

Trachoma Virus Isolation Studies on Taiwan*

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Studies on trachoma have been carried out on Taiwan since 1958 by the US Naval Medical Research Unit No. 2. Routine methods for the collection of virus specimens, storage and processing of successful virus isolation are presented in this paper.

A special virus isolation study on clinically active cases has shown that 49% positive diagnoses were made at the first examination, 58% at the second and 63% at the third. Detection of virus positive specimens by inclusion bodies alone would have diagnosed only 19%, 30% and 34% of the cases. Combining results of both tests increased the positive diagnosis to 57%, 64% and 68% respectively. Administration of oxytetracycline ophthalmic ointment for two series of intermittent treatments reduced virus isolations by 48% in a single test. The clinical findings in the treated cases showed only minor changes.

The successful isolation of the trachoma virus in the yolk sac of embryonated eggs was first reported by T'ang et al. in 1957. This work was later confirmed by Collier and his associates in the Lister Institute in London (Collier & Sowa, 1958). Since then, the isolation technique has been applied by many laboratories and trachoma viruses have been isolated in numerous countries, such as Saudi Arabia (Snyder et al., 1959), Israel (Bernkopf et al., 1959), Taiwan (Grayston, Wang & Johnston, 1960), the USA (Hanna et al., 1959), India (Agarwal et al., 1960) and Australia (Mann et al., 1960).

An improved technique for trachoma virus isolation was published in 1960 by Sowa & Collier. Their main improvements were to reduce bacterial contamination in conjunctivitis cases by treatment with antibiotic ophthalmic ointment, and to inoculate conjunctival scrapings treated with streptomycin directly into chick embryo yolk sacs.

Since 1958 a series of studies on trachoma have been carried out at the US Naval Medical Research Unit No. 2 (NAMRU-2) on Taiwan (Grayston et al., 1960). This paper presents our routine procedures for collection of virus specimens, storage and processing, with special emphasis on certain essential factors used for successful isolation. The results of a special virus isolation study from clinically active trachoma on Taiwan are presented.

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MATERIALS AND METHODS

Collection of specimens

During the clinical examination care was taken by the ophthalmologist to ensure strict aseptic procedures, especially in the collection of eye swab specimens. Most of our failures to isolate viruses have been due to contamination by saprophytic bacteria, which are quite resistant to the concentration of antibiotics used. These bacteria are present in areas other than the upper conjunctivae. In our surveys, the ophthalmologist everts the upper lid of the eye and holds it steady with both hands while a second person takes the specimens. An eye scraping is taken, first from the upper conjunctivae with a small platinum spatula. This material is spread on a slide for staining with Giemsa-May-Grünwald stain and microscopic examination. A small swab is drawn across and rotated back and forth at the same time over the palpebral conjunctivae and upper fornix. The end of the swab is broken off into a small screw-cap vial containing 2.5 ml of cold Snyder's No. 1 medium. The vial is shaken to free tissue cells and is placed in a dry-ice chest for transport to the laboratory after the wax-lined cap has been firmly secured. In the laboratory the specimens are stored at -65°C in a mechanical deep-freeze.

Antibiotics

To control bacterial contamination of the initial inoculum, streptomycin (10 mg per egg) and poly-

myxin B (0.02 mg per egg) are used. Just prior to inoculation the antibiotics are added to the specimen, which is then placed in a refrigerator for four hours. This gives time for the antibiotics to act on any bacteria present in the inoculum.

Egg inoculation

Five- and six-day-old embryonated hens' eggs are used for the first and second passage. The yolk sac of each egg is inoculated with 0.4 ml of the inoculum. It has been our frequent observation that only one or two of the eggs first inoculated demonstrate elementary bodies in the yolk sacs, and that some, but not all, of the harvested yolk sacs show bacterial contamination. The use of as many eggs as possible and careful checking of each individual yolk sac for elementary bodies are important in successful isolation. A long incubation period is usually expected for the first inoculation. It is our routine practice to keep the inoculated eggs as long as possible, for instance, up to 13 or 14 days of observation. The demonstration of elementary bodies by Macchiavello's stain and a positive isolation are more frequently obtained if the eggs are incubated 13 or 14 days, than if the eggs are harvested earlier and blind passages are made. With this procedure, two egg passages for each specimen usually give a conclusive answer. Attempts at further serial passages have not revealed significant additional information.

RESULTS

Applying the method of virus isolation to the specimens collected on Taiwan we have isolated 123 viruses from 412 non-contaminated specimens. These results are shown in Table 1, where it can be seen that the highest isolation rate (44%) was obtained from the specimens of trachoma stage II (103/236). This was followed by trachoma stage III, which had 32% positive, and the trachoma dubium, where 5% of the specimens were positive. No trachoma virus so far has been isolated from the specimens of other trachoma or non-trachoma eye conditions.

A special virus isolation study was carried out on 67 cases of clinically active trachoma on Taiwan. These cases were in first- or second-grade primary-school children, 59 being of trachoma II and 8 of trachoma III. Thirty-eight cases were from Lung-ching area and 29 from Ta-an area of Taichung County. Three consecutive collections of eye swab specimens were made from the same individuals

TABLE 1
VIRUS ISOLATIONS FROM DIFFERENT STAGES
OF TRACHOMA, TAIWAN,
15 OCTOBER 1959 TO 31 MARCH 1961

Clinical diagnosis	No. specimens	No. virus isolates	Percentage positive
Tr-IV	1	0	0
Tr-III	54	17	32
Tr-II	236	103	44
Tr-I	12	0	0
Tr-D	60	3	5
Non-trachoma	49	0	0
Total	412	123	30

at 1½-2-month intervals. A slide of the conjunctiva scraping was concurrently made for each eye when the eye swabs were taken. The first collection of specimens was made from one eye of each of the 67 cases during the period 26 October to 5 November 1960. In the second collection, which was made from 12 to 14 January 1961, the outside lids of the left eye of each individual were washed with a 2% boric acid solution prior to everting the upper lid for specimen collection. Washing of the eyelids was performed in an attempt to see whether minimum contamination of specimens could be obtained. In about two-thirds of the same individuals, specimens were also collected from the unwashed right eye as controls. Sixty-four of 67 children were examined in the second isolation attempts. The third collection of eye cultures was made between 12 February and 4 March 1961, when cultures were made from 63 of the 67 children. When the third collection of specimens was taken, it was learned that the children in Ta-an area had been receiving antibiotic ophthalmic treatment. The ophthalmic therapy consisted of two 5-day courses of oxytetracycline ointment applied two times daily at 3-4 week intervals. The children had just finished their second course of treatment when the third specimens were collected.

A consideration of the results of the virus isolation and slide examination for inclusion bodies in this series of specimens (Table 2) reveals a number of interesting facts. Altogether 231 sets of specimens both for virus isolation and inclusion body examination were collected on three consecutive attempts

TABLE 2
RESULTS OF REPEATED VIRUS ISOLATION STUDIES ON 67 CASES^a OF CLINICALLY ACTIVE TRACHOMA

Virus isolation	First attempt ^b (26 Oct.-5 Nov. 1961; 67 patients)	Second attempt ^b (12-14 Jan. 1961; 64 patients)		Third attempt ^b (25 Feb.-4 March 1961; 63 patients)		Total ^b (67 patients)
		Eyelid washed ^c	Eyelid not washed	Treated ^d (Ta-an)	Not treated (Lung-ching)	
Positive specimens	33 (49%) [8]	19 (31%) [3]	17 (43%) [5]	6 (21%) [2]	18 (53%) [2]	93 (40%) [20]
Negative specimens	25 (37%) [2]	21 (34%) [1]	15 (38%) [2]	19 (66%) [0]	14 (41%) [1]	94 (41%) [6]
Contaminated specimens	9 (13%) [3]	21 (34%) [0]	8 (20%) [1]	4 (14%) [0]	2 (6%) [0]	44 (19%) [4]
Total specimens	67 (100%) [13] (19%)	61 (100%) [4] (7%)	40 (100%) [8] (20%)	29 (100%) [2] (7%)	34 (100%) [3] (9%)	231 (100%) [30] (13%)

^a The 67 cases consisted of 59 cases of Tr-II and 8 of Tr-III; 38 cases were from Lung-ching area and 29 from Ta-an area.

^b The italicized figures in square brackets represent the numbers of smears positive for inclusion bodies in concurrent tests.

^c Eyelid washed with 2% boric acid before specimen collection.

^d Ta-an area was treated with antibiotics after the second course of intermittent therapy.

from the 67 trachomatous children. In 19% bacterial contamination prevented successful isolation of virus from the eye swabs. Washing the eyelid prior to collection of the specimen increased the rate of contamination. In the eyes that were washed 34% of the specimens were contaminated, as opposed to 20% in the eyes that were not washed.

When the numbers of specimens positive for inclusion bodies were compared with the results of concurrent virus isolations, it was found that 10 out of 30, or one-third of the positive inclusion specimens, fell into the undetected virus group of specimens. In other words, our current technique of virus isolation detected only two-thirds of the inclusion-positive specimens. Even though our current technique for virus isolation is sensitive, it failed, in a single examination, to detect one-third of the children excreting trachoma virus. This generalization was supported by the results of the three consecutive examinations.

On the basis of the results of virus isolation and demonstration of inclusion bodies in the smears in the three consecutive surveys, a laboratory diagnosis of the 67 cases was calculated. These data are shown in Table 3. The virus isolation alone gave a 49% positive diagnosis on the first examination, 58% by two examinations and a cumulative positive diag-

nosis of 63% with three examinations over the four-month period. A diagnosis made on the basis of inclusion bodies alone showed only a 19%, 30% and 34% positive laboratory diagnosis. When the results of the two laboratory tests were combined, the corresponding rates of positive diagnosis were 57%,

TABLE 3
LABORATORY DIAGNOSIS BASED ON THREE
ACCUMULATED RESULTS^a OF VIRUS ISOLATION AND
INCLUSION BODY STUDIES ON 67 CASES OF CLINICALLY
ACTIVE TRACHOMA

Diagnosis	No. persons tested: ^b		
	Once	Twice	3 times
Positive virus isolation	33 (49%) [8]	39 (58%) [16]	42 (63%) [20]
Virus negative or unknown	34 (51%) [5]	28 (42%) [4]	25 (37%) [3]
Positive for inclusion bodies	13 (19%)	20 (30%)	23 (34%)

^a Tested during four-month period.

^b The italicized figures in square brackets represent the numbers of smears positive for inclusion bodies in concurrent tests.

TABLE 4

REPRODUCIBILITY OF POSITIVE VIRUS ISOLATIONS AND INCLUSION BODY DEMONSTRATIONS IN THREE ISOLATION ATTEMPTS ON 67 CASES OF CLINICALLY ACTIVE TRACHOMA

Result	Positive ^a at least:		
	Once	Twice	3 times
Positive virus isolation			
Lung-ching area	25/38 (66 %)	18/37 (49 %)	9/33 (27 %)
Ta-an area	17/29 (59 %)	10/28 (36 %)	5/28 (18 %)
Total	42/67 (63 %)	28/65 (43 %)	14/61 (23 %)
Positive for inclusion bodies	23/67 (34 %)	4/65 (6 %)	2/61 (3 %)

^a Expressed as number of persons positive/actual number of persons tested (followed by percentage positive).

64% and 68% respectively. The combination of the two tests and the three attempts gave the highest rates for a positive diagnosis.

In the three virus isolation attempts, shown in Table 4, three successive positive isolations were obtained in 23%, or 14 of the 61 persons tested. Two or more isolations were obtained in 43%, or 28 of the 65 persons, and 63%, or 42 of 67 children, were positive in one of the three isolation attempts. It should also be noted that the virus isolation attempts gave not only a higher percentage positive rate but a more reproducible result than the demonstration of inclusion bodies on eye smears. The presence of inclusion bodies on the slides actually established the diagnosis in 3%, 6% and 34% of the trachoma cases respectively. A perusal of the trachoma virus isolation data in NAMRU-2 revealed that five of the children in the present study had trachoma virus isolated from previous eye cultures. Two of the five children had trachoma virus isolated in January, June and November of 1960 and in January of 1961. From the other three children virus was isolated in June and November of 1960 and again in January 1961. It was interesting to note that the trachoma virus was shed over a period of one year. This could be interpreted as the infectious period and these

TABLE 5

INFLUENCE OF LOCAL ANTIBIOTIC TREATMENT ON LABORATORY-CONFIRMED CASES OF CLINICALLY ACTIVE TRACHOMA

Area	No. of laboratory-confirmed cases ^a	Cases virus-positive after treatment ^b	
		No.	%
Ta-an (treated)	16	6	38
Lung-ching (control)	21	18	86

^a Cases confirmed by positive virus isolation, demonstration of inclusion bodies, or both.

^b After second five-day cycle of intermittent treatment with 1% oxytetracycline ophthalmic ointment.

children could have been spreading the virus to other contacts. All of the viruses isolated from each child over the period of one year were the same, as determined by the mouse toxicity test.

The third isolation attempt in this group of 67 trachomatous children was the most interesting of the three. In the third attempt, as shown in Table 2, there was a definitely lower virus isolation rate from Ta-an area, which received antibiotic treatment, than from Lung-ching area. When the percentages of positive virus isolations in both areas were calculated on the basis of the numbers of laboratory-confirmed cases, Lung-ching area had an 86% isolation rate as compared with only 38% for Ta-an area (Table 5). A laboratory-confirmed case was defined here as a case positive on virus isolation and/or positive for inclusion bodies in the two previous surveys. There was a 48% reduction in a single test of the virus positive cases treated in Ta-an area as compared with Lung-ching area. This may be ascribed to the effect of a two-month period of antibiotic treatment given to these children. Even though there was a reduction in isolation of viruses from Ta-an area, it was evident that two periods of intermittent treatment were insufficient. Also, it was found that the clinical findings for these cases were not greatly changed. These data demonstrate that virus isolations may be useful in evaluation of treatment not readily assessed by clinical observations alone.

RÉSUMÉ

Depuis que la culture sur œuf embryonné du virus du trachome permet de compléter les observations cliniques, elle est appliquée à l'étude épidémiologique de la maladie

et à l'évaluation des effets du traitement par les antibiotiques.

C'est dans ce but que les auteurs ont procédé à une

enquête, auprès d'enfants, à Taïwan: 123 virus ont été isolés de 412 prélèvements (non contaminés par des bactéries); 44% provenaient de trachome au stade II, 32% du stade III. Les cas qui ont fourni les virus étaient plus aigus, cliniquement, que la moyenne de ceux que l'on rencontre à Taïwan. Parmi ces cas, on a étudié de façon plus approfondie 59 cas de trachome II et 8 de trachome III, en relation avec les observations cliniques. Les résultats montrèrent que la culture du virus n'était positive que pour deux-tiers seulement des cas avec inclusions. L'isolement du virus, dans ces 67 cas, a été positif pour 49% lors du premier examen, 58% pour deux

examens, et 63% pour trois examens; 14 sur 61 furent positifs aux trois examens, 28 sur 65 à deux examens.

Une pommade ophtalmique à l'oxytétracycline fut administrée en deux séries de traitement aux enfants d'un des groupes étudiés. Une réduction de 48% a été observée sur le nombre des virus isolés, après traitement, des mêmes enfants, alors que, cliniquement, on n'observait que de faibles changements. La technique d'isolement du virus peut donc apporter un complément d'information utile, les résultats du traitement ne pouvant être facilement évalués par le seul examen clinique.

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