EXAMINATION OF MULTIPLE SPUTUM SPECIMENS IN A TUBERCULOSIS SURVEY

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SUMMARY

During an epidemiological survey, four specimens of sputum were collected within seven days from each person with an abnormal chest x-ray in 30 villages in a district of South India. Each specimen was examined by fluorescence microscopy, Ziehl-Neelsen technique and culture.

There were 34 culture-positives among the 2,164 persons for whom all the four culture examinations were available. Out of these, on average, 21(62 %) were found from examination of one specimen only. An estimate of prevalence obtained from only one sputum specimen will have to be multiplied by 1.67 to get the prevalence that would be obtained from many specimens. Similarly, the correction factor for estimates based on two sputum specimens will be 1.26.

Ziehl-Neelsen positives not confirmed by culture (mostly with less than four bacilli reported in the smear) increased from 7 from the first specimen to 18 from all four specimens, while positives confirmed by culture showed only a marginal increase from 13 to 15. Fluorescence microscopy did not have this disadvantage.

More than 80 % of the smear-positives (confirmed by culture) could be found from examination of one specimen only. Examination of two specimens by fluorescence microscopy detected about 95 % of the cases demonstrable by this method. But with the Ziehl-Neelsen technique additional specimens can do more harm than good because more ‘false positives’ are added. Multiple specimens are more rewarding for detecting cases positive on culture only.

RÉSUMÉ

Au cours d’une enquête épidémiologique on a recueilli chez tous les malades présentant une image radiologique anormale, dans 30 villages d’un district du Sud de L’Inde, quatre échantillons de crachats recueillis dans la même semaine. Chaque échantillon a été examiné par microscopic en fluorescence, coloration de Ziehl-Neelsen et culture. Parmi celles 2,164 personnes chez qui les quatre cultures ont été faites, on a trouvé 34 cultures positives. Parmi celles ci, en moyenne, 21 (62%) ont été trouvées positives par examen d’un seul prélèvement. Une estimation de la prévalence obtenue par examen d’un seul prélèvement doit être multiplié par le coefficient de 1,67 pour obtenir la prévalence qui serait obtenue par examen de plusieurs prélèvements. De la même façon, le facteur de correction pour l’estimation de la prévalence basée sur deux prélèvements serait de 1.26.

Le nombre des examens positifs au ziehl, mais negatifs a la culture (la plupart avec moms

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de quatre bacilles par lame) qui était de sept par examen du premier prélèvement est monté à 18 par examen des quatre prélèvements, tandis que les examens positifs au ziehl et confirmés par culture ont eu seulement un faible accroissement de 13 à 15. La microscopie en fluorescence n’a pas montré ce désavantage.

Plus de 80% des examens positifs sur lame et positifs a la culture pourraient être trouvés par examen d’un seul prélèvement. L’examen de deux prélèvements en microscopie par fluorescence a détecté environ 95% des cas positifs par cette méthode. Mais avec la coloration de Ziehl-Neelsen la répétition des prélèvements fait plus de mal que de bien, car on peut alors inclure des ‘faux positifs’. Des examens répétés sont beaucoup plus utiles pour la détection des cas positifs en culture seulement.

**RESUMEN**

Durante una encuesta epidemiológica en 30 pueblos de un distrito de Sud India se recogieron cuatro muestras de esputos en siete días de todas las personas que tenían una radiografía de tórax anormal. Cada muestra fue examinada con el microscopio fluorescente, con la ténica de Ziehl-Nielsen y con cultivos.

Hubo 34 cultivos positivos entre las 2164 personas que fueron sometidas a los cuatro cultivos de esputos. De ellos, hubo 21(62 %) en que el hal!azgo se hizo en una sola muestra. La prevalencia que se obtiene de una sola muestra de esputos debe ser multiplicada por 1,67 para obtener la que rinden las muestras multiples. En forma similar, el factor de corrección cuendo se examinan dos muestras es 1.26.

Los positivos con el Ziehl Nielsen no confirmados por el cultivo (la mayoría con menos de cuatro bacilos) aumentaron de siete en la primera muestra a 18 en las cuatro muestras, mientras que los cultivos positivos solo aumentaron de 13 a 15. La microscopía fluorescente no presenta esta desventaja.

Más del 80 % de las baciloscopias positivas (confirmadas por cultivo) se hallaron al examinar solo una muestra. El examen de dos muestras por microscopía fluorescente permitió descubrir alrededor del 95 % de los casos demostrados. Con la ténica de Ziehl-Nielsen el agregado de más muestras puede ser contraproducente porque agrega más ‘falsos positivos’. Las muestras múltiples son más rentables para descubrir los casos positivos mediante el cultivo.

**ZUSAMMENFASSUNG**


Für 2.164 Personen liegen alle vier Kulturergebnisse vor; bei 34 Personen wurden Tuberkulosebakterien nachgewiesen. In 21(62%) Fällen war nur eine Probe positiv. Wenn man auf Grund nur einer Untersuchung die Prävalenz schätzen will, so muß mit einem Faktor 1,67 multipliziert werden. Der Korrekturfaktor für Schätzungen auf Grund von zwei Sputumproben lautet 1,26.


Mehr als 80% der im Ausstrich positiven Fälle (mit kultureller Bestätigung) konnten durch Untersuchung nur einer einzigen Sputumprobe gefunden werden. Fluoreszenzmikroskopische Untersuchung von zwei Proben führte zum Herausfinden von 95% aller Fälle, die mit dieser Methode überhaupt erfassbar waren. Aber mit der
Introduction

Prevalence surveys are useful for estimating the tuberculosis problem in different countries. They make it possible to formulate the long-term strategy for control. Three techniques are commonly used in such surveys: the tuberculin test, mass miniature x-ray and sputum examination. Each technique has its own limitations. A limitation of sputum examination is that all the sputum-positive cases in the community cannot be diagnosed when only one sample of sputum is examined from each person eligible. Barton (1958) reported the addition of 10% positives through examination of a second specimen. Such additions were also observed in surveys conducted at the National Tuberculosis Institute, Bangalore (NTI), (Raj Narain, Jambunathan and Subramanian, 1962). Even among a highly selected group of patients attending the Tuberculosis Chemotherapy Centre, Madras, after a provisional diagnosis of pulmonary tuberculosis had been made at a local chest clinic, a second sample added about 4% to the culture-positive cases detected by the first specimen (Andrews and Radhakrishna, 1959). While in tuberculosis clinics a number of sputum specimens could be collected from an individual, such multiple examinations are often not possible under field conditions of surveys covering the whole community or for patients attending general health institutions in rural areas. Quite often, sputum examinations may have to be limited to one or two specimens only.

It would be worthwhile having some idea of the extent of under-diagnosis which results from these restrictions. For this purpose, four specimens were collected from each eligible person in some villages covered by the Longitudinal (repeat) Survey being conducted by NTI since 1961.

Material and Methods

The study was conducted during the second round of the Longitudinal Survey in 30 out of the 133 villages randomly selected for the survey. Briefly, the survey techniques* were:

All persons were offered a tuberculin test with ITU RT23 with Tween 80. Those five years old and above were also referred for a 70 mm. chest x-ray. Persons with abnormal shadows read by either of two independent readers were eligible for one spot and one overnight (i.e., collected) sputum specimen. The interval between these two samples varied between 12 and 24 hours. Further spot and overnight sputum specimens were collected two to seven days after the first set. The specimens were examined first by fluorescent microscopy (FM) and then by the Ziehl-Neelsen (ZN) method. The first set of two specimens was then cultured using the sodium hydroxide method and the second set was cultured by the swab technique (Nassau, 1954) because of limited centrifuge capacity.

All positive cultures were identified by sub-culturing and observing growth at room temperature, rate of growth at 37°C, production of pigment in the dark and after exposure to light, peroxidase and catalase reactions and niacin production. Only those cultures considered to be of human type were reported as culture positives.

The population of the 30 villages was about 21,000 and 3,639 of them were referred for sputum examination. At least one specimen of sputum was collected from 3,245 (89.2%) and at least two specimens from 3,170 (87.1%). All the four specimens were collected from 2,401 (66%) of the eligibles, but among these, one or more specimens from the same individual were contaminated in 237 (99%). Thus, results for all the four culture examinations were available for only 2,164 (59% of the eligibles) and constitute the study population for detailed analysis.

* Further details can be obtained from the first report on the Longitudinal Survey (to be published).
To study whether or not the eligibles excluded from the study population formed a selected group in respect of factors other than the number of specimens examined, the results of examination of each specimen (1st, 2nd, 3rd and 4th) were considered independently. The percentage of positives from any one specimen did not differ significantly between the study group (in which results of all four specimens were available) and the remainder (in whom less than four results were available). Thus, the study group could be considered fairly representative of the total eligibles.

**Result**

In all, 75 bacillary cases were found. Of them, 53 were positive on culture, and 22 negative on culture but positive on microscopy. The study group included 54 bacillary cases, 34 positive on culture and 20 on direct smear only. The majority of those positive on direct smear only were most probably ‘false cases’ (Raj Narain and others, 1968). Therefore, only culture positives have been considered as ‘true cases’. ‘All direct smear positives’ are also considered separately in order to study the effect of multiple sputum examination in surveys using direct microscopy only.

**CULTURE-POSITIVE RESULTS**

From the 2164 individuals in the study group 24 (1.11 %) of the first specimens were positive on culture, 23 (1.06%) of the second, 16 (0.74%) of the third and 20 (0.92%) of the fourth specimens. The mean number of positives was 21(0.96 %) with 95 % confidence limits of 0.72 % and 1.21%. All the observed proportions in the four specimens were within these limits; so the differences between them were not statistically significant.

At least one positive culture was obtained from 34 individuals. The first sample yielded 70 % of the total (Table I). There was a substantial increase from the second specimen (18 %). Each specimen could be considered the ‘first’, as they were collected independently. If specimen IV were taken as the ‘first’ sample, then only 58 % of the total 34 would be classed positive. The additional positives detected by the ‘last sample, in this case specimen I, would be substantial (18 %), even more than the additional positives from specimens II or III. On average, one specimen detected 62 % (21 of 34) of the total culture-positive cases detected by examining all four specimens.

Correction Factors to be Applied to Prevalence Estimates of Culture-positive Cases Based on One or Two Sputum Specimens.

Generally, either only one or sometimes two sputum specimens are examined in prevalence surveys. The resulting under-estimation could be reduced if suitable correction factors could be applied to the disease rates (prevalence or incidence) calculated on the basis of one or two specimens.

**TABLE 1. - ADDITIONS OF CULTURE-POSITIVE CASES BY SUCCESSIVE SPECIMENS OF SPUTUM.**

<table>
<thead>
<tr>
<th>Positive from specimens</th>
<th>Additional positives from specimens</th>
<th>Total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Positive on culture</td>
<td>24 (70%)</td>
<td>6 (18%)</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td>Positive on culture</td>
<td>20 (58%)</td>
<td>4 (12%)</td>
</tr>
</tbody>
</table>
To arrive at correction factors, it is necessary to estimate as precisely as possible, the total number of cases in the community. The present material provides information on the number of cases which could be detected by examination of four specimens from each eligible person. To get even closer to the ‘true’ figure, attempts were made to fit mathematical curves to the additional yields of cases from successive specimens (i.e., 2nd, 3rd and 4th specimens). This curve showed that additions may continue even up to the 40th specimen. Most probably this result is due to certain limitations of the present data (discussed later).

Correction factors were worked out on the basis of the best estimate of the total cases in the community as provided by the examination of the four specimens. These correction factors could be calculated on the basis of any order of the 4 specimens i.e., addition by 2nd, 3rd and 4th to 1st; 1st, 3rd and 4th to 2nd and so on. In order to arrive at a single estimate, the correction factors were first calculated on the basis of each possible permutation and then a set of average correction factors was worked out. These average correction factors are:

1. To be applied to the estimates based on one specimen – 1.67.
2. To be applied to the estimates based on two specimens — 1.26.

It is likely that these correction factors are minimum and the estimates of prevalence obtained by applying any of these would still be an underestimate to some extent. Thus, if a prevalence rate of 4 per 1,000 is obtained on the basis of examination of a single specimen, a closer approximation to the ‘true’ prevalence rate will be at least 6.7 per 1,000 (4 x 1.67= 6.68). Similarly, a rate of 5 per 1,000 based on two specimens would imply a minimum prevalence of 6 per 1,000 (5 x 1.26= 6.30).

**SMEAR-POSITIVE RESULTS**

There were 37 with smear-positive results in one or more of the four specimens (Table II); but in only 17 of these was the culture also positive. Of these 17 cases confirmed by culture, the smear was positive on the first specimen in about 80 %. The addition of specimen II increased the proportion to almost 95 %. Thus, for detecting cases both smear- and culture-positive two specimens appear adequate. A third specimen is helpful for detecting cases positive on culture only.

However, there were 20 cases with positive smears but negative cultures. Unlike the culture results, the proportion added by the third and fourth specimens was high (40 % compared with 6 % for culture-positives).

Additions by the ZN and FM techniques are shown separately in Table III. With the ZN method 18 of 33 positives were culture-negative; and more than half of these were added by the second and third specimens. On the other hand, there were only 2 with negative cultures among the 18 with positive smear results by the FM method. All 16 positive on both microscopy and culture were detected by the first two specimens together.

**TABLE II.—SMEAR AND CULTURE RESULTS OF THE FOUR SPECIMENS EXAMINED**

<table>
<thead>
<tr>
<th>Positive from Specimen I</th>
<th>Additional positives from Specimen II</th>
<th>Specimen III</th>
<th>Specimen IV</th>
<th>Total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear positive</td>
<td>22(60%)</td>
<td>6(16%)</td>
<td>7(19%)</td>
<td>2(5%)</td>
</tr>
<tr>
<td>Culture positive</td>
<td>14(82%)</td>
<td>2(12%)</td>
<td>0(0%)</td>
<td>1(6%)</td>
</tr>
<tr>
<td>Culture negative</td>
<td>8(40%)</td>
<td>4(20%)</td>
<td>7(35%)</td>
<td>1(5%)</td>
</tr>
<tr>
<td>Smear negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>culture positive</td>
<td>10(59%)</td>
<td>4(23%)</td>
<td>2(12%)</td>
<td>1(6%)</td>
</tr>
</tbody>
</table>
TABLE III.—SMEAR RESULTS BY THE TWO METHODS AND CULTURE RESULTS OF THE FOUR SPUTUM SPECIMENS

<table>
<thead>
<tr>
<th>Method</th>
<th>Specimen I</th>
<th>Specimen II</th>
<th>Specimen III</th>
<th>Specimen IV</th>
<th>Total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ziehl-Neelsen Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total positive</td>
<td>20</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>33</td>
</tr>
<tr>
<td>Culture positive</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>Culture negative</td>
<td>7</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>Scanty positive(1-3 Bacilli in the whole Smear)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture positive</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Culture negative</td>
<td>7</td>
<td>3</td>
<td>6</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Other positive</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Culture positive</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Culture negative</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Fluorescent microscopy Method</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total positive</td>
<td>15</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>Culture positive</td>
<td>14</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>Culture negative</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Smears in which only one to three bacilli (scanty positive) are reported by the ZN method are most probably ‘false positives’ (Raj Narain and others, 1968). Of the 33 positive by this method the majority (23) were scanty positives. Only 7 of these 23 were culture-positive, compared with 8 of the 10 other positives. Moreover, 9 of the 16 scanty positives with negative cultures were added by the second and third specimens. The ratio of culture-positives to culture-negatives among all ZN positives increased from 13:7 for the first specimen to 13:11 for two specimens, 13:17 for three and 15:18 for four specimens. Thus, additional specimens examined by the ZN method can lead to considerable increase in ‘false positives’ with only slight increase in ‘true positives’ if smear in which less than four bacilli are seen are reported ‘positive’.

The estimate of the prevalence of positive cases by the ZN method was 33. This is close to the estimate for culture-positive cases (34). But it included 23 scanty positives, of which only 16 were positive on culture. The estimate by the FM method was 18.

Follow-up of Culture-positive Cases

Of the 34 culture-positive cases, 12(35 %) were positive in only one of the four specimens (not shown in the table). Only one of the 12 was positive on microscopy of any of the specimens and only five had 20 colonies or more on culture. Eleven were followed-up after 18 months: only one had died. On the other hand, of the 22 with more than one positive culture 13 were smear-positive and 16 had 20 or more colonies on culture. Eighteen were followed up: 7 had died.

Although the causes of death were not known, the former group most probably contained the less serious cases of tuberculosis. All but one of the group were smear-negative. Among the total of 17 smear-negative culture-positive cases six were added by the second and third specimens (Table II). Thus, the composition of the group diagnosed is likely to depend on the number of specimens examined, a group diagnosed
from one single specimen having a smaller proportion of less serious cases than a group diagnosed from several specimens.

**Discussion**

The main objective of tuberculosis prevalence surveys is to define the tuberculosis problem in the community. The methods adopted should be able to provide an estimate which is quantitatively close to the actual number of tuberculosis cases in the community and also give reproducible results under all survey situations. The main finding of the present study is that out of the 34 culture-positive cases, on the average only 21 (62%) could be found by culture examination of one sputum specimen. It was also observed that the four specimens examined did not provide consistent results. These differences emphasize the importance of efficient and uniform standards for sputum collection, processing and examination.

Application of a correction factor to the positives found from one examination by standard techniques may give reasonably good approximations to the number that would be found by multiple examinations. But, for qualitative purposes (composition and distribution of cases) and for the calculation of incidence of disease, breakdown rates in certain groups, fate of cases etc., missing about 40% of the cases by examining only one specimen cannot be considered satisfactory. This is particularly so because the missed cases are likely to be mainly the less serious ones. The above considerations make it clear that it is not worthwhile to economise on methods in the field of tuberculosis epidemiology.

In the sample survey on tuberculosis in India (Indian Council of Medical Research, 1959) sputum examination was confined to sputum (or laryngeal swabs) collected during a single visit. Therefore, the prevalence was under-estimated. Similarly, the estimate of 4.1 cases per 1,000 obtained by Raj Narain and others (1963) for a district in South India may really imply a prevalence of at least 6 per 1,000.

Direct microscopy being simpler and economical, it is natural to ask ‘why tuberculosis surveys should not be conducted using only this method of sputum examination’. Over 80% of the direct smear positive cases could be found by examination of one specimen by either Ziehl-Neelsen or fluorescence microscopy techniques. Examination of a second specimen by fluorescence microscopy could detect about 95% of the cases demonstrable by this method. But in case of Ziehl-Neelsen technique, additional specimens could be harmful as more ‘false positives’ would be added.

The main limitation of such a survey is that a fairly large proportion of the cases cannot be demonstrated by the available methods of microscopy. The simple tool of direct microscopy could be utilized more effectively if more sensitive techniques are developed. One possibility is to improve and standardize the method of cold staining with chloroform, described by Rao, Naganathan and Nair (1966). As suggested by them, it is also worthwhile to improve the technique of fluorescence microscopy itself.

The present study had some limitations. The material provided only four observed points to determine the relationship between specimen number and additional positives from each specimen. Probably about 8 or 10 specimens may have to be collected from each person to find this relationship. Such a study should also cover larger samples and take into account possible differences between spot and overnight specimens and differences due to variations in culture technique. Studies carried out in areas with widely differing prevalence rates and among symptomatic out-patients of health institutions will help to obtain a clearer picture of the limitations of diagnosis based on only one or two specimens in different situations, and in working out correction factors applicable to different situations.

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