J. Hyg., Camb. (1971), **69**, 693
With 1 *plate*
Printed in Great Britain

A comparison of the iodine and fluorescent antibody methods for staining trachoma inclusions in the conjunctiva*

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(*Received* 23 *June* 1971)

SUMMARY

In terms of the rate of positive diagnoses the indirect fluorescent antibody (FA) test was rather more effective than iodine for demonstrating trachoma (TRIC) inclusions in conjunctival scrapings, but the degree of advantage was not statistically significant. In duplicate scrapings stained at random by one or the other method, FA staining yielded the higher inclusion count significantly more often than did iodine. Some inclusions that failed to stain with FA were found on subsequent staining with Giemsa. A method is described for improving the post-FA Giemsa staining of conjunctival smears stored at subzero temperatures. Given adequate facilities, the FA stain is preferable to iodine for demonstrating TRIC inclusions in the conjunctiva; but the iodine method, properly used, holds advantages for field use.

INTRODUCTION

The problem of which is the 'best' method for detecting trachoma/inclusion conjunctivitis (TRIC) agent in the conjunctiva has long been a matter of controversy; and perhaps the reason why there is no clear-cut answer lies in the diversity of ends to which the various means are directed. Whereas one worker may be interested in the rapid screening of large populations, another may be concerned only with an occasional diagnostic test, or with careful observation of an experimental infection in a few human volunteers or monkeys.

The *Chlamydia*, including TRIC agents, are demonstrable by direct microscopy of infected cells, in which they form cytoplasmic inclusion bodies of characteristic morphology and staining reactions; and by isolation in a suitable host. Halberstaedter & von Prowazek (1907) first observed trachoma inclusion bodies in conjunctival scrapings stained with Giemsa, and although other methods have been described, this stain probably remains the most popular. It reveals the varying morphology and staining characteristics of the inclusion at all stages of development, its relation to the cytoplasm and nucleus of the host cell and the presence of free elementary bodies. The disadvantages are that conjunctival smears must be scanned with the higher-power objectives – a slow and laborious process; that inclusions are often missed because they are hidden in densely

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stained clumps of cells; and that it may be difficult or impossible to decide whether a basophilic granule in the cytoplasm is an early inclusion or ingested debris.

During the later stages of development the inclusions of type A chlamydiae contain a carbohydrate matrix that stains brown with iodine and is closely similar to, if not identical with, glycogen (Rice, 1936). It is probably true that the iodine method has been exploited more extensively by the M.R.C. Trachoma Unit than by any other group of workers. In 1958 Gilkes, Smith & Sowa described the use of dry iodine-stained smears covered with a thin layer of immersion oil. This technique gave better definition than the wet preparations used hitherto, and proved particularly useful for detecting faintly stained inclusions; it was used extensively in the study of experimental infections in man (Collier & Sowa, 1958; Collier, Duke-Elder & Jones, 1958, 1960; Jones & Collier, 1962) and in baboons (Jones, Collier & Smith, 1959; Collier, 1961, 1962; Collier & Blyth, 1966*a*, *b*; Collier, Blyth, Larin & Treharne, 1967).

One of us (J.S.) devised a modification in which the use of weak ammonia improved the contrast between iodine-stained inclusions and the background, and minimized staining of red cells and leucocytes (Sowa, Sowa, Collier & Blyth, 1965). This method was used in the investigation described in the present paper. The iodine method has several advantages: staining takes but a few minutes, and can easily be done in the field; it reveals inclusions buried within clumps of epithelial cells, so that thick smears containing large numbers of cells can be examined with ease; the characteristic colour and granular morphology of the inclusions leaves but little room for error in interpretation; and with experience a conjunctival scraping can be scanned completely in 10 min. with a $\times 10$ or $\times 20$ objective, with virtually no risk of missing even a single stained inclusion. The only disadvantage is that at some stages in their development inclusions do not stain with iodine; but it must be rare for all the inclusions in a given scraping to fall into this category.

The indirect fluorescent antibody method was used 16 years ago to study the growth cycle of a chlamydia in cell cultures (Buckley, Whitney & Rapp, 1955) but it was not until 1961 that Vozza & Balducci attempted to stain trachoma inclusions in conjunctival scrapings by this method, with inconclusive results. In the following year Siboulet, Galistin & Huriez (1962) published a short note on the use of this technique for diagnosing inclusion blennorrhoea. The application of immunofluorescence to the diagnosis of TRIC infections was first placed on a firm footing by Nichols and his colleagues (Nichols & McComb, 1962; Nichols, McComb, Haddad & Murray, 1963); these workers exploited the technique for large-scale studies of trachoma in Saudi Arabia (Nichols, Bobb, Haddad & McComb, 1967). Immunofluorescence was used by investigators at the University of California Medical Center to diagnose TRIC infections in patients and in artificially infected volunteers (Hanna *et al.* 1965; Hanna, 1968), and for assessing the results of treatment (Dawson, Hanna & Jawetz, 1967). The California workers also claimed that immunofluorescence revealed inclusions in a surprisingly high proportion of American Indian schoolchildren with no signs of trachoma, or with signs of inactive disease only (Jawetz *et al.* 1967).

Although several workers compared immunofluorescence with the Giemsa method for detecting conjunctival inclusions, we have found only one report of its comparison with the iodine technique (Hanna *et al* 1965); this is referred to in the Discussion. The researches we describe were designed primarily to assess the relative efficacy of the fluorescent antibody and iodine stains in terms of their suitability both for limited studies and for large-scale screening procedures; they formed part of a series of investigations of trachoma in young Gambian children.

MATERIALS AND METHODS

General plan

The investigations were undertaken in the large Gambian village of Salekini (Sowa, Sowa, Collier & Blyth, 1969). During 1968, preliminary tests were made on 36 children with active trachoma in order to perfect techniques (study no. 1). In January 1969 a group of 99 children aged 3 years and under was assembled. Of these, 37 had normal (N) eyes, 34 had active trachoma (Tr I or II), 10 had physical signs suggestive of early trachoma (Tr D), and 18 had minor conjunctival abnormalities not suggestive of trachoma (Ab). The children were tested 5 times between January and October 1969; a few were absent from one or more examinations. On each occasion their eyes were carefully examined with a slit-lamp, and conjunctival scrapings were taken for microscopy. In addition, samples of conjunctival secretions and blood were tested for antibody; these findings will be reported in a further communication.

Conjunctival scrapings

The conjunctivae were anaesthetized by instilling 0·5% 'Amethocaine' and 0·001% adrenalin hydrochloride. The tarsal area of the everted upper lid was then lightly scraped with a small spatula made by flattening one end of a length of $\frac{1}{16}$ in. (1·5 mm.) aluminium welding rod; scrapings from each eye were spread over separate areas on a microscope slide. When comparing 'thick' with 'thin' smears, a light scraping was taken from the medial half of the tarsal conjunctiva for the 'thin' specimen, and a rather more vigorous scraping from the lateral half for the 'thick' smear. Thin smears were made by spreading a small quantity of material over an area approximately 10×2 mm. For thick smears, about 10 times more material was spread over a circular area 10–15 mm. in diameter; this is our routine practice for the iodine method (Sowa *et al.* 1965). Duplicate thin smears for comparing iodine with fluorescent antibody staining were made by dividing a single epithelial scraping from the mid-tarsal area between two slides which were stained at random by one or the other method.

Iodine stain

Air-dried smears were flooded with absolute ethyl alcohol, drained and allowed to dry. They were then treated for 2 min. with 88 vol. ammonia freshly diluted to 1/200. The ammonia was then rinsed off with a small quantity of ethyl alcohol; this step requires caution since the ammonia tends to loosen the material on the

slide. It was found best to stain slides individually with ammoniated iodine (see below) shortly before examination.

After applying iodine for at least 2 min. the excess stain was poured off and the slide was immediately blotted dry on filter paper. After delineating the smear with a wax pencil, the entire area was systematically scanned with a $\times 10$ objective (final magnification $\times 120$) and the total number of inclusions was counted. In the 'dry' method originally described by Gilkes *et al.* (1958) the stained smears were covered with a thin layer of immersion oil. The use of oil to improve contrast depends on the optical properties of the microscope used; it was not necessary in this investigation.

Ammoniated iodine

The following stock solutions were prepared: A, saturated iodine solution in 20% (w/v) potassium iodide; B, potassium iodide 20% (w/v); C, 88 vol. ammonia, diluted 1/20 in distilled water.

To prepare the stain, 0·5 ml. of A was added to 4·5 ml. of B and mixed well. After rapidly adding 5 ml. of C the mixture was shaken vigorously. Any precipitate was removed by sedimentation or filtration. (Note that dried ammonium iodide is fulminant.) This solution is best kept in darkness, and should not be stored more than 3 days.

Giemsa stain

This stain was used to verify the morphology of inclusions previously stained with FA. To 50 ml. of phosphate buffer pH 6·8 were added 1 ml. of Giemsa R 66 and 0·5 ml. of May–Grünwald stain (G. Gurr and Co., London). The slides were immersed in this solution for 1 hr., rinsed in buffer and dried.

Fluorescent antibody (FA) methods

Diluent. All sera and slide antigens were diluted in phosphate-buffered saline (PBS) pH 7·2 (Fothergill, 1964).

Fixation and storage of scrapings. In surveys 1 and 2, slides were fixed for 10 min. in acetone at room temperature and then stored at $-20°$ C. or below until examination. In surveys 3–6 they were stored at $-20°$ C. or below and fixed in acetone just before staining. There was no evidence that this variation affected the results.

Antiserum to TRIC agent grown in yolk sac was prepared by injecting a rabbit intravenously with a partly purified suspension of live TRIC/2/GB/MRC-4/ON (formerly LB4: Jones, 1961; Jones & Collier, 1962) in its 8th chick embryo passage. This is a 'slow-killing' strain (Reeve & Taverne, 1963); the abbreviation is MRC-4*s*. The suspension contained $10^{6·0}$ 50% egg lethal doses and $10^{10·0}$ total elementary bodies in 1 ml.; it was stored in 1 ml. volumes in liquid nitrogen. The rabbit received four 1 ml. doses at weekly intervals; after 10 weeks rest it was given a booster dose. Serum obtained by heart puncture 1 week after the final dose was stored in 0·1 ml. volumes at $-60°$ C. Its complement-fixing titre against *Chlamydia* group antigen prepared from infected HeLa cells (Collier & Blyth, 1966*a*) was 1/1280.

Antisera to TRIC agent grown in cell culture. Strain MRC-4*s* was propagated in BHK-21 cells by centrifuging the agent into monolayers (Taverne & Blyth, 1971), incubating at 35° C. for 48 hr., disrupting the monolayer with ultrasonic vibrations, and using the resulting suspension to infect the next lot of cells. After three preliminary passages to eliminate any yolk-sac antigen, a sonic-treated suspension of heavily infected cells was made and stored at −60° C. in 1 ml. volumes each containing $10^{8\cdot1}$ elementary bodies and $10^{4\cdot3}$ inclusion-forming units. Two rabbits (nos. 366 and 367) each received four 1 ml. doses at weekly intervals and were bled out 1 week after the final dose. The complement fixation titres of the sera against group antigen prepared in yolk sac were 1/160 and 1/320 respectively.

Anti-rabbit serum prepared in goats and conjugated with fluorescein isothiocyanate was obtained from Sevac Laboratories.

Staining of conjunctival scrapings. Preliminary chess-board titrations with conjunctival scrapings containing many inclusions indicated that the optimum dilutions of anti-TRIC and anti-rabbit sera were 1/20 and 1/80 respectively, and these dilutions were used as a routine. Smears were covered with diluted antiserum and incubated at 37° C. for 30 min. in a moisture-saturated atmosphere. After rinsing off excess serum, the slides were rinsed in 50 ml. of PBS and dried in a current of cold air. After repeating the procedure with anti-rabbit conjugate the smears were mounted under coverslips in glycerol-PBS, 9:1.

Staining of yolk-sac antigens. Slide antigens were prepared by placing a series of small loopfuls of a 5% yolk sac suspension on a slide, drying in air, and fixing in acetone for 10 min. at room temperature; these slides were stored at −30° C. Sera were titrated by covering each spot with an appropriate dilution of serum and incubating the slides for 30 min. at 37° C. in a humidity box. After washing off the sera with PBS delivered from a wash-bottle, and two further 5 min. rinses in PBS, the drops were dried in an air stream, and then covered with a 1/40 dilution of goat anti-rabbit serum conjugated with fluorescein isothiocyanate; this dilution was chosen by chess-board titration of the conjugate with serum 367 and the homologous yolk sac antigen. The incubation and washing procedures were repeated, except that 5 drops of 0·1% Evans blue were added to 100 ml. of PBS for the final rinse. (The use of this counterstain was described by Nichols & McComb (1964), but in a higher concentration.) The antigen spots were mounted under cover-slips in glycerol-PBS. Each slide included two control spots treated respectively with buffer only, and buffer followed by conjugate. When handling more than a few slides at once, we found it very convenient to mount them in plastic storage holders (Bie and Berntsen, Copenhagen, Denmark). The use of these frames enables 12 slides to be stained, incubated and rinsed together.

Ultraviolet microscopy was done with a Zeiss Photomicroscope equipped with an HBO 200 lamp. Slides were scanned under bright field illumination with exciter filter BG 12/4 and barrier filter 53; the objective was a ×40 apochromat. Unless there were more than 50 inclusions, the entire area of each conjunctival scraping was scanned. The brightness of stained yolk-sac antigens was assessed with a set of graded density filters (Collier, 1968). The titration end-point was taken as the first doubling dilution (reading from the lowest) to give a score of 1.

Verification of FA stained inclusions with Giemsa

In study no. 1 and in the 1st survey of study no. 2, all inclusions or probable inclusions stained with fluorescent antibody were photographed on 35 mm. Kodak Tri-X film after recording their position on the stage micrometer. The slide was then stained with Giemsa; to identify the inclusions previously photographed under u.v. light, the photographic negative was projected on a screen and compared directly with the corresponding Giemsa-stained field under the microscope.

Coding of slides

Throughout this investigation all conjunctival scrapings were identified by code number only; one microscopist examined the FA stained smears and another the iodine-stained preparations. Neither was aware of his colleague's findings, or of the results of the clinical examinations.

RESULTS

Tests on TRIC antiserum

Specificity for TRIC antigen. From each eye of two trachoma patients, a thick smear and two thin smears of conjunctival scrapings were made. The thick smear was stained with iodine. One of the thin smears was stained by the routine FA method; the other was stained in the same way, except that normal rabbit serum was substituted for the anti-MRC-4*s* serum. Inclusions were found only in the iodine-stained scrapings, and in those stained with the anti-MRC-4*s* serum (Table 1).

Table 1. *Conjunctival scrapings from two trachoma patients stained by the immunofluorescence (FA) method or by iodine*

	No. of inclusions in scrapings stained by		
	FA		
Patient and eye	Normal serum	Anti-TRIC serum	Iodine*
135 R	0	0	6
135 L	0	4	20
166 R	0	5	10
166 L	0	2	16

* Thick smears containing about 10 times more material than FA-stained smears.

Heterogeneity for TRIC serotypes. The anti-TRIC serum used for staining inclusions was prepared against strain MRC-4*s*, a type 2 TRIC agent. Since the rabbit was immunized by repeated doses, and the serum was not cross-absorbed, it should have been capable of staining inclusions resulting from infection with any TRIC serotype. Because this serum reacted strongly with yolk sac, it proved difficult to verify this supposition by testing against our set of antigens represent-

ing the various serotypes, all of which had been propagated in yolk sac. Accordingly, sera were prepared against MRC-4*s* grown in BHK-21 cells and titrated against the antigens indicated in Table 2. The titres of serum 367 were higher than those of 366 (which also had the lower titre of complement-fixing antibody); but it is apparent from Table 2 that both sera reacted adequately with all the major TRIC serotypes, and it seems reasonable to assume that the anti-MRC-4*s* serum actually used for staining conjunctival material possessed a similar degree of heterogeneity.

Table 2. *Fluorescent antibody titrations of anti-MRC-4s sera with various TRIC serotypes*

		Serum no.	
Serotype	Strain	366	367
1	SAU/HAR-13/OT	20*	160
1b	SAU/HAR-32/OT	20	160
2	SAU/HAR-36/OT	40	160
2	GB/MRC-4*s*/ON	80	320
D	GB/MRC-1/OT ('G 1')	20	160
E	USA-Cal/Cal-1/OT ('BOUR')	40	160
F	USA-Wash/UW-6/GCx	20	40
.	Normal yolk-sac control	< 10	< 10

* Reciprocal of end-point dilution.

Criteria for validity of FA staining of inclusions

No scraping was scored as positive by FA staining unless it contained morphologically characteristic inclusions that were contained within the intact cytoplasmic boundaries of epithelial cells, and that stained brightly with a yellow-green fluorescence (Plate 1 *a–d*). Almost all patients diagnosed as inclusion-positive by FA staining were also positive by the iodine or Giemsa methods. In study no. 1 for example, of 15 patients diagnosed as inclusion-positive by FA, 12 were also positive by both the other methods.

Relative efficacy of iodine and FA stains

Study no. 1. Comparison of the FA method with the iodine technique used by us as a routine is not straightforward; the former demands a thin smear, whereas a much larger sample of cells is examined in the thick smear used in the iodine method. It was thus desirable in the first instance to compare the results of staining duplicate thin smears by both methods with those of tests on thick smears stained by iodine alone. At this stage the numbers of inclusions were not counted, and results were recorded merely as positive or negative. Tables 3 and 4 give the findings in 36 children with active trachoma. Somewhat to our surprise the three methods (thick smear stained with iodine, thin smear stained with iodine and thin smear stained with FA) yielded similar numbers of positive tests (Table 3) although the individuals diagnosed as inclusion-positive by one or the other method did not correspond exactly. The proportion of inclusion-positive patients was about 15/36 (42%) with any one method. With a combination of any two

methods about 50% were positive; and with all three methods this figure rose to 21/36, or 58% (Table 4).

The percentages of positive and of false negative results were similar with all three methods, so that failure of correspondence in a proportion of findings was probably attributable to sample variation; in other words, use of thin smears only for comparing the diagnostic efficacy of the iodine and FA methods would not bias the results unduly against the iodine test.

Table 3. *Study no.* 1*: comparison of results of tests for inclusions in thick and thin smears stained with iodine, and thin smears stained with fluorescent antibody; numbers of inclusion-positive eyes*

	No. of eyes		
	Tk I positive	Tk I negative	Totals
Tn I positive	12	8	20
Tn I negative	10	42	52
Totals	22	50	72
FA positive	12	9	21
FA negative	10	41	51
Totals	22	50	72
	Tn I positive	Tn I negative	
FA positive	13	8	21
FA negative	7	44	51
Totals	20	52	72

Tk I = thick smear stained with iodine. Tn I = thin smear stained with iodine. FA = thin smear stained with fluorescent antibody.

Table 4. *Study no.* 1*: numbers of patients diagnosed as inclusion-positive by combinations of the three methods*

		Additional methods		
	Alone	FA	Tn I	FA and Tn I
Tk I	15	18	18	21
Tn I	14	19	.	.
FA	15	.	.	.

Abbreviations as in Table 3.

Study no. 2. As described under Materials and Methods, duplicate thin smears were made on 5 occasions from the 99 children participating (Table 5), and stained at random either with iodine or FA. Table 6 classifies the results according to clinical diagnosis, and Table 7 summarizes the findings in all patients who were inclusion-positive by either or both methods in each of the five surveys. In 448 examinations of individuals, at which Tr I or Tr II was diagnosed 189 times

Table 5. *Study no. 2: dates of surveys undertaken in* 1969

Survey	From	To
1	6 January	21 January
2	2 March	8 March
3	17 April	5 May
4	7 June	14 June
5	25 September	10 October

Table 6. *Study no. 2: comparative efficacy of iodine and FA staining for detecting trachoma inclusions; combined results for surveys* 1–5 *analysed by clinical diagnosis*

Clinical diagnosis*	Total no. of examinations	Tn I−, FA−	Tn I+, FA+	Tn I−, FA+	Tn I+, FA−	% inclusion-positive (either method)
N	111	111	0	0	0	0·0
Ab	88	86	2	0	0	2·3
Tr D	44	42	2	0	0	4·5
Tr I	62	53	7	2	0	14.5
Tr II	127	103	17	5	4	20·5
Tr III	16	13	1	2	0	18·8
Totals	448	408	29	9	4	9·4

* See text for meaning of these abbreviations; other abbreviations as in Table 3.

Table 7. *Study no. 2: comparative efficacy of iodine and FA staining for detecting conjunctival inclusions*

(Surveys 1–5 analysed by numbers of positive subjects and numbers of positive eyes.)

Survey no.	Positive (+) or Negative (−)	No. of patients		No. of eyes	
		Tn I	FA	Tn I	FA
1	+	7	9	11	11
	−	3	1	9	9
2	+	1	1	1	1
	−	1	1	3	3
3	+	4	5	5	9
	−	2	1	7	3
4	+	8	8	13	15
	−	0	0	3	1
5	+	13	15	23	28
	−	3	1	9	4
Totals	+	33	38	53	64
	−	9	4	31	20
		42	42	84	84
χ^2 (with Yates's correction)		1·456		2·815	
P		$>0·10$		$<0·10$, $>0·05$	

Abbreviations as in Table 3.

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(Table 6), the result was positive both by iodine and FA on 29 occasions; on 9 occasions the result was FA-positive but iodine-negative and on 4 it was iodine-positive but FA-negative. In 84 tests on individual eyes there were 31 false negative results with iodine, and 20 with FA. Although the proportion of positive

Table 8. *Study no. 2: comparison of numbers of inclusions in duplicate thin smears stained by iodine or by FA*

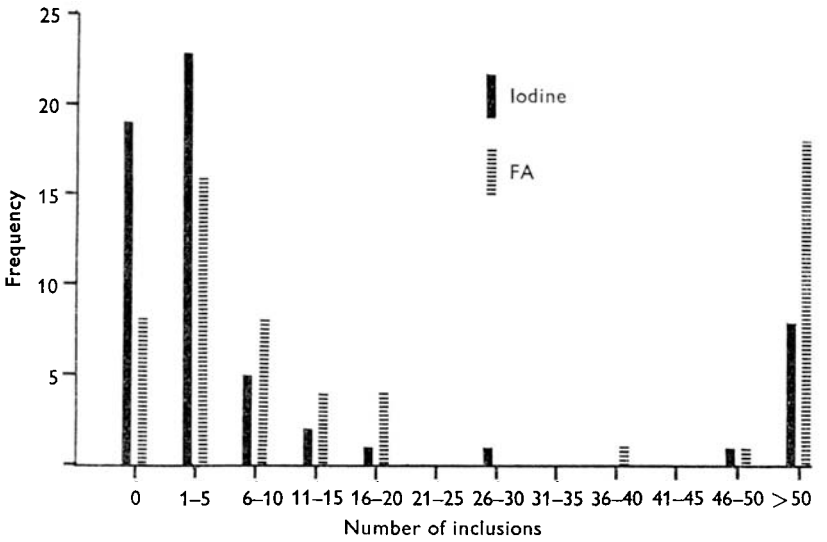
Survey no.	Patient no.	Nos. of inclusions in Right eye Tn I	Right eye FA	Left eye Tn I	Left eye FA
3	3	0	1	0	0
	7	0	0	1	0
	20	4	8	0	1
	27	5	5	1	> 50
	54	0	6	0	1
	73	0	5	2	> 50
4	2	3	9	1	2
	7	27	6	0	1
	10	3	12	2	4
	20	19	20	5	52
	27	7	46	14	10
	31	3	18	0	0
	61	0	2	1	14
	73	> 50	> 50	6	40
5	1	0	6	0	12
	3	0	2	0	1
	7	0	16	0	1
	10	0	2	3	2
	18	3	0	11	2
	22	> 50	> 50	> 50	> 50
	31	1	2	4	> 50
	32	> 50	> 50	> 50	> 50
	41	> 50	> 50	> 50	> 50
	46	0	0	1	0
	61	1	9	0	14
	73	3	> 50	6	> 50
	80	3	6	5	17
	88	46	> 50	1	0
	89	3	> 50	8	> 50
	93	10	> 50	> 50	> 50

Abbreviations as in Table 3.

results with FA was higher, it was not significantly so (Table 7). In surveys 3, 4 and 5 the number of inclusions in each smear was counted. When it exceeded 50, the result was usually recorded as '> 50' since the accurate counting of large numbers of inclusions was very time-consuming. Table 8 gives the actual inclusion counts made in surveys 3, 4 and 5 and Text-fig. 1 their distribution in histogram form. In this series of 60 scrapings taken in duplicate, the inclusion count in the FA-stained scrapings was recorded as the same as in the iodine-stained sample in

https://doi.org/10.1017/S0022172400021963 Published online by Cambridge University Press

13 instances, including 4 in which both samples were negative. The count was greater in the FA-stained sample on 39 occasions, and less on 8 occasions; if there were no differences between the two techniques these numbers would be equal or nearly so. The observed departure from equality is highly significant ($\chi^2 = 19{\cdot}4$; with 1 D.F., $P < 0{\cdot}001$), so that in terms of the *numbers* of inclusions revealed, the FA method holds a considerable advantage.



Text-fig. 1. Frequency distribution of numbers of inclusions in duplicate thin smears stained by iodine or by FA.

Failure of inclusions to stain with FA

Comparative inclusion counts were made on slides stained first by FA and then by Giemsa. The second count was usually the higher, sometimes by as much as twofold; and in a few of our many paired photographs it was apparent that some typical inclusions that stained with Giemsa had not previously stained with FA (Plate 1*e*, *f*).

Sequential staining with FA and Giemsa

Efforts were made in the early part of these researches to verify by subsequent Giemsa staining all inclusions that were detected by the FA method. At that time, slides were stained immediately after fixation, and the quality of subsequent Giemsa staining was good. Later on, slides were stored for some time before FA staining, and both cells and inclusions stained poorly with Giemsa. Table 9 shows the effects of varying the time and temperature of storage on replicate slides from two patients. The ability to stain with FA was best preserved at 4° C. or lower; at −60°C. (not shown in the table) it was unimpaired after 3 years. By contrast, subsequent Giemsa staining was best in preparations held at room temperature. For stored slides the most satisfactory results were eventually obtained with the following procedure, to which unsuccessful attempts to follow the FA stain with

iodine made an unexpected contribution. The unfixed scrapings were stored at −60° C. For FA staining they were thawed, fixed at room temperature in acetone for 10 min. followed by 5% formalin for 2 min., rinsed quickly in PBS and air-dried. After FA staining, the smears were rinsed well in 5% formalin, treated for 10 min. in ammoniated iodine, immersed in methyl alcohol for 30 min., and then stained with Giemsa/May–Grünwald; the best results were obtained by omitting the customary post-stain alcohol rinse.

Table 9. *The influence of time and temperature of storage on the sequential staining of conjunctival scrapings with FA and Giemsa*

Time stored (days)	Patient and eye	Temperature of storage: Room FA*	Room G†	4° FA	4° G	−30° FA	−30° G
1	11 R	3	3	ND		ND	
	11 L	3	3				
	13 R	3	3				
	13 L	3	3				
5	11 R	2	3	3	2	ND	
	11 L	0	0	3	2		
	13 R	2	3	3	2		
	13 L	2	3	3	2		
10	11 R	1	3	3	2	3	1
	11 L	1	3	3	2	3	1
	13 R	2	3	3	2	3	1
	13 L	1	3	3	2	3	1
15	11 R	0	3	3	2	ND	
	11 L	0	3	3	2		
	13 R	0	3	3	2		
	13 L	0	3	3	2		
30	11 R	0	3	3	2	3	1
	11 L	0	3	3	2	3	1
	13 R	0	3	3	2	3	1
	13 L	0	3	3	2	3	1

ND, not done.

* FA stain: inclusions not seen (0), faint (1), moderately bright (2), very bright (3).

† Giemsa/May–Grünwald stain: scored from 0 (cell architecture lost, inclusions unrecognizable) to 3 (cells and inclusions well stained).

DISCUSSION

Before comparing the efficacy of the iodine stain with that of Giemsa, a discussion of certain technical considerations may be of interest.

The advocates of the FA method rightly claim that it has the advantage of serological specificity; on the other hand, the morphology of an iodine-stained inclusion is so characteristic that it can be identified with almost no possibility of error. This statement is based on many years' experience of the confirmation of iodine-stained inclusions by subsequent staining with Giemsa.

We may here refer again to the difficulty – which appears to be a common

https://doi.org/10.1017/S0022172400021963 Published online by Cambridge University Press

experience – of verifying the morphology of inclusions with Giemsa after they have been stained with FA. Storage of the slides at subzero temperatures and their treatment with serum during FA staining both seem to impair the quality of subsequent Giemsa staining. Conversely, occasional inclusions failed to stain with FA, but were proved by serial photography to stain with Giemsa applied afterwards. Perhaps the most likely explanation is that penetration of antiserum or conjugate into the cell was prevented by a layer of mucus or other substance. The heterogeneous reactions of unabsorbed anti-MRC-4*s* sera make it improbable that the failure to stain some inclusions is attributable to infections with mixtures of serotypes, which in any event appear to be very rare (Bell, McComb, Nichols & Roca-Garcia, 1970). For the same reason, it is unlikely that false negatives resulted from the use of a serum prepared against a type 2 TRIC agent. The antiserum to MRC-4*s* grown in BHK-21 cells stained antigens representing the main trachoma serotypes 1, 1b and 2 identified by the Harvard/ARAMCO workers (McComb & Bell, 1967), and serotypes D, E and F that consist mostly of strains isolated from ocular and genital tract syndromes other than trachoma (Alexander, Wang & Grayston, 1967). In this connexion it is interesting to note that in an investigation still in progress, 21 of 52 trachoma agents isolated in Gambian villages were type 1 and 31 were type 2; no type 1b strains were encountered. In Bathurst, the capital of The Gambia, type F agents were isolated from the eye of an infant with neonatal TRIC conjunctivitis, and from the urethra of the father (Sowa, Sowa & Collier, 1968; Collier, Sowa & Sowa, 1969).

We had supposed that the ability to examine much thicker smears with iodine than is possible with FA would increase the chance of finding inclusions; but the comparison of thick and thin iodine-stained smears in study no. 1 suggested that this does not hold true, at least in terms of the number of positive diagnoses.

In considering our findings, the important point must be made that results obtained with the iodine stain are greatly influenced by the technique employed. Hanna and co-workers (1965) found that in 13 experimentally infected volunteers, TRIC inclusions were demonstrated in 11 by FA, in 3 by Giemsa and in none by iodine; but in this investigation the iodine-stained slides were wet-mounted (L. Hanna, personal communication). For best results, it is essential to employ dry preparations; depending on the optical properties of the microscope used, it may be advantageous to cover the smear with a thin coating of immersion oil.

It is apparent that the FA and iodine techniques for staining TRIC inclusions in the conjunctiva each have their advantages and disadvantages. In study no. 1, both methods yielded the same proportion of inclusion-positive specimens; in study no. 2 the proportion demonstrated as positive by FA was higher, but not significantly so. There was, however, a very significant increase in the numbers of inclusions revealed by FA as compared with iodine, probably because antibody stains inclusions at all stages of their formation, whereas iodine stains only mature inclusions that possess a carbohydrate matrix.

The skew distribution of the numbers of inclusions (Text-fig. 1) deserves comment. The frequency rapidly diminished from a peak value of 1–5 inclusions per scraping, and there were few instances of specimens containing from 21–50 inclu-

sions. As determined by the FA test, however, there was a fair number of scrapings with counts of more than 50 inclusions; in some instances, several hundred were present. The finding that certain children are liable to produce large numbers of inclusions recalls observations in artificially infected baboons; some of these animals – not necessarily those with the most severe inflammatory response – respond similarly to infection with TRIC agent (Collier, 1967).

Our conclusions are that where good laboratory facilities are available, FA staining is somewhat preferable to iodine for demonstrating conjunctival inclusion bodies. It should, however, be remembered that the technique demands comparatively elaborate reagents that must be carefully prepared, a first-rate fluorescence microscope, and observers with a high standard of training. Iodine-staining can be done with simple reagents and equipment, is much less time-consuming, and requires less in the way of skilled personnel. Since slides can even be stained and examined in villages, the method is valuable when large numbers of scrapings have to be screened under primitive conditions. It is unlikely that the proportion of positive diagnoses will be much less that with the FA method.

We wish to thank Professor R. Nichols (Harvard School of Public Health) and Dr J. Treharne (Institute of Ophthalmology, London) for providing antigens of known serotype; Dr I. A. Sutherland (M.R.C. Statistical Research and Services Unit) for his advice on the statistical aspects; Mr M. Race for his excellent technical assistance; Mrs Anne Mogg and Mr R. Harris for making the anti-TRIC sera; and Messrs Bie and Berntsen of Copenhagen for kindly providing the plastic slide holders. We are also grateful to Dr I. A. McGregor and his staff at the Medical Research Council Laboratories for the provision of various facilities in The Gambia.

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EXPLANATION OF PLATE

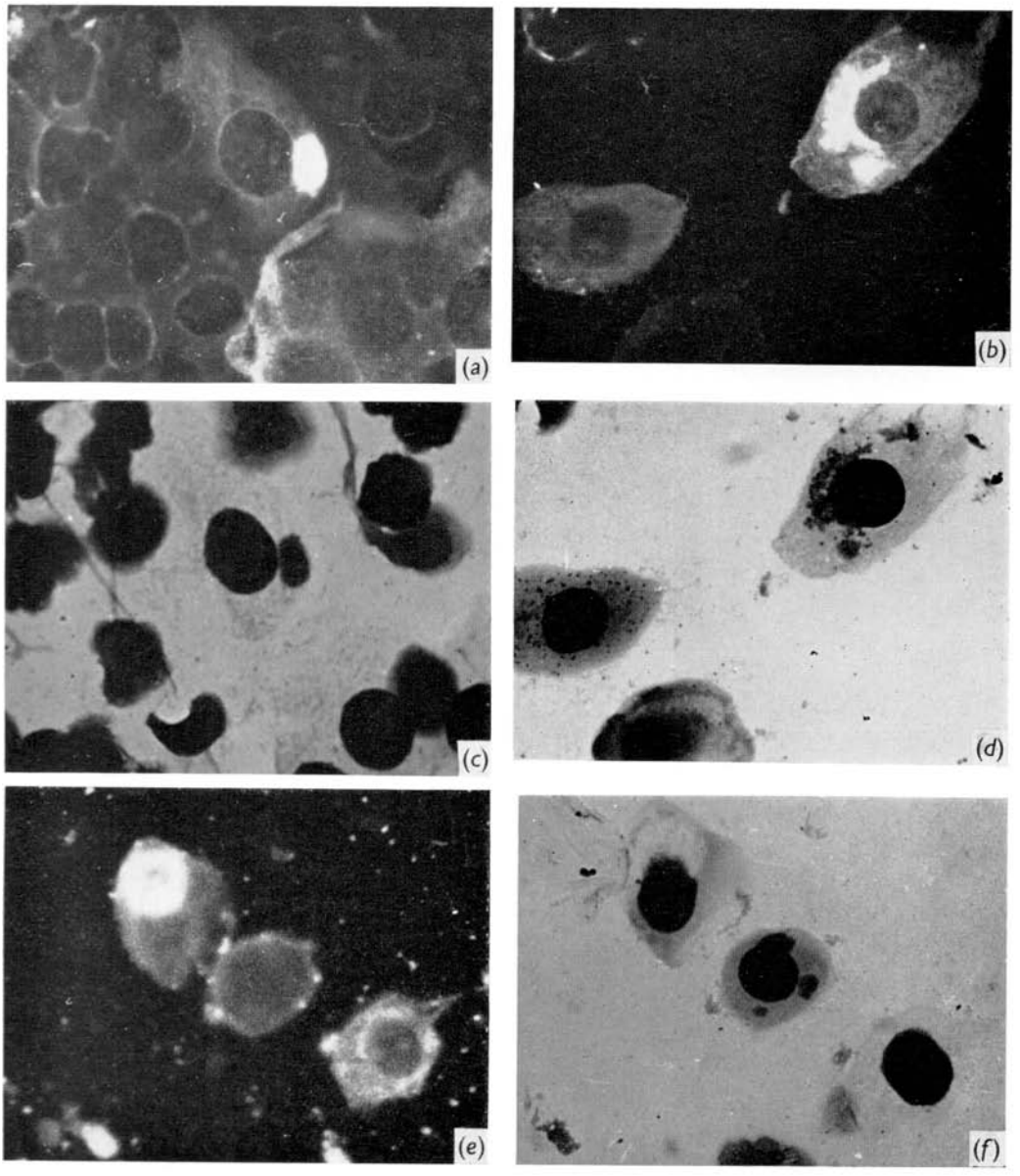
Conjunctival scrapings from trachoma patients, × 2000.

(*a*, *b*) Inclusion bodies within epithelial cells, stained with fluorescent antibody (FA). Bright field illumination; the dark background is due to the filter combination used.

(*c*, *d*) The same cells stained subsequently with Giemsa/May–Grünwald.

(*e*) Staining and illumination as (*a*), (*b*).

(*f*) The same field stained with Giemsa/May–Grünwald; three inclusions that did not stain with FA are now visible in the middle epithelial cell.



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https://doi.org/10.1017/S0022172400021963 Published online by Cambridge University Press