead and dying eggs presumably dilute out those eggs which are at an optimal stage of maturity for the COPT. It is not known whether maturation (embryonation) of eggs is also inhibited in long-term infected hosts (see Discussion).

Influence of maturity and/or viability of eggs on COPT

The results presented in Fig. 1 suggest that a period of egg maturation in tissues (? development of a miracidium) is required before an optimal COP reaction is obtained with sera. If this is so then it is predicted that unembryonated eggs obtained from the uteri of *S. japonicum* female worms or dead or dying ova should be less suitable for the test than eggs obtained from the tissues of judiciously-chosen infected hosts. The notion that immature eggs are not suitable (for the COPT) was tested by using fresh eggs dissected from worms harvested from 47 to 62 day infected mice and 5 sera of known high COP reactivity. Reactions over a 24 and 48 hour incubation period were compared with those obtained from fresh eggs harvested from the macerated, enzyme-treated livers of 47 to 62 day infected mice and lyophilized eggs from 60 day infected rabbit liver. The data in Table 1 indicate that freshly collected, unembryonated, immature uterine eggs of *S. japonicum* are totally unsatisfactory for the COPT with blebs only as the resulting precipitate.

Influence of number of incubated eggs on COPT

It might be anticipated that, if antibodies are limited, an excessively high number of eggs used in the COPT may result in a

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Table 1

24 hour COPT results using 5 sera from patients with schistosomiasis japonica and *S. japonicum* eggs of 3 sources*

Type of Egg	Percentage of eggs positive for COP reactions using serum Nos:—				
	1153	1136	1139	1060	1167
Fresh uterine	0	0	1.6 ⁺	1.8 ⁺	3.0 ⁺
Fresh mouse liver (days 47-62)	22.5	26.5	32.7	35.2	31.6
Lyophilized from rabbit liver (day 60)	25.0	24.3	31.4	25.3	11.5

*Number of eggs per slide varied from 13 to 100 with a mean of 56.

+Small blebs only.

reduction in the proportion ultimately positive or in the size of precipitate associated with each egg. In Fig. 3, results are presented on the effects of incubating (at 37°C) samples of approximately 40, 80, 160 and 320 eggs from a single batch with 2 drops of sera from 22 patients with schistosomiasis japonica. The "sensitivity" of the test was greater when using 50 eggs in that a higher mean percentage of eggs was positive but variation was also considerable. Sizes of precipitates did not vary significantly between the 4 groups (Fig. 3). In order to conserve a particular batch of eggs to be used in a standardized test, and to ensure that several eggs are seen to be positive when the proportion positive may only be 10%, approximately 100 eggs for 2 drops of serum is sufficient for the COPT.

Influence of duration of incubation on COPT

In Fig. 4 is depicted the time course of appearance of COP reactions using 1 batch of eggs and 4 representative sera (out of 12 tested) of high, intermediate or low COP reactivity. It can be seen that a reaction was well advanced at 1 hour using sera of high "titer" (i.e. high ultimate proportion of positive eggs in the COPT) but with other

sera, strong positivity developed later (longer than 6 hour). Differences in the proportion of eggs which are ultimately positive using different sera presumably reflect differences in amount of COP antibodies whereas differences in the rate of appearance of precipitates presumably reflect both amount and avidity of COP antibodies in sera. It is also conceivable that antibodies or other factors in some sera cause the accelerated export of COP antigens from the egg.

Other sera were reacted with a single batch of eggs and the development of a precipitate associated with a single egg on the slide measured from 6 min to 48 hour (at room temperature, approximately 28°C). Again rate of appearance of precipitates varied between sera and the ultimate size of the precipitates also varied widely (Fig. 5). With one of the sera, a large precipitate was clearly apparent at 6 minutes and the size of the precipitate increased over the following 24 hours. With 2 other sera, the precipitates only increased markedly in size between 6 and 24 hours; with a further 2 sera, the size of the precipitates reached a reasonable size (10-20 microns) only after 24 hours incubation (Fig. 5). Using 2 of the sera of high COP

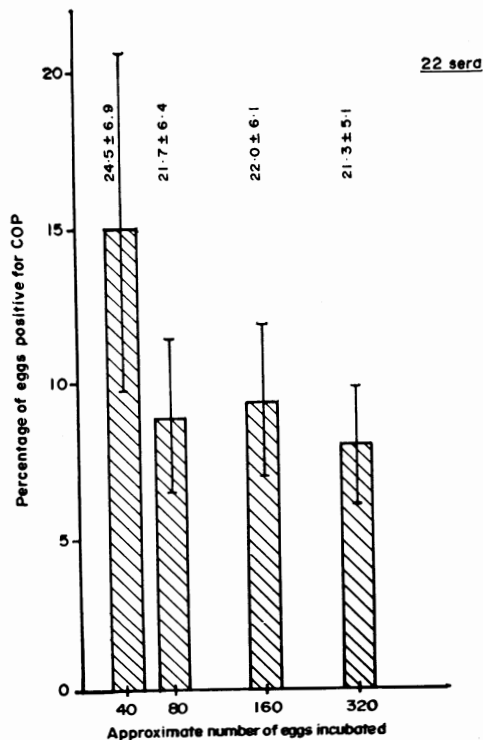


Fig. 3—Effect of incubation of various numbers of eggs harvested from 60-day infected rabbits and reacted with 2 drops of sera from 22 schistosomiasis japonica patients for 24 hours at 37°C. The actual mean number of eggs used in each of the 4 categories was determined to be 35 ± 4 , 79 ± 6 , 155 ± 12 and 315 ± 23 and the arithmetic mean of the percentage of eggs positive for a COP reaction and the 95% confidence intervals are indicated by the histograms and bars. Values for 50 and 300 eggs are significantly different. For each egg preparation and each serum, an arithmetic mean size of precipitate (in microns) was determined and the mean of this figure for the 22 sera (and the 95% confidence interval), is indicated above each histogram.

reactivity, a difference in the size and rate of development of precipitates was noticed when single eggs in two batches of eggs (from 55 and 60 day infected rabbits) were followed (but not when the 3 sera of lower COP reactivity were used). These results further emphasize the importance of the egg source in the COPT.

Influence of incubation at room temperature versus 37°C

The data presented in Fig. 6 indicate that incubation at room temperature does not result in any loss of sensitivity of the COPT. This makes it particularly suitable for field stations in endemic areas where a 37°C incubator may not be available.

Results of COPT using sera from patients coming for diagnosis of schistosomiasis: Data collected from patients presenting at the Department of Parasitology, IPH, during 1979-80 which had not been drug treated and from which both fecal egg counts and COPT results are available, are presented in Table 2. No evidence of schistosomiasis japonica was found in 42% of patients presenting. No patient was found with eggs in feces but whose serum was negative in the COPT (i.e. the false negative rate of the serological test, considering any reaction with the eggs as positive, is zero). 44% of COPT positive patients were stool negative according to the results of a single determination; if COPT positivity indicates infection then this result is in line with the known unreliability of a single or even 3 stool examinations particularly in chronic infection (e.g. Garcia *et al.*, 1968). In this COPT positive/stool negative subgroup, approximately 90% of individuals were from known endemic areas and this figure applied to the 38 small bleb COPT reactors (see footnote to Table 2). The proportion of small bleb reactors in the COPT positive/stool negative group is greater than that in the COPT positive/stool positive group (33% versus 4%). Thus, small bleb reactions presumably reflect low COP antibody titers in patients, many of whom may have low worm burdens. However, it is also possible that some are false positive reactions. If the false negative rate using 3 stool examinations for schistosomiasis japonica is as high as 67% in chronic infections (Garcia *et al.*, 1968),

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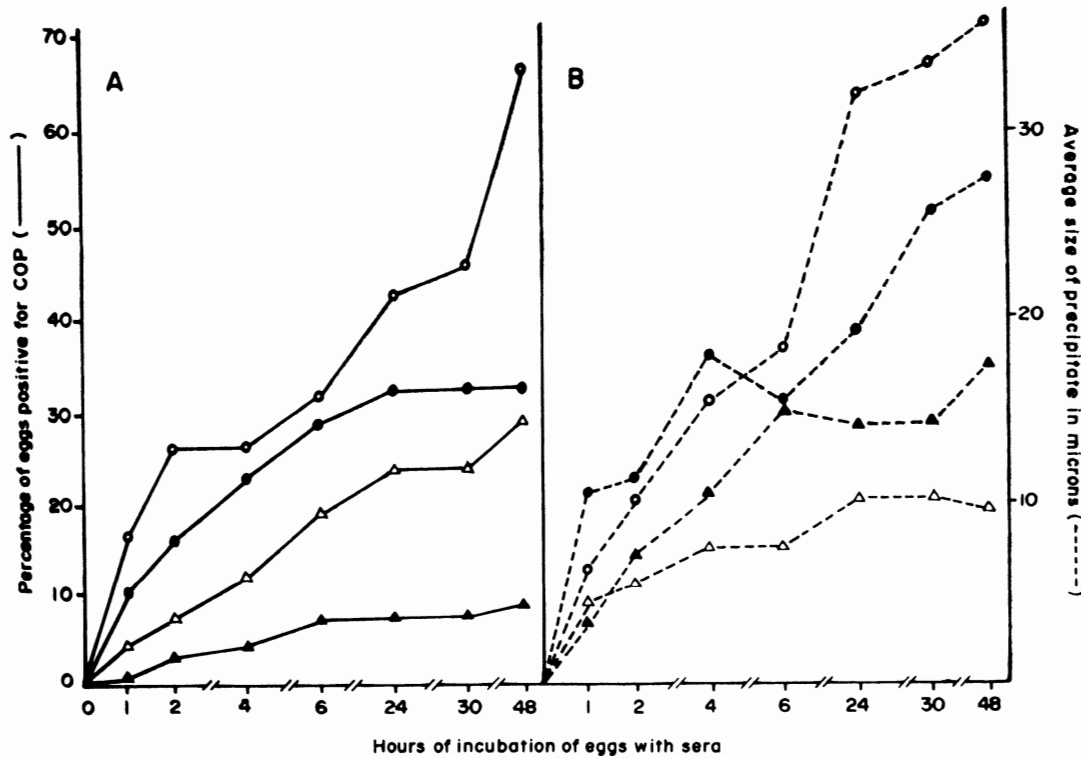


Fig. 4—Effect of duration of incubation (from 1 to 48 hours) on the percentage of eggs positive for precipitation (solid lines, panel A) and the size of the precipitates measured in microns (broken lines, panel B) using 4 sera from patients with schistosomiasis japonica (o, serum No. 269, ●, serum No. 282; △, serum No. 412, ▲, serum No. 414). Sera were incubated at 37°C with approximately 70 eggs obtained from livers of 60 day infected rabbits. The data with these 4 sera represent the spectrum seen with 12 sera. Fecal egg counts in donors of sera 269, 282 and 412 were 46, 161 and 84 eggs, respectively, and ages were 14, 16 and 32 years.

and if false positives are only contained in the small bleb COPT reactor group, then the absolute maximum false positive rate in stool negative/small bleb COPT reactors, relative to all COPT positives, is 10%. In the unequivocally infected patients (i.e. stool positive), only 4% (6/147) showed small bleb COPT reactions. Five of these 6 sera were still available in early 1981 for retesting with eggs optimized for detection of COPT reactions (see above). Four of these 5 sera were again small bleb reactors, the remaining serum showing an unequivocal positive reaction (i.e. some segmented precipitates). Data in Table 3 indicate that no strong correlation exists between segmented precipitates in the COPT and presence of severe disease (splenomegaly and hepatomegaly) in 95 patients.

DISCUSSION

At the individual patient level in the laboratory, and for seroepidemiological purposes, the utility of the COPT for the immunodiagnosis of schistosomiasis japonica and mansoni has been demonstrated on numerous occasions (see Introduction). However, there is no doubt that the test suffers from variability and a lack of standardization such that results obtained by different workers are difficult to compare. The experiments present-

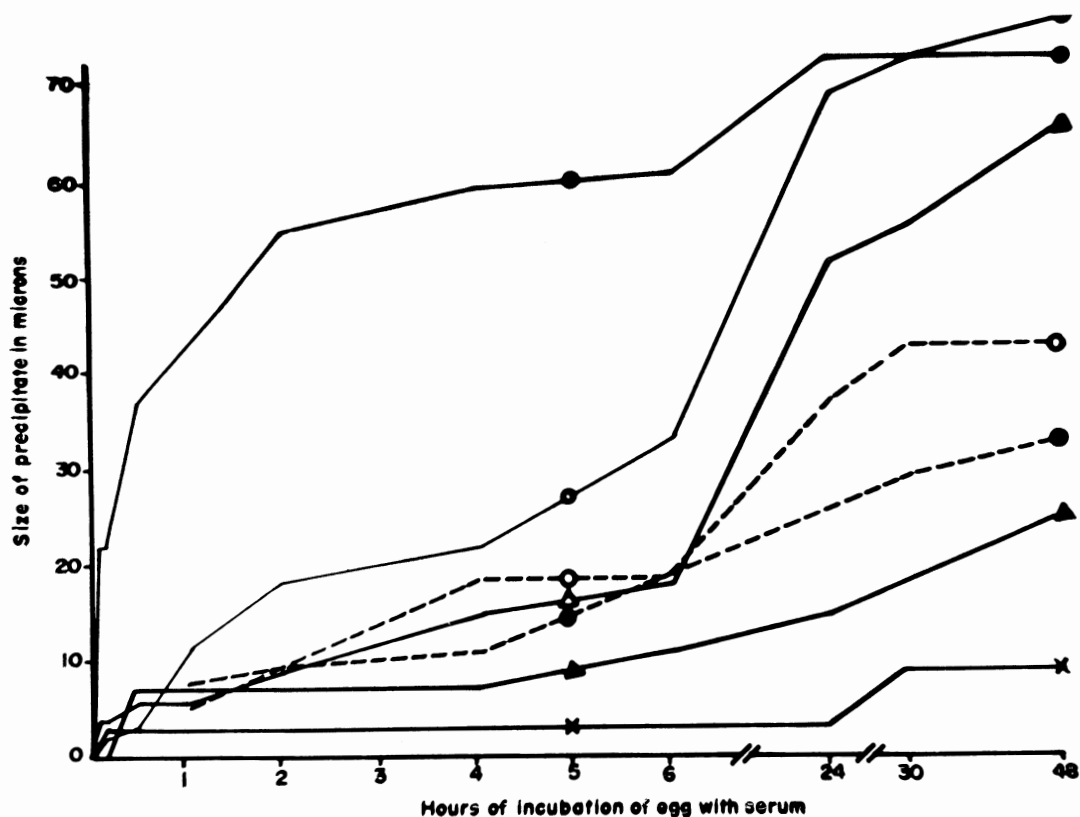


Fig. 5—Effect of duration of incubation (from 6 minutes to 48 hours) on the size of precipitates associated with one egg in approximately 50 incubated at room temperature with 2 drops of sera from 5 patients with schistosomiasis japonica (●, serum No. 46, ○, serum No.80; △, serum No.45, ▲ serum No. 90; X, serum No. 78). Eggs were obtained from livers of 60-day infected (solid lines) or 55-day infected rabbits (broken lines); two of the sera (● and ○) showed a difference in size of precipitates which developed in association with the two eggs under observation (●—● versus ● . . . ● and ○—○ versus ○ . . . ○) whereas precipitate sizes using the other 3 sera (△, ▲ and X) were identical following incubation with the two sources of eggs (data not shown). In these experiments, an egg which displayed a reaction at the earliest time point was chosen for subsequent observation. Fecal egg counts in donor of sera 46 and 45 were 483 and 1,173 egg, respectively and ages were 12 and 16 years. The donor of serum 80 was also a teenager whereas serum donors 90 and 78 were 33 and 29 years old, respectively.

ted in this paper were designed to provide optimization data for this simple, inexpensive test of high sensitivity and specificity and to supplement data previously reported from this laboratory (Garcia *et al.*, 1968; Garcia, 1975).

It is worth emphasizing that the popular term, "circumoval precipitin" (COP) and the test to detect the presence of such precipitating antibodies in serum (i.e. the COPT),

is unfortunate. "Circumoval" provides a misleading impression of the visible precipitates formed in association with the egg. The antigen-immunoglobulin aggregate is localized and often appears as an appendage, which is usually segmented when fully formed, rather than as a precipitate around the egg (Oliver-Gonzales, 1954).

Results obtained in the experiments reported indicate that a 24 or 48 hour incubation

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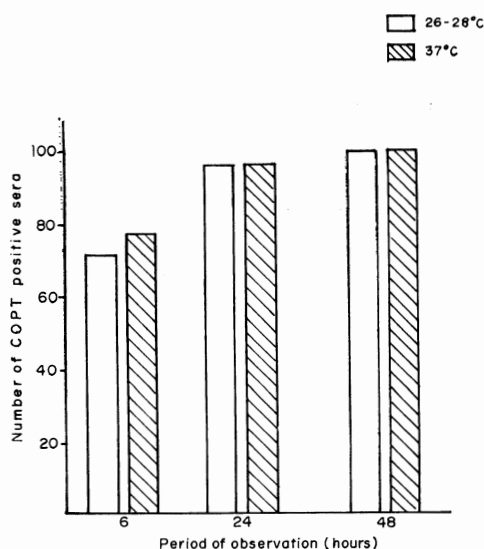


Fig. 6—Effect of incubation temperature (room temperature 26-28°C and 37°C) on the sensitivity of COPT as measured by the number of COPT positive sera at different observation periods. The COPT slides were prepared using 2 drops of previously tested positive sera of 100 patients.

period at 37°C with approximately 100 eggs from 55-60 day infected rabbits and 2 drops of serum in a sealed chamber will provide maximum sensitivity. Unequivocally positive reactions involving segmented precipitates in association with a relatively high proportion of eggs are obtained using such conditions. Data in Fig. 1 indicate that eggs harvested from livers of infected rabbits at time points earlier or later than the 55th to 60th day of infection are less suitable for performance of the COPT. Differences presumably reflect immaturity of eggs at early time points (and it was shown that fresh uterine eggs are most unsatisfactory) and loss of viability at later time points. There is the additional possibility that embryonation of eggs is retarded or embryonated eggs are destroyed more rapidly in long-term infected animals. The available data does not differentiate between this and a simple dilution of eggs (at an optimal stage of maturity for COPT) by

Table 2

Summary of results using the COPT on sera from patients presenting at IPH and for which fecal *S. japonicum* egg counts are available.

COPT results		Fecal <i>S. japonicum</i> eggs*	No. of individuals	Percentage of unequivocally infected individuals
+/-	Size of precipitates			
+	Seg + blebs†	+	130	96
+	Larger blebs	+	11	
+	Small blebs	+	6	4
+	Small blebs	-	38‡	
+	Larger blebs	-	12‡	
+	Seg + blebs	-	65‡	
-		+	0	0
-		-	190	
Total =			452	

*Single determination.

†Seg = segmented precipitate associated with egg (i.e. strong positive reaction); small blebs are reactions which amount to less than 1/8th the diameter of the egg (i.e. less than approximately 5 microns).

‡Of the 38, 12 and 65 patients in the COPT positive/stool negative group, 89.2, 91.7 and 93.7%, respectively, were from known endemic areas for schistosomiasis japonica.

Table 3

Information on splenomegaly (S) and hepatomegaly (H) in 95 patients with COPT positive sera.

Appearance of precipitate in COPT	Fecal <i>S. japonicum</i> eggs*	Number of patients in disease categories:			
		S + H +	S - H -	S + H -	S - H +
Seg + blebs†	+	27	14	8	7
	-	6	8	4	0
Larger blebs	+	2	2	1	2
	-	1	1	2	1
Small blebs	+	2	1	2	0
	-	1	2	1	0

* † See Table 2 for footnotes.

accumulated dead and dying eggs in the tissues of more chronically infected donors. The possibility that accelerated loss of egg viability, or reduced efficiency of embryonation, leads to reduced granuloma size in long-term infected hosts is the subject of current experiments in mice. If such influences on eggs are demonstrable then this would provide an alternative explanation to the modulation of granuloma formation ("endogenous desensitization") seen in chronically-infected mice (Domingo and Warren, 1968; Warren, 1977). Accelerated destruction of eggs or compromised egg development (which prevent production of adequate amounts of antigen for large granuloma formation) could be mediated by several immunological mechanisms including eosinophil plus antibody-mediated anti egg effects (James and Colley, 1977). It is of some interest that a mouse strain which is a poor COP antibody producer (i.e. CBA/H) is also a poor lung granuloma producer following injection of *S. japonicum* eggs into egg-sensitized mice (Mitchell *et al.*, 1981a). The CBA/H may be a genetic low responder to egg antigens responsible for COP reactions and granuloma formation, or these mice may be capable of destroying eggs more rapidly than other mouse strains.

Three observations are pertinent to possible reasons underlying the formation of segmented precipitates versus blebs: 1) the proportion of segmented precipitates (versus blebs) decreased markedly when eggs from long-term infected rabbits (versus short-term infected rabbits) were used in the COPT, 2) only blebs were formed on incubation of freshly recovered uterine eggs with highly reactive sera, and 3) the incubation of highly optimized eggs (from 60 day infected rabbits) with monoclonal anti-egg hybridoma antibody also resulted only in bleb formation (Cruise *et al.*, 1981). A bleb probably results from the reaction of one specific antibody against a particular antigen. This partly explains the formation of blebs only with immature uterine eggs or aged or dead eggs and also why sera from recently infected rabbits (Garcia, 1975) gave only the bleb reaction. In early infection, the host is not yet primed against the majority of the antigenic components of the mature egg while immature or dead eggs are able to export only a limited number of antigens for COP. As a corollary to this, *segmented* precipitates are formed with sera from fully sensitized hosts in association with mature or fully embryonated eggs since such eggs are able to export from

localized sites (micropores) a larger number of antigens, responsible for the COP, in greater quantity. Thus, each segment of a segmented precipitate may be a separate antigen-antibody reaction.

Taken together, the data in this series of experiments involving a large number of human sera, indicate that the COPT does suffer from variability but that this variability can be reduced using standardized conditions of incubation and, in particular, a selected batch of eggs from infected rabbits. Recent observations by Hillyer *et al.*, (1979) in schistosomiasis mansoni, and Tapales *et al.*, (1981) in schistosomiasis japonica, strongly suggest that the more sensitive, quantitative and expensive assays such as the radioimmunoassay using egg antigens do not provide anymore useful data than that already available from the simple, inexpensive and standardized COPT. Nevertheless, it is unlikely that the COP test will provide reliable information on presumed infection levels in patients although it is a general observation that teenagers have high anti-egg antibody responses or COPT scores or titers (Tapales *et al.*, 1981 and Figs. 4 and 5) and presumed worm burdens are high in this age group (Pesigan *et al.*, 1958). Recent experiments using a hybridoma-derived antibody directed against *S. japonicum* adult worms suggest that development of an immunodiagnostic test, capable of providing information on infection level or disease status, is attainable (Mitchell *et al.*, 1981b; Cruise *et al.*, 1981b).

SUMMARY

The circumoval precipitation test (COPT) is a simple and inexpensive immunodiagnostic test for schistosomiasis japonica which, in the Philippines, has high sensitivity and specificity. Lack of standardization does, however, increase the variability of the test. Parameters

which influence the COPT have been examined using large numbers of sera from known *S. japonicum* infected individuals. In this series of experiments, optimal conditions were determined to be as follows using 2 drops of neat serum and incubation at 37°C in a sealed slide chamber; - approximately 100 eggs from 55 or 60 day infected rabbits for a 24 to 48 hour incubation period. COP reactions (i.e. precipitates associated with eggs) were much less obvious when either immature eggs or eggs obtained from long-term infected rabbits were used. The results emphasize the prime importance of the source of *Schistosoma japonicum* eggs in the performance of a standardized COPT.

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