

Isolation of TRIC agent (*Chlamydia*) in irradiated McCoy cell culture from endemic trachoma in field studies in Iran

Comparison with other laboratory tests for detection of *Chlamydia**

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The technique of cultivation of TRIC agent, and other *Chlamydia* (*Bedsonia*) by centrifugation onto irradiated McCoy cells, developed by Gordon, Magruder, Quan, and Arm (1963), Magruder, Gordon, Quan, and Dressler (1963), and Gordon, Dressler, and Quan (1967), has been used to recover laboratory-adapted isolates of TRIC agent from the infected eyes of simians (Gordon and others, 1963) and of man (Magruder and others, 1963).

Studies recently completed in this laboratory on clinical specimens from genital or genitally-related ocular infections in London showed that isolation in irradiated McCoy cells is more sensitive for the detection and isolation of chlamydial agents than cultivation in the yolk sac of embryonated hen's eggs or inclusion-finding in Giemsa-stained scrapings (Gordon, Harper, Quan, Treharne, Dwyer, and Garland, 1969; Dunlop, Hare, Darougar, Jones, and Rice, 1969). It was also shown that TRIC agent could be isolated in the cell culture system from endemic trachoma in Iran, but the number of specimens tested was too small to give an assessment of the sensitivities of the two methods of isolation in relation to the finding of inclusions in conjunctival scrapings.

Isolates of TRIC agent from endemic trachoma in the Middle East and the Far East have shown biological differences from TRIC isolates of genital origin in London, USA, and Taiwan (Alexander, Wang, and Grayston, 1967; McComb and Bell, 1967; Wang and Grayston, 1970, 1971). It was, therefore, not clear whether the irradiated McCoy cell system could provide a satisfactory alternative to the yolk sac of embryonated hen's eggs for isolation of TRIC agent from endemic trachoma.

This paper compares the results of isolation of TRIC agent in cell culture with those of isolation in the yolk sac of embryonated eggs from scrapings collected during a field study of trachoma in certain villages in Iran. It also relates these isolations to the findings of inclusions in conjunctival scrapings stained by iodine or Giemsa stain, and the results of microimmunofluorescence typing of the isolates.

Material and methods

SELECTION OF CASES

Specimens used in this project were collected during a field survey in Iran in May, 1967, from four villages: Baba Rais, Varchagh, and Galeh Asad in the Malayer area, and Daylam Sofla in the Dezful area.

Clinical examinations were carried out in a field station using a modified Haag-Streit slit lamp microscope for the cornea, tarsal conjunctiva, and lower fornix, and Keeler $\times 2.5$ telescopic spectacles with integrated illuminator for the upper fornix of the conjunctiva.

Of 49 cases selected for this project, 46 had clinical signs of active trachoma (43 children and 3 adults). The clinical picture and the intensity of signs of disease in these patients will be described and discussed in subsequent papers. The other three patients were babies between 3 and 6 months of age with acute papillary conjunctivitis.

CONJUNCTIVAL SCRAPINGS

The conjunctiva of each eye was anaesthetized by two or three drops of Jocaine N (cocaine hydrochloride 4 g., adrenalin acid tartrate 0.18 g., sod. metabisulphite 0.1 g., neomycin sulphate 0.5 g., chlorhexidine acetate 0.1 per cent., 5 ml. distilled water to 100 ml.). Smears for detection of inclusion bodies were prepared from the conjunctiva of the left eye. In this survey the conjunctiva was divided into four different areas: upper tarsus, upper border of upper tarsus, upper fornix, and lower lid (including both lower tarsus and fornix). Conjunctival scrapings were collected from each area with sterile aluminium scrapers which were flattened at both ends. This material was placed on slides that had been marked by diamond pencil with circles 6 mm. in diameter: one circle for each area of the conjunctiva. Smears collected from each of 49 patients were fixed with methyl alcohol for 10 min., dried, and kept at room temperature until stained by iodine solution and subsequently by Giemsa stain.

SPECIMENS FOR ISOLATION

Material was collected from the anaesthetized right eye of each of the 49 patients. Conjunctival scrapings were taken from each of the four areas in turn, and were placed in a single vial as a combined specimen.

Conjunctival scrapings collected from 24 cases in three of the villages, Baba Rais, Varchagh, and Galeh Asad, were suspended in 2.4 ml. 2SP medium with antibiotics (M/15 phosphate buffer at pH 7.2 with 0.2M sucrose, 50 $\mu\text{g./ml.}$ streptomycin, and 100 $\mu\text{g./ml.}$ vancomycin). The specimens were kept for 6 to 7 hrs on wet ice or in a $+4^\circ\text{C}$ refrigerator until they were ampouled and stored in a Linde liquid nitrogen refrigerator at -180°C . Another 25 specimens from the village of Daylam Sofla were collected in 1.5 ml. SPG (Bovarnick, Miller, and Snyder, 1950), containing 20,000 $\mu\text{g./ml.}$ streptomycin and 20,000 u./ml. neomycin. These specimens were kept at $+4^\circ\text{C}$. for about 24 hrs until they were ampouled and stored in the liquid nitrogen refrigerator for 4 to 10 mths before cultivation.

CELL CULTURE AND YOLK SAC CULTIVATION OF *CHLAMYDIA*

The technique for cell culture and for culture in embryonated eggs were those of Gordon and others (1969).

MICROIMMUNOFLUORESCENCE TYPING OF ISOLATES

The isolates that had been stored in this study were subsequently typed using the microimmunofluorescence test (micro-IF test) described by Wang and Grayston (1970, 1971) and confirmed by Treharne, Katzenelson, Davey, and Gray (1971).

DETECTION OF INCLUSIONS IN CONJUNCTIVAL SCRAPINGS

Iodine staining Smears previously fixed with methyl alcohol were stained with Lugol's solution (Iodine 1 g., potassium iodide 2 g., water 100 ml.) for 10 min. Slides were rinsed in water and dried with blotting paper, since it has been our experience, confirmed by other workers (Schachter and Meyer, 1969), that rinsing in water washes out excess iodine and prevents crystallization without interfering with the staining of inclusions. The smear in each circle was examined for 5 to 10 min., using an oil immersion objective $\times 40$ and $\times 8$ oculars; the number of inclusions was recorded. Smears that were heavily blood-stained were treated with 0.5 per cent. ammonia solution before staining with iodine (Sowa, Sowa, Collier, and Blyth, 1965).

Giemsa staining After examination, smears stained with iodine were decolorized by immersion in methyl alcohol overnight. They were then stained with 10 per cent. Giemsa solution for 1 hr. The smear in each circle was examined for 15 to 20 minutes, using an oil immersion objective $\times 40$ and $\times 8$ oculars; the number of inclusions was recorded.

Results*Isolation of TRIC agent in cell culture*

PRIMARY ISOLATION IN CELL CULTURE

Of 49 specimens inoculated into cell cultures, thirteen produced typical inclusion bodies in infected cells (Table I; Figs 1 and 2, overleaf). All thirteen isolates produced inclusions that stained with iodine (Fig. 3, overleaf), thus establishing that they were subgroup A *Chlamydia* (Gordon and Quan, 1965).

Table I Incidence of positives among 49 cases of trachoma or conjunctivitis in certain villages in Iran, by various methods of detecting *Chlamydia*

Source of specimens	No. of specimens	Isolation				Conjunctival inclusions				Total positive by any method	
		Cell culture		Egg culture		Giemsa stain*		Iodine stain*		+	Per cent.
		+	Per cent.	+	Per cent.	+	Per cent.	+	Per cent.		
Trachoma											
Stage I	18	6	33.3	1	5.5	2	11.1	4	22.2	7	39
Stage II	17	5	29.4	1	5.8	3	17.6	1	5.8	7	41.1
Stage III	11	2	18.1	2	18	1	9	1	9	4	36.3
Total	46	13	28.2	4	8.6	6	13	6	13	18	39.1
Conjunctivitis	3	0	0	0	0	0	0	0	0	0	0

*All four areas of conjunctiva examined, see text

+ = Positive

Of the total of thirteen positives in cell culture isolation, only six were also positive by one or more other methods of detecting *Chlamydia* (Table II, overleaf).

Cells inoculated with the specimens from Daylam Sofla, that had been collected in the higher concentration of antibiotics, developed intracytoplasmic granules. This granulation caused difficulty in identifying inclusion bodies in the first passage, but was not transmitted to subsequent passages. However, the rate of contamination with bacteria was very low throughout this study (one specimen in second passage and another specimen in third passage were contaminated). All cell cultures inoculated with coded uninfected control specimens remained negative through three passages.

Table II *Distribution of positive results using different methods of detection of Chlamydia*

Specimen code	Stage of trachoma	Isolation		Conjunctival inclusions	
		Cell culture	Egg culture	Iodine stain	Giemsa stain
271B*	I	+	-	-	-
100V*	I	+	-	+	-
102V	I	+	+	+	+
269V	I	+	-	-	-
25D*	I	+	-	-	-
28D	I	-	-	+	-
64D	I	+	-	+	+
36A*	II	-	-	+	+
297B	II	+	-	-	-
299B	II	+	-	-	-
20V	II	-	+	-	-
114V	II	+	-	-	+
227V	II	+	-	-	+
14D	II	+	-	-	-
72A	III	-	+	-	-
174V	III	-	+	-	-
68D	III	+	-	+	+
94D	III	+	-	-	-
Total 18		13	4	6	6

*A = Village of Galeh Asad (Malayer area)

V = Village of Varchagh (Malayer area)

B = Village of Baba Rais (Malayer area)

D = Village of Daylam Sofa (Dezful area)

+ = Positive - = Negative

SERIAL PASSAGE OF ISOLATES IN IRRADIATED MCCOY CELLS

Six of the thirteen chlamydial agents isolated in cell culture were stored at the time of isolation: five of these were revived and serial passage in irradiated cells was subsequently attempted. At each passage the harvested cell suspension was sonicated and diluted with CMGA (Gordon and others, 1969) to contain approximately ten to twenty mature inclusions per ml. Of this dilution 1 ml. was inoculated onto each fresh monolayer using the standard technique. All five isolates were passaged successfully six to nine times; the average number of inclusions formed at each passage was 100 to 200 per coverslip, indicating 10-20-fold serial multiplication.

PASSAGE FROM CELL CULTURE TO YOLK SAC

Passage of tissue culture-isolates to the yolk sac of embryonated hen's eggs was attempted with all six of the isolates that had been stored. The cell culture isolate was passaged in McCoy cells until the number of mature inclusions in the harvest reached at least 500 per ml. Of this material 0.3 ml. was inoculated into each of three 7-day-old embryonated hen's eggs. In each case Giemsa-stained smears from harvested yolk sacs of the first passage eggs contained numerous elementary bodies. Thereafter each agent could be propagated by serial passage in the yolk sac without difficulty, and has been available for immunofluorescence typing and other studies.

ISOLATION OF TRIC AGENT

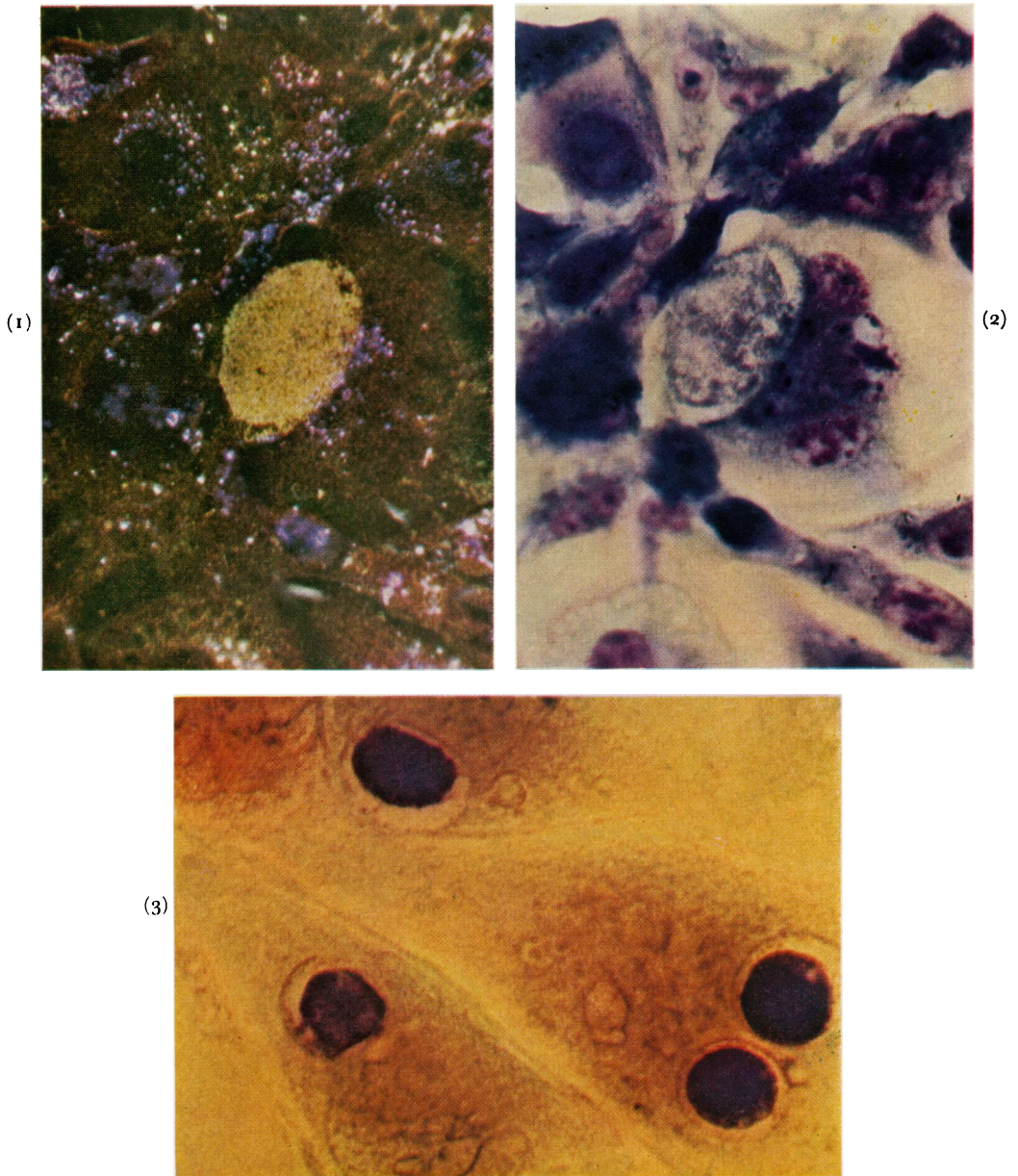


FIG. 1 TRIC inclusion body in irradiated McCoy cell, 60 hrs, Giemsa stain, $\times 1280$, darkfield illumination. The elementary bodies show as bright golden fluorescing particles within the inclusion body

FIG. 2 TRIC inclusion body in irradiated McCoy cell, 60 hrs, Giemsa stain, $\times 1280$, bright field illumination (same field as Fig. 1). The contrast between the elementary bodies in the inclusion bodies and other material in the monolayer is much less striking than with darkfield illumination

FIG. 3 TRIC inclusion bodies in irradiated McCoy cells, 48 hrs, iodine stain, $\times 1920$. The inclusion bodies, which are typical subgroup A Chlamydia, are stained dark brown or mahogany and contrast well with the light yellow background

Isolation of TRIC agent in yolk sac

PRIMARY ISOLATION IN YOLK SAC

Of 49 specimens inoculated into the yolk sac of embryonated eggs, four were shown to be positive by producing typical elementary bodies (Table I).

Of the four positive in egg culture isolation, one was also positive by other methods of detecting *Chlamydia* (Table II).

The rate of contamination with bacteria was very low; only one specimen in the first passage and another specimen in the second passage was contaminated. In eight cases it was not possible to complete three passages because the embryos were undeveloped. In four cases only one passage was completed and in another four cases two passages were completed. All eggs inoculated with coded uninfected control material remained negative throughout three passages.

PASSAGE FROM YOLK SAC TO CELL CULTURE

In order to ascertain whether embryonated eggs might successfully support the growth of some strains of TRIC agent that would not grow readily in McCoy cell cultures, the three yolk sac isolates that were obtained from specimens that did not yield isolates in cell culture were later inoculated into the cell culture system. The isolates from Galeh Asad (72A) produced inclusions in the second passage, the other two specimens from Varchagh (20V and 174V) showed typical inclusions in the first passage in cell culture. There was thus no evidence suggesting selective growth in eggs of any isolate in this series.

MICROIMMUNOFLUORESCENCE TYPING OF ISOLATES

The three isolates obtained only in the yolk sac and the six tissue culture isolates that had been stored were typed in the micro-IF typing test (Table III). With the exception of

Table III Designation and microimmunofluorescence type of 16 isolates in relation to primary isolation in cells or eggs

<i>Specimen code</i>	<i>Egg inoculation</i>	<i>T.C. inoculation</i>	<i>Designation of isolate</i>	<i>Micro IF type</i>
72A*	+	—	TRIC//IR/IOL-223/OT	C
271B*	—	+	TRIC//IR/IOL-231/OT	C
297B	—	+	TRIC//IR/IOL-251/OT	B
299B	—	+	TRIC//IR/IOL-250/OT	ND**
20V*	+	—	TRIC//IR/IOL-225/OT	C
100V	—	+	TRIC//IR/IOL-206/OT	C
102V	+	+	TRIC//IR/IOL-205/OT	C
114V	—	+	TRIC//IR/IOL-227/OT	ND
174V	+	—	TRIC//IR/IOL-234/OT	C
227V	—	+	TRIC//IR/IOL-232/OT	ND
269V	—	+	TRIC//IR/IOL-233/OT	ND
14D*	—	+	TRIC//IR/IOL-235/OT	D
25D	—	+	TRIC//IR/IOL-245/OT	ND
64D	—	+	TRIC//IR/IOL-237/OT	ND
68D	—	+	TRIC//IR/IOL-236/OT	ND
94D	—	+	TRIC//IR/IOL-243/OT	B

*A = Village of Galeh Asad (Malayer area)

B = Village of Baba Rais (Malayer area)

+ = Positive

— = Negative

V = Village of Varchagh (Malayer area)

D = Village of Daylam Sofla (Dezful area)

**ND = Not done

14D (TRIC//IR/IOL-235/OT), which serotyped as Type D, all the other isolates from the Malayer and Dezful areas of Iran which were examined serotyped as Type B or C. In order to check the validity of the typing of this isolate 14D, a further aliquot of the stored third passage level culture material was inoculated into eggs and typed in the micro-IF test. Three repeat test typings consistently identified it as Type D.

Inclusions in conjunctival scrapings

IODINE STAIN

By examination of iodine-stained smears from all four areas of the conjunctiva, six out of 49 patients were shown to be positive by demonstration of typical inclusion bodies (Table I). Examination of scrapings from the upper tarsus alone revealed only two positive in these 49 cases.

Of the six positive by iodine stain, four were positive by other methods (Table II), and only three were positive with Giemsa. It should be noted that, in those cases positive only by iodine, the inclusion bodies were detected in the thicker parts of the smears.

GIEMSA STAIN

By examination of Giemsa-stained smears from all four areas of the conjunctiva, six out of 49 patients were shown to be positive by the demonstration of typical inclusion bodies (Table I). Examination of the upper tarsus alone revealed only one positive out of 49 cases.

All six positive by Giemsa stain were also positive by other methods of detecting *Chlamydia* (Table II). Of these cases, two were negative by iodine stain. The inclusion bodies detected by Giemsa in these two cases were immature and contained either initial bodies only or were of early intermediate type particles (pink or red).

The totals of positives for each method of detecting *Chlamydia* shown in Table I indicate that the cell culture system (13 positives) was over three times more sensitive than egg culture (4 positives). This difference is significant at a level of $P < 0.02$. Cell culture was also twice as sensitive as finding inclusions by iodine or by Giemsa-staining techniques. This difference is significant at a level of $P < 0.10$.

By combining the positive results of different methods of detecting chlamydial infection (Table II), it may be seen that eighteen out of 46 patients with trachoma (39 per cent.) were found to be positive by at least one of the four methods used.

Discussion

In other studies of trachoma carried out in the Malayer area in North-Western Iran an extremely low rate of positivity was found when scrapings from the upper tarsal conjunctiva alone were examined after staining with iodine. In the present study the proportion positive by this method was again very low: in 46 active cases only two such scrapings were positive with iodine and one with Giemsa stain. When additional scrapings from the conjunctiva of the upper border of the upper tarsal plate, from the upper fornix, and from the lower lid were also examined, there were six positives with iodine and six with Giemsa stain. The iodine stain had some advantage in thick areas of the smears, and the Giemsa stain was better for staining immature inclusions.

Inclusion finding with these stains was thus too insensitive a method of demonstrating the presence of TRIC agent in endemic trachoma in North-Western Iran to provide a satisfactory microbiological parameter for the study of the effect of preventive or therapeutic measures. In our laboratory, fluorescent antibody staining of inclusions in con-

conjunctival scrapings has not been found to be more sensitive than Giemsa staining when applied to TRIC infection of the eye in London (Darougar, Dwyer, Treharne, Harper, Garland, and Jones, 1971).

For these reasons we investigated the sensitivity of isolation of TRIC agent from conjunctival scrapings by inoculation into McCoy cell cultures and into embryonated hen's eggs. Even when applied to conjunctival scrapings after 4 to 10 months' storage in liquid nitrogen, the McCoy cell isolation system gave thirteen positives, as compared with two inclusion-positive upper tarsal scrapings and six positives when all areas were examined by iodine or Giemsa. Aliquots of the same conjunctival scrapings gave only four isolations in yolk sac.

The liquid nitrogen refrigerator used in this study offered a very satisfactory means of preservation of clinical specimens in the field where electrical supplies were inadequate, and provided a reliable method for transportation of specimens from the field to the laboratory in London. It may be possible in future investigations to increase the proportion of positive cultures by reducing the time between collection and snap-freezing; in this study the interval was between 6 and 24 hrs.

The view has been expressed in several discussions that the McCoy cell system fails to yield viable isolates that can be serially passaged in the laboratory. To answer this criticism, six available isolates secured in cell culture were passaged serially six to nine times on McCoy cells. They were then passaged serially in eggs without difficulty, and have subsequently been typed by the micro-IF typing test.

An earlier study had shown that the irradiated McCoy cell culture system was superior to the yolk sac of chick embryos for primary isolation of subgroup A *Chlamydia* from genital disease, or from ocular disease associated with genital infection in London, from which the isolates have so far mainly serotyped as D, E, or F in the micro-IF test (Treharne and others, 1970). The isolates from endemic trachoma in Iran in the present study have mainly serotyped as B or C, thus resembling other isolates from endemic trachoma in the Middle East that have been typed as A, B, or C (Wang and Grayston, 1970, 1971; Treharne and others, 1971). It is clear, therefore, that the irradiated McCoy cell culture system offers a superior method of isolation for each of the two main groups of serotypes of TRIC agent, namely A, B, and C from endemic trachoma in Iran and D, E, and F from genital or ocular disease in London.

However, it is to be noted that one Iranian isolate (TRIC//IR/IOL-235/OT) from the eye of a 1-year-old girl from Dezful suffering from typical trachoma stage II (with moderate papillary hypertrophy, a number of necrotic follicles on the upper tarsal conjunctiva, and pannus) serotyped as Type D. This type has hitherto been found mainly in areas where it is postulated that TRIC agent is spread primarily by genital transmission. Hence the finding of TRIC Type D agent in a trachoma-endemic area of Iran might suggest that a low incidence of ocular infection associated with genital infection occurs in that population; or alternatively that TRIC Type D may sometimes be spread by eye to eye transmission causing endemic oriental trachoma. In this context it is of interest to note that an isolate (TRIC/WAG/MRC-1/OT) from the Gambia (Collier and Sowa, 1958) from the eye of a 10-year-old child with typical trachoma, has serotyped as Type D (Wang and Grayston, 1970, 1971; Treharne and others, 1971). On inoculation into the ophthalmic socket of a volunteer, this isolate produced a severe picture of chronic cicatricial trachoma in the conjunctiva (Collier, Duke-Elder, and Jones, 1958). In this case, although the Gambia is a trachoma endemic area, it should be noted that TRIC isolates have been secured from the genital tract of certain patients there (Sowa and others, 1965).

The present study has also confirmed the following general advantages of the McCoy cell system over the yolk sac system for the isolation of TRIC agent:

- (1) A shorter time is required in which to report a clinical specimen as either positive or negative: 2 to 9 days as compared with 6 to 36 days for egg culture.
- (2) The maintenance of the cell line in the laboratory avoids dependence on supplies of eggs not under laboratory control.
- (3) The costs of isolation are greatly reduced.
- (4) Unlike diagnosis by finding inclusions in conjunctival scrapings, the irradiated McCoy cell system secures isolates for typing and other methods of study.

It is also possible to use the cell culture technique to measure the amount of viable TRIC agent in the clinical specimen (Gordon and others, 1969; Darougar and Jones, 1971).

It is therefore concluded that an irradiated McCoy cell culture system offers a more sensitive method for the detection of *Chlamydia* in field studies of trachoma than has hitherto been available, and furthermore that this technique is truly a method of serial cultivation that yields isolates for biological characterization.

The need for a suitable source of irradiation may constitute a difficulty for some laboratories; but it has been shown that different types of ionizing irradiation, including gamma ray, X ray, electron beam, and beta ray, in doses ranging from 4500 to 6000 r can be successfully employed (Darougar and others, in preparation). This has led to the design of a simplified source of irradiation that will enable this procedure to be carried out in any laboratory.

Summary

The results of this study have established that isolation of TRIC agent in an irradiated McCoy cell culture system is three times as sensitive as isolation in the yolk sac of embryonated eggs when applied to endemic trachoma in Iran. This system is also at least twice as sensitive as the detection of inclusion bodies in conjunctival smears stained by iodine or Giemsa, or both. The sensitivity of the technique is achieved by the use of cells which are rendered more suitable to support the growth of TRIC agent by exposure to ionizing irradiation, and by the centrifugation of clinical specimens onto these cells. The results in this study clearly demonstrate that this technique does provide a method of serial cultivation in cell culture which yields isolates that can be transmitted in egg culture and are available for biological study.

This technique, applied to material transported on liquid nitrogen, would therefore appear to have considerable advantages for field studies of endemic trachoma. It is of interest that, although most of the isolates from endemic trachoma in Iran in this study have been serotyped as B or C, one has serotyped as D.

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