*J. Hyg., Camb.* (1971), **69**, 709
*Printed in Great Britain*



# Sensitivity of trachoma agent to streptomycin and related antibiotics*

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(*Received* 23 *June* 1971)

## SUMMARY

For isolating trachoma (TRIC) agent from conjunctival scrapings, both streptomycin and neomycin were effective in preventing bacterial contamination, but at high concentrations neomycin was rather more inhibitory to TRIC agent. Prolonging storage of scrapings with neomycin at 4° from 30 min. to 24 hr. reduced the bacterial contamination rate, but also diminished the chance of isolating TRIC agent at the 1st passage. Two freshly isolated TRIC agents differed in their susceptibility to neomycin and streptomycin. Kanamycin and framycetin appeared to be less suitable than streptomycin for use in isolation of TRIC agent. In isolation studies, the possibility of inhibiting TRIC agent by high concentrations of antibotics, including streptomycin, should be borne in mind.

## INTRODUCTION

Trachoma is so frequently associated with bacterial infection of the conjunctiva that isolation of its aetiological agent was possible only by using streptomycin (T'ang, Chang, Huang & Wang, 1957; Collier & Sowa, 1958; Sowa & Collier, 1960), neomycin (Sowa, Sowa, Collier & Blyth, 1965) and other antibiotics singly or in combination to inhibit bacterial contaminants (e.g. Collier, Duke-Elder & Jones, 1960; Hanna *et al.* 1962; Holt *et al.* 1967). These drugs did not seem to inhibit trachoma/inclusion conjunctivitis (TRIC) agents propagated in yolk sac; but the comparatively low isolation rates often reported suggested the possibility that some failures at least were due to inhibition of TRIC agents by drugs to which they were supposedly completely resistant. The investigation described here was undertaken to test this assumption and to determine whether other antibiotics related to streptomycin would be more suitable for isolating TRIC agents.

## MATERIALS AND METHODS

*Diluent.* Sucrose potassium glutamate (SPG) was used as diluent (Bovarnick, Miller & Snyder, 1950). It was sterilized by autoclaving at 5 lb./in.$^2$ pressure for 10 min.

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*Antibiotics* were obtained as sterile powders for injection and were dissolved in diluent. Streptomycin sulphate (Streptomycin); Diston Products Ltd.; potency 740,000 $\mu$g./g. Neomycin sulphate (Mycifradin); Upjohn Ltd. potency 700,000 $\mu$g./g. Framycetin sulphate (Framygen); Genatosan Division of Fison Pharmaceuticals Ltd.; potency not stated. Kanamycin sulphate (Kantrex); Bristol Laboratories Ltd.; potency not stated.

*Conjunctival scrapings* from children with active trachoma were examined for the presence of inclusions by the ammoniated iodine method of Sowa *et al.* (1965). For isolating TRIC agent pooled scrapings from both eyes were collected into 2 ml. diluent, disrupted in a mechanical homogenizer (Measuring and Scientific Equipment Co. Ltd.) at full speed for 1 min. and divided into 4 aliquots. To each, 0·5 ml. of the appropriate antibiotic solution was added after which it was kept at 4° C. for 30 min. In comparative experiments some specimens with neomycin were tested after storage at 4° C. for 24 hr.

Each sample was inoculated into three embryos; three blind passages were made before any result was accepted as negative.

*Chick embryo inoculation.* Fresh hens' eggs were collected in villages, washed briefly in soapy water, rinsed quickly in running tap water and dried on a clean towel. They were incubated at 37° C. and 50% relative humidity with one daily turning, and were candled at 3 days and again just before inoculation on the 6th day. The embryos were inoculated by the yolk-sac route and were incubated at 35° C. and 50% humidity. After inoculation they were first candled at 48 hr., when dead embryos were discarded, and thereafter daily until the 12th or 14th day, depending on the experiment. All yolk sacs harvested after 48 hr., including those of survivors, were examined for elementary bodies by staining smears with Giemsa/May–Grünwald.

For passage the entire yolk sac was placed in a screw-cap bottle with an equal weight of SPG. After homogenization as described above, the suspension was inoculated into the next set of eggs immediately, or after storage at $-60°$ C.

*Suspensions of TRIC agent for use in testing antibiotics.* Partly purified stocks were prepared from two recently isolated strains and from an earlier stored strain, all in their third egg passage. Their full designations (Gear, Gordon, Jones & Bell, 1963) are WAG/MRC-51/OT, WAG/MRC-84/OT and WAG/MRC-60/OT. They are referred to in the text by their MRC numbers only. These strains were grown in the presence of the concentration of the antibiotic with which they were originally isolated. Chick embryos were harvested on the day of death. After shaking each yolk sac by hand in 10 ml. SPG the membranes were discarded; the fluids were pooled and centrifuged for 10 min. at 200***g*** at 10° C. The deposit was discarded and the supernatant was centrifuged at 4500***g*** for 1 hr. at 10° C.; the deposit containing the TRIC agent was resuspended in SPG to give half the volume of the original suspension, i.e. about 20% dilution of the yolk sac. The stock suspensions were stored at $-60°$ C. in 1 ml. amounts. When required they were thawed rapidly in a water bath at 37° C. and suitably diluted.

*Infectivity titrations.* The suspensions were titrated without antibiotic by inoculating tenfold dilutions into groups of 10–12 embryos which were then incu-

bated for 11 days. The 50% infective dose (EID 50) was calculated by the method of Reed & Muench (1938).

*Single dilution titrations.* Inocula were prepared by diluting the stock suspension 1/100. Samples treated with a 'high' concentration of an antibiotic (see under 'Results') for 30 min. at 4° C. and controls without antibiotic were inoculated into large groups of embryos, usually about 30 in number. They were candled every 12 hr. for 14 days. In calculating the average day of death embryos still alive on day 14 were counted as dying on day 15.

*Assays of antibiotic activity.* Tenfold dilutions of each drug were each mixed with a 1/100 suspension of MRC-60 and inoculated immediately into groups of about 12 embryos. The eggs were incubated for 12 days thereafter; embryos surviving for this time were counted as dead on day 13. The index of protection was calculated according to the following formula, which allows for embryos surviving uninfected for the maximum period:

$$\text{index of protection} = \frac{MS_t - MS_c}{MS_m - MS_c} \times 100,$$

where $MS_t$, $MS_c$ are the mean survival times of the test and control embryos respectively and $MS_m$ is the maximum possible survival time after inoculation (13 days in these tests).

## RESULTS

### *Isolation of TRIC agent from conjunctival scrapings*

#### *Influence of neomycin and streptomycin*

The amount of TRIC agent present in conjunctival scrapings was insufficient to permit titrations of sensitivity to antibiotics. In consequence sensitivity was assessed by comparing isolation results from replicate samples of conjunctival

Table 1. *Isolations of TRIC agent from replicate scrapings inoculated into chick embryos together with high and low concentrations of streptomycin and neomycin*

| Concentration of antibiotic (μg./egg) | No. of replicate specimens | Specimens positive at passage no. 1 | 2 | 3 | Total no. of isolations | Isolation failures due to bacterial contamination | Isolation rate |
|---|---|---|---|---|---|---|---|
| Neomycin | | | | | | | |
| 11,250 | 83 | 21 | 6 | 2 | 29 | 2 | 29/81 (35·8%) |
| 112 | 83 | 31 | 2 | 0 | 33 | 4 | 33/79 (41·7%) |
| Streptomycin | | | | | | | |
| 10,713 | 39* | 15 | 2 | 0 | 17 | 0 | 17/39 (43·6%) |
| 107 | 39* | 17 | 0 | 0 | 17 | 1 | 17/38 (44·6%) |

* Common to the neomycin series.

scrapings to which either 'high' or 'low' concentrations of the antibiotics were added. To assure even dispersion each specimen was homogenized for 1 min.; infectivity was not increased by extending this time. The 'high' concentrations

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of neomycin (11250 μg./egg) and of streptomycin (10713 μg./egg) were the greatest that could be obtained; the 'low' concentrations were 1/100 dilutions of the high doses.

Thirty-nine specimens were common to the streptomycin and the neomycin series; another 44 were tested in the presence of neomycin alone. At the high concentration of neomycin, which none the less was insufficient to eliminate all bacterial contamination, there were four fewer isolations than from replicate specimens treated with the low dose of the drug. Streptomycin under similar conditions was less detrimental. The inhibitory effect was also evident from the delayed appearance of the TRIC agent, sometimes until the second or third embryo passage (Table 1).

Table 2. *The effect of neomycin and streptomycin on infection of the individual embryos with TRIC agent at the first passage*

| Concentration of antibiotic (μg./egg) | No. of embryos inoculated with 'potentially positive' specimens* | No. of valid inoculations† | Embryos infected with TRIC agent |
|---|---|---|---|
| Neomycin | | | |
| 11,250 | 39 × 3 = 117 | 107 | 37 (34·6 %) |
| 112 | 39 × 3 = 117 | 95 | 61 (64·2 %) |
| Streptomycin | | | |
| 10,713 | 19 × 3 = 57 | 53 | 32 (60·4 %) |
| 107 | 19 × 3 = 57 | 51 | 35 (68·6 %) |

* I.e. from which TRIC agent was isolated at any concentration of either antibiotic.
† I.e. excluding deaths due to non-specific causes or bacterial contamination.

Table 3. *Isolation of TRIC agent from inclusion-positive and inclusion-negative specimens in the presence of neomycin and streptomycin*

| Concentration of antibiotic (μg./egg) | Nos. of specimens: Total | Isolation-positive | Contaminated | Isolation rate |
|---|---|---|---|---|
| | (*a*) Inclusion-positive patients | | | |
| Neomycin | | | | |
| 11,250 | 17 | 14 | 1 | 14/16 (87·5 %) |
| 112 | 17 | 15 | 1 | 15/16 (93·8 %) |
| Streptomycin | | | | |
| 10,713 | 6 | 6 | 0 | 6/6 (100·0 %) |
| 107 | 6 | 5 | 1 | 5/5 (100·0 %) |
| | (*b*) Inclusion-negative patients | | | |
| Neomycin | | | | |
| 11,250 | 22 | 15 | 1 | 15/21 (71·5 %) |
| 112 | 22 | 18 | 3 | 18/19 (94·7 %) |
| Streptomycin | | | | |
| 10,713 | 13 | 11 | 0 | 11/13 (84·6 %) |
| 107 | 13 | 12 | 0 | 12/13 (92·3 %) |

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*Decrease in number of positive embryos in the primary passage.* The figures in Table 2 are based only on embryos inoculated with 'potentially positive' specimens, i.e. those from which TRIC agent was isolated at any concentration of either antibiotic. With the low concentration of neomycin nearly twice as many embryos became infected as with the high; with both high and low concentrations of streptomycin the percentages of positive chick embryos were similar to that obtained with the lower dose of neomycin (Table 2).

Table 4. *Isolation of TRIC agent in embryonated eggs after storage of the scrapings at 4° C. in the presence of neomoycin*

| Concentration of neomycin (μg./egg) | No. of replicate specimens | Exposure to neomycin at 4° C. before inoculation (hr.) | No. of isolations: At passage 1 | 2 | 3 | Spoilt by contamination | Isolation rate |
|---|---|---|---|---|---|---|---|
| 11,250 | 44 | 0·5 | 14 | 1 | 2 | 1 | 17/43 (39·6%) |
| 112 | 44 | 0·5 | 18 | 0 | 0 | 2 | 18/42 (43·8%) |
| 11,250 | 44 | 24·0 | 10 | 2 | 3 | 0 | 15/44 (34·1%) |
| 112 | 44 | 24·0 | 13 | 3 | 0 | 0 | 16/44 (36·4%) |

*Isolation rates from inclusion-positive and inclusion-negative patients.* From inclusion-positive patients two isolations were lost at high and one at low concentrations of neomycin (not counting losses from bacterial contamination). Only one isolation was lost, and this by contamination, from material treated with streptomycin. The inhibitory effect of both antibiotics on TRIC agent was somewhat higher, but not significantly so, in the inclusion-negative series (Table 3).

*Treatment of scrapings with neomycin at 4° C.* The scrapings used in the experiments described above were obtained from patients at the laboratory; in field work it is often necessary to store them on ice from 6 to 24 hr. before inoculating chick embryos. These conditions were simulated by comparing the isolation rates from specimens left in contact with two concentrations of neomycin for 30 min. with that from replicate scrapings stored for 24 hr. at 4° C. It was impracticable to test for isolations without any antibiotic on account of bacteria which are always present in these specimens. With either concentration of neomycin prolonging storage from 30 min. to 24 hr. diminished the chance of isolating TRIC agent in the first passage, but the influence of storage is less pronounced if subsequent passages are taken into account (Table 4). With specimens stored for 30 min., three attempts at isolation were spoiled by bacterial contamination, whereas none were spoiled with those stored for 24 hr.

## *Inhibition by antibiotics of TRIC agent* in ovo

*Prolongation of mean survival times by neomycin and streptomycin.* Strain MRC-51 was isolated only at the 'low' concentration of streptomycin but not with the 'high' concentration, and with neither concentration of neomycin; likewise, strain MRC-84 was isolated only with the lower concentration of neomycin and with neither 'low' nor 'high' concentrations of streptomycin. To test the assumption

that failure to isolate them at the higher concentrations of these drugs was due to unusually pronounced sensitivity, they were titrated in eggs with the 'high' concentrations by the single dilution method. Four replicate determinations were made with each antibiotic, and four without, for each strain. Fig. 1 compares the results with serial dilution titrations of the two strains without antibiotic. The effects of neomycin and streptomycin were similar and are not shown separately; the vertical bars show the ranges of results obtained in the single dilution titrations. The prolongation of survival time afforded by these antibiotics to embryos inoculated with MRC-84 was comparable to that obtained by diluting the inoculum a hundred-fold; the effect of these drugs on MRC-51 was much less.

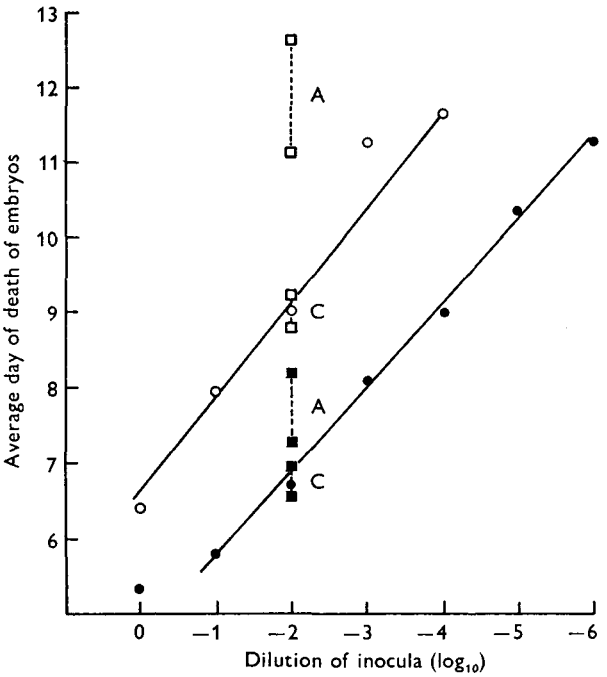


Fig. 1. Dose–response curves of MRC-51 ($10^{4\cdot6}$ EID 50 undiluted) and MRC-84 ($10^{4\cdot3}$ EID 50 undiluted), compared with single dilution titrations in the presence of high concentrations of neomycin or streptomycin. Closed symbols, MRC-51; open symbols, MRC-84; circles, dose–response curves. Squares, single dilution titrations; A, with antibiotics; C, controls. The dotted vertical bars represent the ranges of four separate determinations for each antibiotic, and four control tests without antibiotic.

*The effect of framycetin and kanamycin.* Isolation of TRIC agents would be facilitated by the use of an antibiotic to which they were completely resistant, but which was active against a wide range of bacteria; with this in mind framycetin and kanamycin were compared with streptomycin and neomycin, to which they are related. For this experiment, strain MRC-60 was used; it was isolated in the presence of neomycin, 700 $\mu$g. per embryo. Fig. 2 shows that both kanamycin and framycetin were more inhibitory than neomycin and streptomycin. From this

point of view, the two last-named drugs appear to be the more suitable for use in isolating TRIC agents; but in preliminary experiments neither of them in a dose of 160 $\mu$g. per embryo protected against a 'streptomycin-sensitive' staphylococcus injected at the same time. Low doses of these antibiotics are thus unlikely to suppress bacterial contamination effectively.

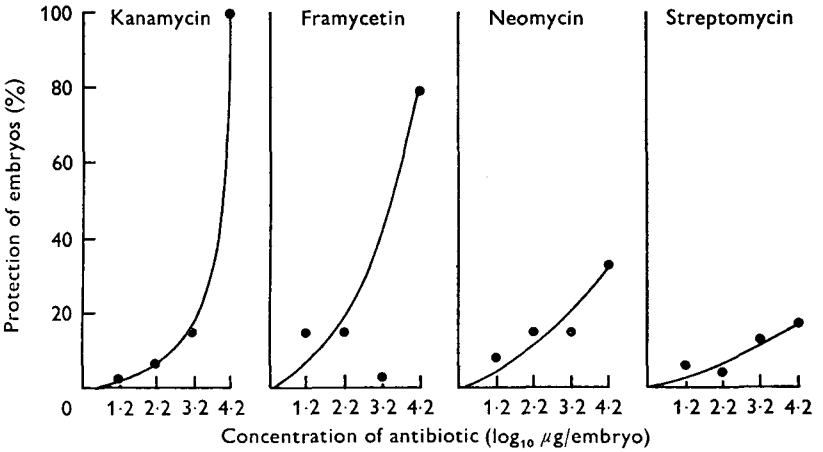


Fig. 2. Dose–response curves representing protection of chick embryos inoculated with $10^{3\cdot2}$ EID 50 of TRIC agent MRC-60. At the $10^{3\cdot2}$ $\mu$g dose of framycetin, 8/10 embryos died within 2 days, probably because of bacterial contamination.

## DISCUSSION

T'ang and co-workers (1957) were the first to point out the 'viricidal' action of several drugs on TRIC agent in egg culture. A notable exception was streptomycin, which they found 'frankly negative'; other workers reached the same conclusion and high concentrations of this drug alone were often used to assist isolation (Collier & Sowa, 1958; Grayston *et al.* 1960; Murray, Guerra, Abbot & McComb, 1962). Bacterial contamination was still troublesome however, and neomycin (Sowa *et al.* 1965) or other antibiotics in various combinations were used (e.g. Holt *et al.* 1967). Streptomycin, which it was generally agreed did not inhibit TRIC agent in embryonate eggs, reduced the number of inclusions formed in chick entodermal culture (Gordon & Quan, 1962). As the embryonate egg still remains in general use for isolating TRIC agents from natural and experimental infections and the success rate is often comparatively low, an improvement of the method would be of considerable value.

The inoculum, consisting of conjunctival epithelium, inflammatory cells, tears and mucus usually contains a variety of bacteria that multiply in the embryo more rapidly than TRIC agent, and as both may be affected by antibiotics, the TRIC agent isolation success rate will depend on differences in sensitivity. Tarizzo & Nabli (1963) tested a number of antibiotics against a variety of TRIC agents, all of which were relatively insusceptible to neomycin, framycetin and kanamycin; however, our experience suggests that none of these drugs was more suitable than

streptomycin, and even this should be used in moderate concentration and not added until the time of inoculation. By way of practical recommendation we may here refer to a recent study (unpublished) in which 53 scrapings from trachoma patients were cultured in the presence of streptomycin 400 $\mu$g./egg. The rate of isolation was 70% and only two attempts failed because of bacterial contamination; this compares favourably with our best results in the past (Sowa *et al.* 1965).

We are grateful to Dr Shiona Sowa for undertaking the clinical examinations and for collecting the conjunctival scrapings; and to Professor L. H. Collier for his help in preparing this paper for publication. We also thank Dr I. A. McGregor and his staff at the Medical Research Council Laboratories, Fajara, The Gambia for various facilities.

## REFERENCES

BOVARNICK, M. R., MILLER, J. C. & SNYDER, J. C. (1950). The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsiae. *Journal of Bacteriology* **59**, 509.

COLLIER, L. H., DUKE-ELDER, S. & JONES, B. R. (1960). Experimental trachoma produced by cultured virus. Part II. *British Journal of Ophthalmology* **44**, 65.

COLLIER, L. H. & SOWA, J. (1958). Isolation of trachoma virus in embryonate eggs. *Lancet* i, 993.

GEAR, J. H. S., GORDON, F. B., JONES, B. R. & BELL, S. D. (1963). Nomenclature of isolates of virus from trachoma and inclusion blennorrhoea. *Nature, London* **197**, 26.

GORDON, F. B. & QUAN, A. L. (1962). Drug susceptibilities of the psittacosis and trachoma agents. *Annals of the New York Academy of Sciences* **98**, 261.

GRAYSTON, J. T., WANG, S.-P., WOOLRIDGE, R. L., YANG, Y.-F. & JOHNSTON, P. B. (1960). Trachoma: studies of etiology, laboratory diagnosis, and prevention. *Journal of the American Medical Association* **172**, 1577.

HANNA, L., ZICHOSCH, J., DAWSON, C., THYGESON, P. & JAWETZ, E. (1962). Isolation of viruses from inclusion conjunctivitis in the newborn. *American Journal of Ophthalmology* **53**, 774.

HOLT, S., PEDERSON, A. H. B., WANG, S.-P., KENNY, G. E., FOY, H. M. & GRAYSTON, J. T. (1967). Isolation of TRIC agents and mycoplasma from the genito-urinary tracts of patients of a venereal disease clinic. *American Journal of Ophthalmology* **63**, 1057.

MURRAY, E. S., GUERRA, P., ABBOTT, A. G. & MCCOMB, D. E. (1962). Isolation of viruses of trachoma from patients in Ethiopia. *Annals of the New York Academy of Sciences* **98**, 14.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating 50% end points. *American Journal of Hygiene* **27**, 493.

SOWA, J. & COLLIER, L. H. (1960). Isolation of trachoma virus from patients in West Africa. *Journal of Hygiene* **58**, 99.

SOWA, S., SOWA, J., COLLIER, L. H. & BLYTH, W. A. (1965). Trachoma and allied infections in a Gambian village. *Special Report Series, Medical Research Council*, no. 308.

T'ANG, F.-F., CHANG, H.-L., HUANG, Y.-T. & WANG, K.-C. (1957). Studies on the etiology of trachoma with special reference to isolation of the virus in chick embryos. *Chinese Medical Journal* **75**, 429.

TARIZZO, M. L. & NABLI, B. (1963). Études sur le trachome. 7. Action comparée des antibiotiques sur différents virus du groupe du trachome cultivés dans les œufs embryonnés. *Bulletin of the World Health Organization* **29**, 289.