Severe endemic trachoma in Tunisia

1. Effect of topical chemotherapy on conjunctivitis and ocular bacteria

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In areas where trachoma occurs as an endemic, blinding disease, it is often associated with bacterial conjunctivitis which occurs in spring and autumn epidemics (Wilson, 1932; W.H.O., 1973; Maxwell-Lyons, 1953; Huet, 1958). It is accepted that trachoma is due to infection with a member of the Chlamydia (psittacosis-lymphogranuloma) group of organisms, but when it occurs in the absence of bacterial conjunctivitis, as is now the case in American Indians, the disease rarely leads to major visual loss (Hoshiwara, Powers, and Krutz, 1971). Although the ways in which bacterial conjunctivitis and trachoma interact have not been determined, it has been suggested that one of the principal mechanisms of topical chemotherapy in severe endemic trachoma is the suppression of bacterial conjunctivitis which reduces the blinding complications of trachoma (Reinhards, Weber, Nižetić, Kupka, and Maxwell-Lyons, 1968).

In Tunisia, trachoma appears to have diminished considerably in severity in recent years, although it continues to be a public health problem in certain regions where economic development has been slow. In two villages in the south of Tunisia with severe trachoma and seasonal epidemics of purulent conjunctivitis, we have studied the bacterial flora of the conjunctiva and the changes in ocular bacteria before and during a chemotherapy trial for trachoma with topically applied chlortetracycline or erythromycin or boric acid.

Material and methods

POPULATION STUDIED

The subjects lived in two pre-Saharan oases (villages A and B) about two kilometres apart in southern Tunisia. The climate is dry throughout the year with temperatures varying from 21° to 44°C. in summer and from −3° to 24°C. in winter. Sand storms lasting one or more days are frequent throughout the year and result in considerable superficial ocular trauma. The traditional economic base of these communities is the cultivation of dates and vegetables in the irrigated oasis, each family owning about half a hectare (one acre) of irrigated land. Many adult men work in other parts of Tunisia or

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in Europe and remit part of their earnings to their families who remain in the villages. Most of the houses are constructed of earth, and are located outside the shaded, cultivated part of the oasis. Drinking water is obtained from public fountains from artesian wells. Above ground latrines are located near most houses and in the fields.

Acute mucopurulent conjunctivitis among young children begins in May and reaches an epidemic peak in October each year, when it subsides with the onset of cooler weather. Flies are particularly numerous from May to October and they appear to cluster on the eyes of children, particularly those with purulent conjunctivitis. The principal programme for the control of trachoma consists of the administration of chlortetracycline ointment to the conjunctivae of first and third grade schoolchildren twice daily for five consecutive days each month from January to June.

During the year before the study reported here, the entire population of village B (1,880 persons) had been examined with a slit lamp for disease of the external eye by an ophthalmologist experienced in trachoma diagnosis. In this village, all children had evidence of trachoma by age 2 and in adults over 30 years of age severe corneal scarring was found in 15 per cent. and economic blindness (visual acuity of 20/400 or less) in 14 per cent.

In village A, 6- to 9-year-old children entering school for the first time in October, 1970, were examined; 85 per cent. (151 individuals) were found to have active trachoma, and were included in the study. In nearby village B, a group of families comprising 200 individuals had been randomly selected for special study. From these families 44 children under 15 years of age were selected at random for the study.

**SPECIMEN COLLECTION, CULTURE MEDIA, AND PLATING PROCEDURE**

To obtain bacterial cultures of the eyes, both lower conjunctival sacs (but not the lid margins) of each patient were swabbed with a single cotton-tipped applicator moistened with nutrient broth. Swabs were streaked immediately onto one plate of sheep’s blood agar and one plate of Fildes’ peptic digest of sheep’s blood, which was used specifically for the isolation of *Haemophilus sp.* (Fildes, 1920). One-half of each plate was streaked with the initial swab, and a second sterile, dry applicator was then passed through the initial streak and used to inoculate the remaining half. Each inoculated plate was sealed with masking tape and placed in a 37°C. incubator within 1 to 6 hours. All plates were incubated for 48 to 72 hrs before being read.

**GRADING GROWTH ON CULTURE PLATES**

The amount of bacterial growth on a plate was graded as follows:

- **No growth** None to two colonies
- **Light growth** Three to 30 colonies
- **Moderate growth** Thirty to 100 colonies
- **Heavy growth** More than 100 colonies to completely confluent growth

This grading system was applied to each individual species on the plate and to the overall bacterial growth.

**IDENTIFICATION OF BACTERIAL SPECIES**

In addition to identifying the organisms by their colonial characteristics and Gram staining, we used the following special preparations or tests when indicated: Optochin discs for pneumococcus; oxidase discs for *Neisseria*; coagulase tests for staphylococcus, and haemagglutination tests for *Haemophilus aegyptius* (Pittman and Davis, 1950; Davis, Pittman, and Griffitts, 1950; Smith, 1954). *Haemophilus* strains originally isolated from cases of seasonal conjunctivitis in California were tested later in San Francisco using the same haemagglutination technique and the same donor of O-positive red cells (Dawson, 1960).

**DIRECT EXAMINATION OF CONJUNCTIVAL SCRAPINGS**

Before treatment (on November 25 and 26), conjunctival smears were taken from the anaesthetized lower conjunctival sacs of 117 children in village A. These smears were air dried and fixed in absolute
methyl alcohol for 5 min. at room temperature, and later stained with Giemsa. Each slide was examined for at least 30 min. for the presence or absence of chlamydial inclusion bodies and bacteria.

**Grading of the Conjunctivitis**

The clinical severity of the purulent conjunctivitis was estimated according to the following scale:

*None or minimal conjunctivitis:* Normal conjunctiva, or minimal hyperaemia and follicular reaction in lower conjunctiva; no discharge.

*Mild conjunctivitis:* Some hyperaemia; minimal mucoid discharge or thread of purulent discharge in inferior cul-de-sac.

*Moderate conjunctivitis:* Marked hyperaemia; purulent discharge on lid and in inferior cul-de-sac.

*Severe conjunctivitis:* Marked bulbar hyperaemia with swelling of lids and periorbital area; copious purulent discharge.

**Medications**

The children were treated topically with ointments containing either 1 per cent. erythromycin,* or 5 per cent. boric acid,† or 1 per cent. chlortetracycline.‡

**Drug Administration**

The 151 children with active trachoma in village A were divided into three groups: 50 received boric acid ointment, 49 received chlortetracycline ointment, and 52 received erythromycin ointment.

The 44 children in village B were followed without any specific therapy.

In village A the treatments were administered twice during the single 3-hr school period, 6 days a week for 10 consecutive weeks from 2 December, 1970, to 13 February, 1971. About 0·1 g. ointment was given in each dose. The ointment was given by assistants trained as part of the government's trachoma control programme. To assure accuracy, each assistant dispensed only one kind of ointment. The children were distributed in six classes, and at least two ointments were used in each class.

**Schedules of Bacterial Culture and Clinical Examination**

The collection of specimens, estimation of conjunctivitis, and treatment is summarized in the following schedule:

<table>
<thead>
<tr>
<th>Date</th>
<th>Procedure</th>
<th>Weeks from start of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov 26–28, 1970</td>
<td>Bacterial cultures, Giemsa smears, Estimate conjunctivitis</td>
<td>-1</td>
</tr>
<tr>
<td>Dec 2–12</td>
<td>Treatment</td>
<td>0 to +2</td>
</tr>
<tr>
<td>Dec 14–15</td>
<td>Bacterial cultures, Estimate conjunctivitis</td>
<td>+2</td>
</tr>
<tr>
<td>Dec 16, 1970 to Feb 13, 1971</td>
<td>Treatment</td>
<td>+2 to +10</td>
</tr>
</tbody>
</table>

All bacterial cultures taken during the treatment period were collected between 18 and 36 hrs after the last previous treatment. The severity of the conjunctivitis was estimated at the times of specimen collection in November and again in December after 10 days' therapy.

* Erythromycin ophthalmic ointment—Eli Lilly Co.
† 5 per cent. boric acid ointment—Eli Lilly Co.
‡ Prepared by the Pharmacie Centrale in Tunis.
Results

Changes in clinical conjunctivitis

While the incidence of overt conjunctivitis in the group treated with boric acid declined only slightly after 10 days of treatment, there was a dramatic decrease in conjunctivitis in the two antibiotic-treated groups with two cases persisting in the chlortetracycline group and only one in the erythromycin group (Table I). This decrease suggested that most of the conjunctivitis observed was caused by micro-organisms sensitive to the action of antibiotics. It was the purpose of this study, then, to examine the role of bacteria in this epidemic purulent conjunctivitis in endemic trachoma.

Table I  Effect of boric acid and antibiotic ointments on the prevalence of purulent conjunctivitis in trachomatous children

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Before therapy (Nov., 1971)</th>
<th>After therapy (Dec., 1971)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild (per cent.)</td>
<td>Moderate or severe (per cent.)</td>
</tr>
<tr>
<td>Boric acid</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td>Chlortetracycline</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>30</td>
<td>6</td>
</tr>
</tbody>
</table>

Prevalence of bacteria in the conjunctiva

The prevalence of the various bacterial species recovered in late November, 1970, from the eyes of 277 Tunisian children, and the relative amounts of growth on the culture plates, are presented in Table II. Bacteria of one species or another failed to grow in cultures from 8 per cent. of these children. The most frequently encountered potential ocular pathogens were *Haemophilus sp.* and *Moraxella sp.* Only four children had other potentially pathogenic

Table II  Prevalence and degree of growth of bacterial species recovered from the eyes of 277 Tunisian children (November, 1970)

<table>
<thead>
<tr>
<th>Species</th>
<th>Distribution according to amount of growth (per cent.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No growth</td>
</tr>
<tr>
<td>All species</td>
<td>8</td>
</tr>
<tr>
<td><em>Haemophilus sp.</em></td>
<td>60</td>
</tr>
<tr>
<td><em>Moraxella</em></td>
<td>84</td>
</tr>
<tr>
<td><em>Streptococcus viridans</em></td>
<td>35</td>
</tr>
<tr>
<td>Coagulase-negative staphylococci</td>
<td>87</td>
</tr>
<tr>
<td>Diphtheroids</td>
<td>48</td>
</tr>
<tr>
<td><em>Neisseria</em></td>
<td>94·5</td>
</tr>
</tbody>
</table>
species (pneumococcus, beta-haemolytic streptococcus, 
*Staphylococcus aureus*, and a Gram-negative rod). Many children had moderate to heavy growth to *Streptococcus viridans*, which has been considered by some to be a potential pathogen in the eyes (Fedukowicz, 1963; Scheie and Albert, 1969). Among organisms not usually associated with eye disease, diphtheroids and *Staphylococcus albus* occurred most frequently.

Changes in potential pathogens and overall bacterial growth with treatment

The prevalence of bacterial species considered to be ocular pathogens declined dramatically after chlortetracycline or erythromycin treatment (Figure). Among the patients receiving an antibiotic, eight of 98 tested yielded potential pathogens on culture after 10 days of treatment. There was a decline in the moderate to heavy growth of pathogens after treatment with boric acid, but not in the untreated group. Nevertheless, when compared with boric acid, the two antibiotics produced a marked suppression of potential pathogens.

Chlortetracycline appeared to be less effective than erythromycin in reducing total bacterial growth, because of the recovery of diphtheroids and *Streptococcus viridans* from chlortetracycline-treated patients. Thus, the suppression of overall bacterial growth from the conjunctiva was not a particularly useful measure of the efficiency of antibiotic administration or of the efficacy of a particular antibiotic against conjunctival pathogens.

Since *Haemophilus sp.* was the single most common ocular pathogen in this population, its response to the various regimens was evaluated separately. After treatment for 10 days, *Haemophilus sp.* was found in 29 per cent. of the untreated group and 35 per cent. of the group treated with boric acid. In the two antibiotic-treated groups, however, only 8 per cent. yielded *Haemophilus sp.* on culture, and in all but one instance *Haemophilus* was recovered as light growths.

The prevalence of *Moraxella sp.* was 16 per cent. of all children before treatment. *Moraxella sp.* was eliminated completely by 10 days' treatment with chlorotetracycline or erythromycin, but appeared in 8 per cent. of cultures of patients treated with boric acid or untreated. Although pneumococcus was found in only one child in November, five of 93 children (5 per cent.) in the untreated or boric acid groups had this ocular pathogen in early December. It was not found, however, in either of the antibiotic-treated groups.

Haemophilus in conjunctival smears and cultures

 Conjunctival smears and bacterial cultures were collected simultaneously from 117 children with clinically active trachoma in village A in November. Organisms presumed to be *Haemophilus sp.* were identified in 79 patients (68 per cent.) by their morphological characteristics on microscopic examination, and in 54 patients (46 per cent.) by culture (Table III); 91 (77 per cent.) yielded *Haemophilus sp.* by one or both methods. Both the slender slightly curved rods described by Koch and Weeks, and the coccobacillary forms, were observed (Koch, 1883; Weeks, 1886). Although *Haemophilus sp.* appeared more frequently in microscopic preparations or cultures of conjunctival material from patients with conjunctivitis, the organism was often demonstrated in both smears and cultures from patients with clinically normal or minimally affected eyes.

**Table III**  Haemophilus-like organisms in conjunctival smears* and in cultures of the conjunctiva from 117 Tunisian children (November, 1970)

<table>
<thead>
<tr>
<th>Smears</th>
<th>Cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>26 (22 per cent.)</td>
</tr>
<tr>
<td>Positive</td>
<td>37 (32 per cent.)</td>
</tr>
</tbody>
</table>

A total of 91 (77 per cent.) was positive by either smear or culture

* Giemsa-stained preparations

Trachoma inclusions

 In the conjunctival scrapings taken in November, the typical Giemsa-stained cytoplasmic inclusions of trachoma were found in twenty (17 per cent.) a higher rate than the 8 per cent. found in another part of Tunisia by Tarizzo, Nabli, and Labonne (1968). Like these previous investigators, we found that only those cases with intense conjunctival inflammation had inclusions and that mild cases were never inclusion-positive in Giemsa-stained smears.

The roles of *Streptococcus viridans*, other bacterial species, and Chlamydia in conjunctivitis

 Since it has been reported that alpha haemolytic streptococci may act as ocular pathogens (Fedukowicz, 1963; Scheie and Albert, 1969), their association here with conjunctivitis was compared with that of known potential pathogens (Table IV). In this analysis, the known pathogens were identified on culture, and trachoma inclusions in conjunctival scrapings. Among the 117 cases analysed in this way, 44 of the 46 cases of conjunctivitis were associated with at least one of the accepted pathogenic agents, one case was associated with neither *Streptococcus viridans* nor any of the known pathogens, and one was associated
with *Streptococcus viridans* only. It seemed clear, therefore, that *Streptococcus viridans* was not an important cause of conjunctivitis in this population. *Haemophilus sp.* was the commonest pathogen found in cases with active conjunctivitis, and there was also a high carrier rate for this species in unaffected eyes—a situation described by Maxwell-Lyons in Egypt (Maxwell-Lyons, 1953; Maxwell-Lyons and Amies, 1949).

**Table IV** Strepococcus viridans compared with known pathogens as a cause of clinical conjunctivitis in 117 Tunisian children (8 categories of cases)

<table>
<thead>
<tr>
<th>Conjunctivitis</th>
<th>Organisms recovered</th>
<th>Cases in each category</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Known pathogens*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. viridans</td>
<td>No.</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>23</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>1</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>41</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>19</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>7</td>
</tr>
</tbody>
</table>

* Pathogens included *Haemophilus sp.*, *Moraxella sp.*, pneumococci, beta-haemolytic streptococci, Gram-negative rods, and trachoma inclusions

**Haemagglutination by Haemophilus isolates**

Since the Koch-Weeks bacillus (*Haemophilus aegyptius*) has been implicated as the most common ocular pathogen in trachoma-endemic areas, we attempted to differentiate this species from *Haemophilus influenzae*. While *Haemophilus aegyptius* was originally identified as a slender, Gram-negative rod in conjunctival scrapings, isolates have been differentiated on the basis of the haemagglutination associated with *Haemophilus aegyptius* and by immunological methods (Huet, 1958; Pittman and Davis, 1950; Davis and others, 1950; Smith, 1954). Of the sixty *Haemophilus* strains we isolated in Tunisia, only one was haemagglutination-positive. In contrast, haemagglutination positivity was found in three of five *Haemophilus* strains originally isolated from the eyes of children with seasonal conjunctivitis in Southern California (Dawson, 1960).

**Discussion**

In the severe endemic trachoma in these two desert communities, bacterial conjunctivitis and trachoma frequently occurred together in the same child. *Haemophilus sp.* appeared to be the major cause of purulent conjunctivitis here—an observation made many times in this part of the world, starting with Koch's original two-line report in 1886 (Wilson, 1932; W.H.O., 1973; Maxwell-Lyons, 1953; Huet, 1958; Koch, 1883; Maxwell-Lyons and Amies, 1949; Haddad and Ballas, 1968). In Egypt and North Africa, sporadic outbreaks of *Neisseria gonorrhoeae* conjunctivitis have been reported but the few *Neisseria* isolated in this study were neither *Neisseria gonorrhoeae* nor *Neisseria meningitidis* (Wilson, 1932; W.H.O., 1973). In addition, the typical inclusions of trachoma agent were found in Giemsa-stained...
scrapings from 17 per cent. of the cases. The severe keratoconjunctivitis in these villages cannot, therefore, be attributed to any single organism, but is clearly the result of eye infection caused by a number of microbial species, particularly *Haemophilus*.

The Koch-Weeks bacillus (*Haemophilus aegyptius*) and *Haemophilus influenzae* were originally classified separately on the bases of the characteristic slender curved rods of *Haemophilus aegyptius* seen in conjunctival scrapings (Koch, 1883; Weeks, 1886). The *Haemophilus* strains isolated in this study have not been fully characterized, but many showed the satellite phenomenon, and conjunctival scrapings and isolated colonies showed both the cocobillary forms and the slender, slightly curved rods; we have assumed that most of these isolates were either *Haemophilus influenzae* or *Haemophilus aegyptius*. Other investigators as well as ourselves have found that ocular *Haemophilus* isolates in the Mediterranean area do not cause haemagglutination as regularly as those strains isolated in North America (Huet, 1958; Pittman and Davis, 1950; Davis and others, 1950; Dawson, 1960; Haddad and Ballas, 1968). It has been stated, however, that *Haemophilus aegyptius* and *Haemophilus influenzae* can be distinguished by fermentation and immunological tests (Pittman and Davis, 1950; Davis and others, 1950; Smith, 1954).

Like Maxwell-Lyons, we found many patients who were carrying *Haemophilus* in the eye without overt manifestations of conjunctivitis (Maxwell-Lyons, 1953; Maxwell-Lyons and Amies, 1949). In other conditions, such as otitis media and chronic bronchitis, *Haemophilus sp.* appear to play an aetiological role in manifest disease, but also occur frequently in apparently healthy carriers. Such high rates of conjunctival infection with *Haemophilus sp.*, however, have been found only in areas where epidemics of seasonal conjunctivitis occur (W.H.O., 1973; Maxwell-Lyons, 1953; Huet, 1958; Maxwell-Lyons and Amies, 1949; Pagès, 1951). Since *Haemophilus*-like forms were seen more frequently by direct examination of scrapings than in cultures, it may be that the media used in this study were relatively inefficient (Tables II and III). It is apparent that, for the isolation of *Haemophilus* from the eye under field conditions, other types of media and techniques should be evaluated.

The severity and high prevalence of trachoma in communities with seasonal epidemics of bacterial conjunctivitis, and the relative mildness of trachoma in the absence of conjunctivitis, have long suggested that ocular bacterial infections play an important role in the pathogenesis and epidemiology of trachoma (Wilson, 1932; W.H.O., 1973; Maxwell-Lyons, 1953; Reinhardts and others, 1968; Pagès, 1951). Their exact role in severe, blinding trachoma has not yet been determined, but it is very likely that the copious exudate from bacterial conjunctivitis facilitates transmission of the trachoma agent. Moreover, it has recently been observed that the irritation caused by scraping the conjunctiva for diagnostic purposes increases the amount of trachoma agent to be found 2 to 7 days later (Hanna, 1971) and like mechanical trauma, bacterial conjunctivitis may also favour the proliferation of the *Chlamydia* agent. A similar synergistic action of microbial infection has been shown in eggs infected simultaneously with *H. influenzae* and influenza virus (Buddingh, 1963).

Since bacterial infections are so prevalent in areas of severe endemic trachoma such as this, the effect of chemotherapy on the ocular bacterial flora is an important consideration in the evaluation of any anti-trachomatous drug used in public health trachoma control programmes. It is clearly apparent that both the chlorotetracycline and erythromycin given in this study markedly reduced the prevalence of the prevailing bacterial pathogens and of purulent conjunctivitis (Figure; Table 1). Although strains of *Haemophilus* are considered to be somewhat less sensitive to erythromycin than to tetracycline when tested in *vitro* (Sell, 1967), in our study the two drugs had essentially the same effect on the prevalence of
*Haemophilus* in the eye after 10 days of treatment. While the persistence of potential ocular pathogens (predominantly *Haemophilus* sp.) in the eyes of eight of the 98 antibiotic-treated children may have represented persisting infection despite antibiotic therapy, the possibility of re-infection from the child's own nasopharynx or from family and other community contacts must be considered.

In controlled trials of systemic sulphonamides or tetracycline given for 3 weeks for the relatively mild trachoma in American Indians, the prevalence of bacterial pathogens in the eyes was very little changed (Wood and Dawson, 1967; Dawson, Hanna, and Jawetz, 1967; Dawson, Ostler, Hanna, Hoshiwara, and Jawetz, 1971). A recent therapy trial, however, using doxycycline administered over a 5-week period, has shown that potential bacterial pathogens are reduced in the eyes of trachomatosus American Indian children (Hoshiwara, Ostler, Hanna, Cignetti, Coleman, and Jawetz, 1973). Despite the relatively small amounts of antibiotic placed in the conjunctiva in this treatment trial in Tunisia, local chemotherapy appeared to be effective in eliminating pathogenic bacterial species from the eye. Thus, in areas like Southern Tunisia where bacterial conjunctivitis may play an important role in the pathogenesis and spread of trachoma, local chemotherapy appears to be an effective way of reducing conjunctival inflammation due to bacterial pathogens. Moreover, local chemotherapy is less likely to be associated with serious side-reactions, which cannot be monitored adequately in a developing country. Thus it appears that the form of chemotherapy used in trachoma control programmes should take into consideration the epidemiological patterns of the disease—topical therapy for severe trachoma complicated by bacterial conjunctivitis, oral therapy for mild uncomplicated disease.

### Summary

In two villages in Southern Tunisia with severe trachoma and seasonal purulent conjunctivitis, the conjunctival bacterial flora and the changes in ocular flora were studied among 6- to 9-year-old children. The most common potential ocular pathogens in November, 1970, were *Haemophilus* sp. (40 per cent.) and *Moraxella* (16 per cent.); pneumococci first appeared in December in 5 per cent. of patients who did not receive antibiotics. *Streptococcus viridans* was very common (65 per cent.) but did not appear to be a cause of purulent conjunctivitis. Treatment for 10 days with chlorotetracycline or erythromycin suppressed both the overt conjunctivitis and potential bacterial pathogens. In designing public health programmes for the chemotherapy of trachoma, the role of the ocular bacterial flora in the population to be treated should be taken into account.

We wish to thank Mr. Fetuhri Chadgrah for his assistance in the field studies and Mr. Masao Okumoto for his advice on bacteriological problems.

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