FIELD STUDIES ON McCOY CELL CULTURES FOR DETECTION OF *CHLAMYDIA TRACHOMATIS*

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INTRODUCTION

The first reports on the use of McCoy cell cultures for the detection of *Chlamydia trachomatis* revealed a potentially useful method for examination of field specimens. *C. trachomatis* was detected in a human eye following a laboratory accident (Magruder *et al.*, 1963), and was recovered from the eyes of experimentally infected monkeys (Gordon *et al.*, 1963) by use of McCoy cell cultures. In both these studies essentially similar results were obtained whether cell cultures or the yolk sac of embryonated eggs were used as the method of recovering the agent, although the cell cultures provided a result in a shorter time. The observations cited were made with laboratory established strains, and it was still uncertain whether the same result would occur with infectious material from naturally occurring trachoma. Others had reported a limited number of isolations in cell culture (Dzhumanbaeva and Bekleshova, 1963; Li *et al.*, 1963) but their methods evidently have not been applied further. This report is based on our experience with five series of conjunctival specimens, totaling 240, from clinically diagnosed or suspected trachoma. Four of the series (Nos. 1, 2, 4, and 5) were derived from school children in Taiwan, and one (No. 3) from Navajo Indian school children in New Mexico. In the first four series non-irradiated cells were used, and in the fifth, irradiated cells. Our objective was to compare directly the sensitivity of yolk sac and cell culture for detection of trachoma infection, but clear conclusions could not be made due to several factors. Nevertheless the series served to demonstrate the feasibility of cell culture for this purpose, and to reveal potential advantages of the cell culture method. The first three series of tests were almost entirely negative and are described only briefly. Series 4 and 5 are described in greater detail. There was a total number of 22 positives in 240 tests. The laboratory work of Series 1, 2, 3, and 5, was accomplished in the Bethesda laboratory, that of Series 4 in the Taipei laboratory.

MATERIALS AND METHODS

Conjunctival Specimens: These were obtained from the surface of the upper tarsal conjunctiva with the eyelid everted, those collected in Taiwan by means of a dry cotton swab which was then placed in the collection medium, and those taken in New Mexico with a small spatula and transfer of the material to the collection medium. In the latter case the eye was first anaesthetized lightly with Ophthaine (Proparacaine HCl) or Tetracaine ophthalmic ointment, N. F.

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The collection media used were either SPG (0.02 M phosphate buffer, pH 7.2, 0.2 M sucrose, 0.005 M glutamate), SP (the same formula with glutamate omitted but 0.5% bovine albumin added, or the cell culture medium (CM : Eagle's minimal medium with 10% horse serum). All were used for freezing and storage of specimens; CM was also used for preservation up to 24 hours without freezing.

Direct Smears : These were obtained with a small spatula, by scraping the tarsal conjunctiva, usually of the other eye than the one furnishing the specimen for culture, and transferring the material directly to a slide. The preparation was air-dired, stained with Giemsa, and examined by oil-immersion lens.

Cell Cultures : Stock cultures of the McCoy cell line were maintained in T-flasks or prescription bottles in Eagle's minimal medium plus 10% horse serum (MEM, HoS) at 37° C. Suspensions of non-irradiated or irradiated cells in appropriate dilutions in the same medium were placed in flat-bottomed, 1.5×12 cm tubes containing 12 mm circular coverslips, to provide confluent cell monolayers 48 hours later. In the case of the irradiated cells, which no longer multiplied, the monolayer was formed by migration, flattening, and increase in size of individual cells.

(a) Irradiation of cell cultures

Stock cell cultures were exposed to 5000 *r* of gamma radiation from a cobalt-60 source in a Theratron Jr. (General Electric Co., Inc.), and for this assistance we are grateful to personnel of the Naval Hospital, National Naval Medical Center. After exposure to irradiation cells were reseeded into culture bottles, allowed to attach, and one or more changes of medium followed in the course of a few days, which served to remove the cells that did not survive the irradiation. A few days later the surviving cells were seeded onto coverslips as described above and used 2-4 days still later. Routinely, an interval of 7 days occurred between irradiation and inoculation.

(b) Inoculation of cell cultures

From each specimen, collected in 2.5 ml, or diluted to that volume with CM, 1.0 ml was taken for inoculation of cell cultures. An equal amount of CM with double strength antibiotics was added and 4 tubes were inoculated with 0.5 ml each. When the specimen had been preserved in SP or SPG the portion for inoculation of cell cultures was diluted five or tenfold with CM (to avoid exposure of the monolayer to 0.2 M sucrose), and equal portions of the total used to inoculate each of the 4 culture tubes. After centrifugation the inoculum was removed and replaced with 0.5 ml of fresh medium containing antibiotics. All culture tubes contained a final concentration of 100 μg ristocetin and 50 μg streptomycin per ml.

At 48 hours the cell cultures were fixed in methyl alcohol and stained for microscopic examination. At first both iodine solution and periodic acid Schiff's stain (PAS) were used for differential staining of the glycogen-containing inclusions. Later, PAS was found to have no advantage over iodine and its use was abandoned. Examination of the stained cell monolayer was at 200X and entire coverslips were examined before a negative report was made. When present, inclusions were counted, and when in minimal numbers their presence and appearance was verified by a second observer. Positive control specimens (chlamydial strains) and negatives (uninoculated medium) were inserted blindly into the series. In all instances the results of these were as expected.

Embryonated Eggs : Another 1.0 ml portion of the specimen was diluted to 2 ml

and used for inoculation of four eggs, 0.5 ml each. Thus, in every instance, each of 4 tubes and each of 4 yolk sacs received the equivalent of 0.25 ml of the original specimen. At the time of inoculation each egg received 2 mg each of ristocetin and streptomycin either mixed with the specimen or given separately via the air sac (Bloom and Gordon, 1955). It became routine to give another dose of 1 mg of each antibiotic via the air sac on day 2 or 3, and in some cases again on day 5. In some of the first tests performed 1 mg only of each antibiotic was used as an initial dose. This proved to be insufficient and bacterial growth rendered these tests unsatisfactory.

Chick embryos were candled daily and yolk sacs were harvested at death or arbitrarily at 6-9 days (two), and those remaining at day 13 or 14. Examination, storage and passage of yolk sacs were by standard procedures. Ordinarily 3 passages were completed before a negative report was made.

Bacteriological Examination: In series 1 and 3 many of the specimens were examined for bacterial content by culturing 0.1 ml on each of two blood agar plates, one of which was placed in a candle jar to provide approximately 10% CO_2. The predominant types of colonies were recorded as to numbers present and were identified at the generic level by colonial morphology, cultural characteristics, and microscopic morphology after gram strain.

In series 4, examined in the Taipei laboratory, a portion of the specimen was cultured on blood agar and chocolate agar. Results were read as negative unless there were colonies of a single type of bacterium in numbers not normally encountered.

Complement Fixation Tests: Results of CF tests on sera of the patients supplying the specimens of Series 2 were available. The CF tests were performed by a technique described earlier (Woolridge and Grayston, 1962).

RESULTS

Series 1. This consisted of specimens collected in SPG from the conjunctivas of school children in Taiwan. All were from active trachoma types I, II, or III. They were frozen and sent in dry-ice to the Bethesda laboratory. Nineteen of the specimens were satisfactorily processed in a comparative manner in yolk sacs and cell culture. No chlamydial infection was demonstrated by either method. Bacterial cultures were made. It is noteworthy that a number of yolk sac tests in this series were not satisfactory due to insufficient doses of antibiotics resulting in incompletely controlled bacterial contamination. However, the corresponding tests in cell cultures, of shorter duration, were entirely satisfactory. Results of Series 1 and others are tabulated in Tables 1 and 3.

Series 2. Forty specimens were also obtained in Taiwan mainly from children in which the clinical diagnosis was in 22 instances, trachoma stage 2, but only one direct smear was reported as positive. A few cases of stages 1, 3, and 4 were included, as well as 9 from non-trachomatous eyes. (Only specimens from clinically diagnosed trachoma are recorded in the tables.) Thinking that storage in the frozen state in SPG may have rendered the specimens of Series 1 non-infectious, we took 16 of the trachoma specimens in this Series in CM, and 15 in SPG. The distribution of various clinical types was essentially the same in the two groups. All were sent in dry-ice to Bethesda. One specimen only was strongly positive in cell culture (more than 1000 inclusions per coverslip) and proved positive also in the first yolk sac passage. As indicated in Table 1, this was from the one patient of the series whose direct smear was positive. This patient was one of 4 in the

series with a CF serum titer of 1 : 20. A second specimen was positive by yolk sac (2nd passage), but not in cell culture. Both of the positive specimens had been stored in SPG. The patient furnishing the second positive specimen was one of 5 with a CF titer of 1:10. Two others had titers of 1 : 40 and the remainder were less than 1 : 10.

Series 3. Postulating that our difficulty in demonstrating positive specimens was due to an adverse effect of freezing and perhaps inadequate conditions during international shipment, we made arrangements through the U.S. Indian Service to obtain conjunctival specimens from Navajo Indians in New Mexico. The cases were mainly mild although all stages of trachoma were represented. Fifty-one conjunctival scrapings were placed in complete medium and shipped in wet-ice to the Bethesda laboratory for immediate processing. Inoculations were made within 24 hours or less after the specimens were obtained. Twenty-one additional specimens were taken in SP, frozen stored in dry-ice, and processed later. However, no isolates were obtained from any of the specimens, unfrozen or frozen. Evidence from subsequent studies have shown that storage of specimens in SP in the frozen state is entirely satisfactory and lack of success in this and previous series was evidently due to other factors.

Series 4. Sixty-five specimens were obtained from school children in Taipei and near Taichung, Taiwan, and suspended in complete medium. They were kept in wet-ice for transportation to the U. S. Naval Medical Research Unit No. 2 in Taipei. Within hours after collection, inoculation of cell cultures

Table 1

Comparison of non-irradiated McCoy cell cultures and yolk sacs for detection of *C. trachomatis* in conjunctival specimens from clinically diagnosed trachoma.

Series	Total No. specimens	Suspending medium & storage	Specimens negative by both methods		Positive in cell culture	Positive in yolk sac passage			Direct smears of positive specimens
			No.	Direct smears		# 1	# 2	# 3	
1	19	SPG; F	19	ND	0	0	0	0	
2	15	,,	13	13 : —	+	+	+		+
					—	—	+	+	—
	16	CM; F	16	16 : —	0	0	0	0	
3	21	SP; F	21	ND	0	0	0	0	
	51	CM; NF	51	,,	0	0	0	0	
4	65	,,	58	48 : —	+	—	+		—
				10 : ND	+	—	+	+	±
					+	—	—	—	—
					?	—	+	+	—
					?	—	—		—
					—	—	+	+	—
					—	—	+		ND
Total	187		178	negative	4 positive 2 doubtful	7	positive		

SPG = sucrose phosphate glutamate storage medium; SP = sucrose phosphate; CM = complete medium.
F = frozen and stored; NF = not frozen.
0 = none; — = negative test.
? = doubtful; one only inclusion seen, not completely typical.
± = possibly specific inclusions in direct smear; ND = not done.
In each series doubly negative specimens are given in total figures; positive specimens are recorded individually to allow direct comparison of the two methods of examination.

and yolk sacs had been accomplished. This series resulted in a number of positive tests, as indicated in Table 1. In the cell cultures read as positive, the inclusions were low in number (>10, 3, 1) but were identifiable. In the two instances where atypical inclusions were seen in cell cultures, one specimen was positive in yolk sacs, and the other was negative. Of the 54 direct smears read in this series all were negative except one, reported as questionable.

The summarized results (Table 1) from the first 4 series of specimens show a low incidence of positive tests by either the cell culture or yolk sac method of detection. These results were due, at least in part, to inclusion in the several series of many mild clinical infections, as attested by the almost complete absence of direct smears reported as positive. Many, too, were subjected to the uncertainties of international shipment. Nevertheless, the results with non-irradiated cells were encouraging: 4 tests were certainly positive and 2 were doubtful (initial passage only employed) compared with 7 positive in yolk sacs (only 1 positive in initial passage). The one or more blind passages made with yolk sacs clearly gave advantage to that method. We have more recently demonstrated that passage in cell cultures increases the sensitivity of that method (Gordon *et al.*, 1967).

Series 5. These 53 specimens were collected in SPG, mainly from acute stages of trachoma in school children in Taipei, were kept frozen in dry-ice, hand carried to the Bethesda laboratory, and held until methods for employing irradiated McCoy cells were developed (Gordon *et al.*, 1972). The specimens were then examined in a manner similar to all the others in the series except that we used irradiated cells and medium (CMG) to which glucose was added to give 30 mM/ml (Vedros and Gordon, 1963). In this series (Table 2) a higher incidence of positives was encountered by both tests. Again, the cell culture technique (14 positive tests) compared favourably with yolk sacs (12 positive tests) as a means of detecting the infect-

Table 2

Comparison of irradiated McCoy cell cultures and yolk sacs for detection of *C. trachomatis* in conjunctival specimens.

Series	Total No. specimens	Specimens negative by both methods		Positive in cell culture	Average no. inclusions on coverslip	Positive in yolk sac passage			Direct smears of positive specimens
		No.	Direct smears			# 1	# 2	# 3	
5	53	39	2 : — 34 : ND	+	TNTC	+	+		
				+	,,	+	+		
				+	,,	+	+		+
				+	7	+	+		
				+	40	+	+		
				+	15	+	+		
				+	218	?	+		—
				+	approx.100	?	+		—
				+	63	?	+		
				+	23	?	+		
				+	15	—	+		
				+	24	—	+		
				+	7	—	—	—	
				+	7	—	—	—	
Total	53	39 negative		14 positive		12 positive			

TNTC = Too numerous to count, estimated at 500-1000.
? = Insufficient evidence for a clear decision.

ing agent. Also, the fact that 51 of these 53 tests gave the same result in the two types of assay, provides evidence for the basic parallelism of the two methods. The significance of additional passages in yolk sacs beyond the first is again apparent. If yolk sac tests had been limited to initial passages only, 6 specimens of the 12 would have been reported as positive, 4 doubtful, and 2 negative.

Table 2 also indicates the average number of inclusions present in 2-4 coverslips. These represent estimates of the approximate number of infectious particles per 0.25 ml of the specimen after suspension in 2.5 ml of collecting fluid. The coverslip in which the inclusions were "too numerous to count" contained an order of magnitude of 1000 inclusions. The very small amount of material taken from the conjunctival epithelium, when put into 2.5 ml, is diluted at least 1000-fold. It may be seen that very large numbers of infectious particles may be present in the conjunctival specimen.

As might be expected, there is a rough parallel between the level of infectiousness determined by this method, and the infectivity of the specimen for yolk sacs, but exceptions are apparent. Inhomogeneity of the suspended specimen could be a factor; we made no attempt to homogenize the specimens, beyond ordinary mixing, before dividing into portions for inoculations.

Passages in Cell Culture: In 5 instances, when Series 5 specimens proved to be strongly positive in the first one or two coverslips fixed and examined, the remaining tubes were allowed to continue incubation until the 3rd, 4th, or 5th day at which time they were harvested and passed to fresh irradiated cell cultures. This was accomplished by addition of 0.5 ml of fresh medium to the tube and vigorous agitation on a Vortex, Jr. Mixer, by which the monolayer of cells were suspended. The cell suspensions from the 2 or 3 tubes used were pooled, sonicated (Raytheon, Model DF101) for 5 minutes, further diluted and used for inoculation of a second series of 4 tubes by the usual procedure. In all 5 instances in which passage in cell culture was attempted, it was successful, and this aspect of our study has previously been reported (Gordon and Quan, 1965).

Bacterial Cultures: The results of cultures on Series 1 and 3 are summarized in Table 3. Determination of the microflora of trachomatous eyes was not a primary objective in these tests and the specimens were obviously not taken in an optimal manner for such a purpose. Fastidious or labile types were no doubt destroyed by our methods. The objective of the bacterial assays was to determine the bacterial types present in the specimen as it was received in the laboratory, which could complicate the cell culture and yolk sac tests by insufficiently inhibited growth. Actually, the amounts of antibiotics routinely used provided satisfactory control of bacterial growth, and no conjunctival flora were encountered that caused special problems. An exception was noted earlier, in connection with Series 1, when insufficient levels of antibiotics were used in embryonated eggs and a number of unsatisfactory yolk sac tests resulted. The corresponding cell culture tests were entirely satisfactory. In an earlier series of studies on infected monkey eyes (Gordon *et al.*, 1963) cell cultures proved to be satisfactory for detection of chlamydial infection even when heavy bacterial contamination was not entirely suppressed.

It is noteworthy that a different kind of flora was detected in Navajo children than in those of Taiwan. A factor of possible significance was that the Navajo children were attending boarding schools, and the Taiwanese lived at home and attended day schools. The bacterial type most commonly encountered in

Table 3

Number of times bacterial strains of indicated genera were cultured from conjunctival specimens.

Genus	Series 1 School children of Taiwan	Series 3 Navajo school children	Total
Staphylococcus	2 (8%)	31 (50%)	33
Streptococcus	13 (50%)	8 (13%)	21
Corynebacterium	15 (58%)	12 (19%)	27
Neisseria	2	1	3
Sarcina	0	5	5
Gaffkya	1	3	4
Bacillus	2	2	4
Gram neg. rod	0	8	8
Unknown	0	2	2
Total specimens cultured	35	72	107

the former was staphylococcus, and in the latter corynebacteria and streptococci predominated. A greater variety of types was cultured from the Indian children, including gram negative rods. Some of the latter were recorded as resembling *Escherichia*, some *Aerobacter*, and some *Haemophilus*.

The results of cultures from the Chinese children are similar to those recorded in other studies (Arm and Woolridge, 1966) in Taiwan in which *Corynebacterium* and *Streptococcus* accounted for 66 per cent of total isolates from children's eyes.

Sixty-two specimens of Series 4 were cultured on blood agar and chocolate agar. Fifty-one were reported as negative, i.e., only normally encountered species in the usual numbers were observed. Data on the 11 cultures regarded as abnormal are recorded in Table 4, which indicates the species identified by inspection of colonies and gram stain, as required, and the approximate numbers of colonies. There was no apparent relation between the results of bacterial and chlamydial cultures. These results, too, are similar to previously reported data from the same laboratory, except that in the earlier and larger series *Diplococcus pneumoniae* was more frequently isolated (Woolridge and Gillmore, 1962; Woolridge *et al.*, 1964).

Table 4

Data on bacterial cultures of individual specimens of Series 4 reported as positive.

Species	Approximate no. colonies/ml
S. aureus	>2500
S. albus	50
D. pneumoniae	900
,, ,,	50
Strep. (α haemolytic)	100
,, ,,	50
,, ,,	50
,, ,,	50
,, ,,	50
,, ,,	50
G. tetragena	50

DISCUSSION

A principal factor in the failure to obtain isolates in the earlier series of specimens

reported here was probably the inclusion of mild infections. Those of Series 5 were obtained from especially selected cases, and although only a few direct smears were obtained, a larger number of more acute cases than in the previous series were no doubt involved.

It is clear from this study as well as from those reported by others (Murray *et al.*, 1962) that many clinically acute cases are not confirmable by demonstration of the infectious agent by the yolk sac test, and our results show a basic parallelism between yolk sac tests and those in cell culture.

It is noteworthy that several instances were encountered in these series where chlamydial infection was demonstrated, either by cell culture or yolk sac test, in specimens from patients whose direct smears were reported as negative. This, too, has been reported by others (Hanna, 1962). In one instance only in our work (Table 2) were both methods of isolation negative when the direct smear was positive.

The results reported here suggest only a modest advantage of irradiated cells over non-irradiated for detection of the infection, if one assumes that the yolk sac test provides a suitable base-line for comparison, i.e., between Series 4 and 5. Evidence reported elsewhere (Gordon *et al.*, 1972) indicates a markedly greater potential advantage of irradiated cells. A more recent study (Gordon *et al.*, 1969) confirmed the usefulness of irradiated McCoy cells for isolation of chlamydial strains from human infections, and demonstrated their considerably greater sensitivity than the yolk sac.

A distinct advantage of irradiated cells over non-irradiated, readily apparent to the operator who scans the stained coverslip for detection of inclusions, is the larger size of inclusions in irradiated cells, greatly facilitating their recognition.

The advantages of irradiated McCoy cell cultures over yolk sacs for detection of chlamydial infection may be enumerated as follows: a) greater sensitivity resulting in detection of smaller numbers of infectious particles (evidence derived mainly from other studies cited); b) an earlier result, i.e., a minimum of 48 hours for a first reading of cell cultures, 7-14 days for yolk sacs; c) a greater tolerance of the cell culture for bacterial contamination; d) titration of the specimen by counting inclusions in the infected monolayer; e) a technical, and, depending upon location, a logistic advantage in maintaining and controlling the culture medium inside the laboratory (cell line) rather than obtaining it from outside sources (embryonated eggs).

Most of our examination of the potential usefulness of irradiated McCoy cells for this purpose has been conducted with strains of *C. trachomatis*. The few experiments performed with laboratory strains of *C. psittaci* indicate that the method will be valuable for detection of the latter species also.

SUMMARY

Five series of conjunctival specimens (total = 240) from cases of trachoma were examined by inoculation, in parallel, of monolayer cultures of McCoy cells and yolk sacs of embryonated eggs. The total number of positive tests was insufficient to allow a meaningful comparison between the sensitivity of unirradiated cultures and yolk sacs. When irradiated cells were used 14 specimens were positive in cell culture (initial passage only used) and 12 were positive in yolk sac. However, only 6 of the latter were definitely positive in the first passage. There was a suggestion of some advantage of irradiated cells over unirradiated, compatible with their

considerable advantage demonstrated earlier in controlled tests with established strains. Counts of inclusions in monolayers at 48 hours following initial inoculation allowed an estimation of numbers of infectious particles in the inoculum. Bacterial studies on some of the series confirmed previous reports on the types of bacteria found in trachomatous eyes, and revealed no species that were not controlled in cell and yolk sac cultures by the standard levels of antibiotics used. The advantages demonstrated here of irradiated McCoy cells over yolk sacs for detection of chlamydiae are: 1) a much shorter period needed for obtaining the result, 2) a greater tolerance for bacterial contamination, and 3) titration of the specimen by counting inclusions. Other studies have shown a markedly greater susceptibility of irradiated cells in comparison to non-irradiated, and in comparison with yolk sacs.

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