Comparison of Media for the Isolation of *Haemophilus* species from Cases of Seasonal Conjunctivitis Associated with Severe Endemic Trachoma

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Received for publication 21 June 1974

Media for isolation of *Haemophilus* sp. from the conjunctiva were compared in an oasis in southern Tunisia where severe trachoma and seasonal epidemic purulent conjunctivitis are common. Of 89 children tested, IsoVitaleX-supplemented chocolate agar yielded *Haemophilus* in 87%, plain chocolate agar in 75%, sheep blood agar with a stab of *Staphylococcus epidermidis* in 74%, and Fildes medium in 58%. Since other microbial pathogens are easily identified in the modified blood agar, it was the most useful single medium.

Where trachoma is a major cause of blindness, epidemics of purulent conjunctivitis occur in a regular seasonal pattern with a peak incidence in late summer and fall (9, 12). In a previous study we found that ocular bacterial infections accompanying severe trachoma of children in southern Tunisia are most frequently associated with *Haemophilus* sp. (13). In that study, conjunctival smears revealed bacteria with *Haemophilus*-like morphology in many children from whom *Haemophilus* sp. were not isolated on sheep blood agar or Fildes medium. The discrepancy between the recovery of *Haemophilus* by culture and its identification on smears led us to search for more efficient means of demonstrating *Haemophilus* sp. by culture from the eyes of children with severe endemic trachoma. Since blood agar streaked with *Staphylococcus albus* after inoculation and chocolate agar are widely used in isolation attempts from the respiratory tract, we compared these two media as well as Fildes medium and chocolate agar (4, 6).

(This paper was presented in part at a meeting of the Association for Research in Vision and Ophthalmology, April 1972, Sarasota, Fla.)

MATERIALS AND METHODS

The subjects selected for the study were school children 6 to 8 years of age who were entering primary school for the first time in two villages in southern Tunisia. All 132 children included in this study had active trachoma, and 101 of these had overt purulent conjunctivitis diagnosed as described previously (13). The media used included: sheep blood agar that was stabbed with *Staphylococcus epidermidis* after inoculation with a conjunctival swab; Fildes medium prepared with brain heart infusion agar (Difco) with Fildes supplement (3); standard chocolate agar; and chocolate agar with a supplement containing nicotinamide and other factors (IsoVitaleX) (8). A single broth-moistened cotton swab was rubbed over the lower conjunctival sacs of both eyes. The blood agar and Fildes plate were inoculated in sequence with the first swab. A second broth-moistened swab was used to resample the eyes and to inoculate the IsoVitaleX and chocolate agar plates.

Colonies of *Haemophilus* were identified by their appearance as small colonies in satellite formation around *S. epidermidis* on sheep blood agar, as iridescent blue-tinted colonies on IsoVitaleX, as clear creamy colonies on Fildes medium, and as very small, pinpoint colonies on chocolate agar. In addition, Gram stains were done to confirm all questionable colonies and many characteristic colonies. For grading the amount of growth on primary culture, plates with fewer than three colonies were considered to have no significant growth; four to 10 colonies were classified as light growth; more than 30 colonies up to semiconfluent, as moderate growth; and semiconfluent bacterial growth or more, as heavy growth.

Scrapings of the conjunctiva were taken from 131 children after topical anesthesia, and the material was spread thinly on a standard microscope slide. These smears were air dried, fixed for 5 min in methyl alcohol, and Giemsa stained for 1 h. Since the bacterial flora of the human eye is relatively limited, the identification of *Haemophilus*-like organisms was made on morphological appearance in these conjunctival smears (17, 9, 12).

RESULTS AND DISCUSSION

Conjunctivitis and *Haemophilus*. *Haemophilus* sp. were found in 78 of 101 (78%)
cases of overt conjunctivitis on Staphylococcus-inoculated blood agar or Filde's medium, but were also present in 51% of cases without conjunctivitis (Table 1). These specimens were taken in November 1971 at the end of the seasonal epidemic of bacterial conjunctivitis. The significant association of Haemophilus with conjunctivitis cases confirms previous observations on the importance of Haemophilus sp. as a cause of purulent conjunctivitis in areas with endemic trachoma (7,9,12,13).

**Haemophilus recovery on four media.** In comparing the recovery of Haemophilus sp. from 89 children, IsoVitaleX-supplemented chocolate agar, the most sensitive medium, yielded Haemophilus sp. in 78 (87%) of 89 children (Table 2). Staphylococcus-inoculated blood agar and chocolate agar were somewhat less sensitive (74% and 75%, respectively); Filde's medium (58%) was definitely inferior to the other three. In addition, IsoVitaleX had the highest yield of moderate to heavy growths.

**Repeated cultures on 4 consecutive days.** To determine the reliability of a single culture in detecting Haemophilus in the eye, cultures on Staphylococcus-inoculated blood agar only were repeated on 4 consecutive days from 25 children. On the first day, 19 of the 25 (76%) children yielded Haemophilus isolates, and by day 4 all but one had a least one positive culture. Among the 24 children eventually Haemophilus positive, 22 (90%) were positive on at least three of four cultures. Thus, the presence of Haemophilus sp. in the conjunctiva would appear to be long-term carriage rather than occasional spillover from the upper respiratory tract.

**Comparison of isolation with presumed identification in smears.** Among the 131 matched specimens of culture on Staphylococcus-inoculated blood agar and conjunctival smears (Table 3), presumptive Haemophilus were found in one or the other in

| Table 1. Conjunctivitis and Haemophilus species in Tunisian children with chronic trachoma |
|---------------------------------------------|
| Children with: | Haemophilus (Staphylococcus-inoculated blood agar and/or Filde's medium)^* |
|                | Absent | 1+ Growth | > 2+ Growth | Total |
| Conjunctivitis | 22 (22) | 17 (17) | 62 (62) | 101 |
| No conjunctivitis | 15 (48) | 6 (19) | 10 (32) | 31 |

^*Chi square = 9.76; P < 0.01 with 2 degrees of freedom. Numbers in parentheses indicate percentage.

| Table 2. Comparison of media for Haemophilus isolation in 89 children with active trachoma and/or conjunctivitis |
|-------------------------------------------------|
| Medium               | All Haemophilus (%) | Moderate or heavy growth (%) |
|----------------------|---------------------|-----------------------------|
| IsoVitaleX^*         | 87                  | 76                          |
| Blood agar^*         | 74                  | 55                          |
| Chocolate agar       | 75                  | 51                          |
| Filde's              | 58                  | 44                          |

^*Chocolate agar with IsoVitaleX supplement.

^*Sheep blood agar inoculated with Staphylococcus epidermidis.

| Table 3 Comparison of presumed Haemophilus in Giemsa-stained conjunctival smears and in cultures of Staphylococcus-inoculated blood agar in 131 Tunisian children^* |
|-------------------------------------------------|
| Smear | Culture |
|       |         |
|       | Negative | Positive |
| Negative | 32 | 32 |
| Positive  | 8  | 59 |

^*Of the 99 patients positive by either technique, 91 were positive by culture and 67 were positive by smear.

99 cases (76%), and only 8 (6%) of these were by smear alone. In a similar comparison of IsoVitaleX media and smears in 86 cases, 3 of the 76 Haemophilus were found only in smears. Thus, either blood agar or IsoVitaleX appears to detect most of the Haemophilus-like organisms seen in conjunctival scrapings.

**Differentiation of Haemophilus sp.** Gram stains of Haemophilus colonies made in the first 36 to 48 h of growth revealed isolates with one of two distinct forms. The most frequent was a small coccobacillus sometimes associated with long, threadlike forms. Less frequently, slender, slightly curved rods were found that corresponded to the morphology ascribed to Haemophilus aegyptius (7, 9). In initial isolation media, 22 of 23 Haemophilus influenzae-like (coccobacillary) strains appeared on both Filde's medium and blood agar, but four of the eight H. aegyptius-like strains were found only on modified blood agar. Thus, Filde's medium appears to be less sensitive for the ocular pathogen H. aegyptius.

Under the conditions of this field study, IsoVitaleX medium was the most sensitive in detecting Haemophilus sp. in the eye. Whereas the chocolate agar and Staphylococcus-inoculated blood agar were almost as effective
as IsoVitaleX, the Fildes medium was clearly inferior. In addition to its usefulness for primary isolation, the IsoVitaleX was excellent for subculturing Haemophilus strains. A number of other pathogenic bacteria (pneumococcus, Moraxella, gram-negative rods, and others) were easily identified in the Staphylococcus-inoculated blood agar, and its usefulness in isolating other bacterial species certainly makes it the logical choice if only one medium is to be used in such studies.

The occurrence of two distinct morphological types of Haemophilus in areas with severe trachoma was observed by Koch in 1886 in Egypt (7). It has been accepted that the slender bacillus form (H. aegyptius, or the Koch-Weeks bacillus) is associated with a more severe conjunctivitis than H. influenza (1, 9). Although several microbiological tests have demonstrated the difference between these two species, such procedures are difficult to carry out under field conditions (2, 10, 11). For this reason we have used morphology on Gram staining as a preliminary indicator of the two species. In this study the presumed H. aegyptius isolates grew less frequently on Fildes medium.

Although most of the overt conjunctivitis noted during this study was associated with the presence of Haemophilus, a high rate of infection with Haemophilus was found in uninflamed eyes. In a previous study in these villages, we found that only one of 60 Haemophilus isolates hemagglutinated human erythrocytes, a characteristic of H. aegyptius (2, 13). This observation supports previous studies done in Tunisia showing that Haemophilus isolates from the eyes fail to produce hemagglutination (5). Therefore it could not be determined whether cases of conjunctivitis were due to H. influenza or H. aegyptius by this simple test.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants EY 00186, EY 00310, and EY 00427 from the National Eye Institute; Foreign Currency Award (PE 480) no. 07-075; Public Health Service research career development award 1 K3 NB 31, 781 to C.R.D.; and an award from Fight for Sight to D.W.V.

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