CORRESPONDENCE

Comments on the article 'Method of Cultivation of M. Leprae' by N. Veeraraghavan, published in *Curr. Sci.*, January 20, 1983 Vol. 52, No. 2, p. 60–63.

(1) Messrs Vishwa Mohan Katoch and K. V. Desikan of Central Jalma Institute for Leprosy, Taj Ganj, Agra 282 001, write as follows:

Recently Veeraraghavan described a successful method of *in vitro* cultivation of *M. leprae* by using the defined medium[1,2]. The author reported 2–6 fold multiplication of *M. leprae* in 8–60 hr when the cultures were incubated at 8–10° C and recommended this technique for *in vitro* sensitivity of *M. leprae* against various drugs.

We have tried to repeat this work in our laboratory. Medium V was prepared and dispensed as described by Veeraraghavan[2]. The material for cultivation was obtained from 5 untreated cases of multibacillary type of leprosy, processed and inoculation was done by the methods of Veeraraghavan taking all the necessary precautions detailed by him[1,2]. Aliquots from the same specimens were autoclaved and inoculated into medium V to serve as controls. Suspensions of live bacilli were also inoculated into Sauton's medium (medium used for cultivable mycobacteria) and incubation was done at 8–10° C- Counting was carried out at 0, 60 and 120 hr. Subcultures were also done after 60 hr of incubation from a set of tubes containing live bacilli in medium V, killed bacilli in medium V and live bacilli in Sauton's medium and counts were carried out at 60 and 120 hr. All slides were coded and counting was done by double blind method. Counting was carried out by the methods described by Shepard and McRae[3] and also by the method described by Veeraraghavan[2]. The detailed results are being published elsewhere[4]. It was found that no increase in numbers could be recorded after 60 hr and even after 120 hr of incubation either in primary or subcultures from all the specimens investigated. The results were uniformly negative inspite of whether the counting was done by Shepard and McRae or Veeraraghavan's method. Kato[5] also failed to detect any significant multiplication of armadillo derived *M. leprae* in medium V. Regarding another observation of Veeraraghavan that periodical opening of tubes was deleterious to the growth of organism and that counts would drop, no such reduction was observed in sets of culture tubes which were opened for counting at 60 hr.

In order to conclude that the growth of the organism is real, there should be atleast 100 fold increase, growth should be consistent in majority of the specimens and the results should be reproducible[6,7]. The above criteria are not satisfied in the present study. It could therefore be concluded that *M. leprae* could not be cultivated in medium V under the experimental conditions described.

22 July 1983.

1. Veeraraghavan, N., *Studies on leprosy*, V. H. S. Medical Centre, Madras, 1982, p. 9.
2. Veeraraghavan, N., *Curr. Sci.*, 1983, **52**, 60.
3. Shepard, C. C. and McRae, D. H., *Int. J. Lep.*, 1968, **36**, 78.
4. Kotoch, V. M. and Desikan, K. V., *Leprosy in India*, 1982, **55**, 292.
5. Kato, L., *Leprosy Scientific Memoranda*, L-1182, 1983.
6. Report of the Committee 2 workshop on microbiology, *Int. J. Lep.*, 1979, **47**, 291.
7. Stewart, Tull, D. E. S., Biology of Mycobacteria, Vol. I, (eds) J. Standard and Colin Ratledge, Academic Press, London, 1982, p. 288.

(2) Dr. N. Veeraraghavan's reply to Katoch and Desikan's remarks:

It is difficult to give a correct opinion in the absence of details. However, I have the following comments to offer:

I. While employing the slit and scrape method for collection of material from lepromatous cases care should be taken to see that only the dermis is slit and no blood oozes out. Specimens mixed with blood are unsatisfactory probably because of the large amount of antibodies these patients possess.

The number of viable organisms in any specimen varies. In a study of 206 lepromatous cases by cultural methods it has been found that the cultures of about 35% of patients proved to be negative. The bacillary index (BI) among them varied from 0–4.

The possibility has to kept in mind that the cultures may show growth while the number of organisms remains the same. This is due to the fact that the dead organisms would lyse and only the live ones multiply. Therefore, the presence of young dividing forms, sheet formation, increase in the number of cysts, presence of non-acid-fast organisms morphologically resembling *M. leprae* etc are better criteria for growth than mere increase in numbers.

It is found that if a vibrator is used in culture work the orgnisms do not multiply.

It has been repeatedly pointed out that the cultures have to be kept at about 7°C for about 6-8 weeks before subculture can be attempted. So far, tube to tube transfer without storage has not been possible. The authors have not followed this procedure.

The fall in the count on repeated opening of the culture naturally depends on the number of organisms in the culture. If the number is very large they will autolyse. If the number is small they will cease to multiply.

II. Studies during the year with the well characterized armadillo strain of *M. leprae*, which is being used for the production of leprosy vaccine by the WHO, indicate the following:

1. Cultures of the armadillo strain on incubation at 7°C form a thin sheet at the bottom of the tube which gradually spreads upwards. The period required for the formation of the sheet varies from 8-12 weeks depending on the initial count of the culture. The sheet consists of pure bacilli and all the stages in the development of the organism are encountered. The medium can be pipetted out and the sheet suspended in carbol-saline for making lepromin, Hanks' solution for the preparation of a vaccine, or medium V for subculture. The number of organisms in a sheet suspended in medium V equal to that of the original culture, varies from 10^8-10^9 organisms per ml as per Shephard's method of counting. Actually, these sheets are being regularly used for experimental work and subculture.

These sheets are also formed by human strains provided the initial count is high and the period of incubation long enough.

A method using medium V has been developed and is under study for obtaining 100-1000 fold multiplication of the armadillo strain of *M. leprae*. Employing this method it would appear possible to obtain cultures containing 10^{10} organisms per ml, similar to that obtained in infected armadillo tissue.

III. My criticism of Dr Kato's communication has appeared in the June 1983 issue of the Leprosy Scientific Memoranda (L-1199). The note also gives the important guidelines to be followed for the cultivation of *M. leprae*.

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(3) Comments by Dr T. Ramakrishnan, Microbiology & Cell Biology Laboratory, Indian Institute of Science Bangalore, on the preceeding publications:

I have gone through the original paper of Veeraraghavan in *Current Science* 'Method of Cultivation of *M. leprae*', the paper by Kato on 'No growth of *M. laprae*', at + 5° in Leprosy Scientific Memoranda, the present note by Katoch and Desikan and comments on it by Veeraraghavan. Judging from the paper of Kato on the one hand and of Katoch and Desikan on the other hand and my own experience of working with mycobacteria for the last 25 years, I regret to conclude that there is no growth of *M. leprae* in the medium described by Veeraraghavan. My conclusion is based on the following:

1. The characteristic physical property of mycobacteria which distinguishes it from other bacteria is clumping, especially in young cultures. As the cultures become older, there is generally declumping, leading to an apparent 2-6 fold increase in the number of cells. This is what is apparently happening in the medium V. In addition, growth is a kinetic phenomenon and not a static one, and has to be represented as a growth curve, where growth in cell numbers is plotted against time of incubation with a minimum of 3 points on the curve. At least an increase of one log (10 times) in this growth curve is necessary to show growth.

2. The temperature of growth in medium V is given as 10°. While it is true that *M. leprae* grows naturally in the colder parts of the human body, nowhere in the body is the temperature lower than 30-35°. The body temperature of armadillo, the only animal in which *M. leprae* has been shown to grow is 30–36°.

3. The time of incubation in the medium for growth has been given as 3 days. Leprosy is a very slow disease, producing its effects years after infection. Tuberculosis, produced by a related organism, is also a slow disease but faster when compared to leprosy. The rate of growth of a disease-producing organism in culture is normally related to the rate of the progress of the disease. Thus *M. tuberculosis*, in the best liquid medium hitherto devised, takes about 10–12 days to grow. Our studies in this laboratory on the molecular biology of *M. tuberculosis* have shown that it is genetically programmed to grow slower than bacteria like *E. coli*. One would expect *M. leprae* to grow even more slowly in culture. In fact when Veeraraghavan used pure culture of *M. leprae* grown in armadillo in his own medium he finds that it takes 4 weeks to "grow".

4. The essence of microbiology is the purity of the bacterial culture used, and nowhere in the paper has rigorous evidence been presented to show that the organisms grown by Veeraraghavan are *M. leprae* and not some other bacterial contaminants which are known to be present on lepromatous nodules or skin scrapings. The evidence has to be two fold: (a), microscopic staining characteristics of the bacteria under conditions which have been already standardised from *M. leprae*, and (b), inoculation of the cultured bacteria into mouse footpad or armadillo to confirm that it can produce the disease. The latter is absolutely necessary to satisfy Koch's postulates, one of the basic tenets in microbiology.

Numerous investigators all over the world have in the past claimed to have cultured *M. leprae*., but none of these claims have yet been accepted by leprologists. The WHO has given a set of guidelines by which such claims should be tested, and WHO is also willing to test these claims. Till this is done, I am afraid, Veeraraghavan's claim of having cultured *M. leprae in vitro* will be just another claim which will not be accepted by microbiologists or leprologists.

15th Sept. 1983.

ANNOUNCEMENTS

WINTER SCHOOL ON MOLECULAR REACTION DYNAMICS

The Winter School on Molecular Reaction Dynamics is sponsored by the Department of Science and Technology, New Delhi and organized by the Department of Chemistry Indian Institute of Technology, Kanpur, during 1–14 January 1984.

The aim is to introduce the modern area of molecular reaction dynamics to young researchers in the country with the hope of initiating extensive research in this area and the topics covered are as follows: (1) relation between reaction cross section and rate constant and activation energy; (2) experimental methods of state-to-state chemistry; (3) dynamical theories classical trajectories, quantal close-coupling calculations, and quantal time-dependent approach, (4) statistical theories including the information theoretic approach, (5) special topics on lasers, multiphoton processes, electronically excited states etc.

For details please write to Dr N. Sathyamurthy, Department of Chemistry, Indian Institute of Technology, Kanpur 208016.

LECTURE SERIES ON 'RECENT ADVANCES IN DRUGS AND PHARMACEUTICALS INDUSTRY'

The Indian Chemical Manufacturers Association is organising a one-day Lecture Series on "Recent Advances in Drugs and Pharmaceuticals Industry" on Thursday, the *24th November, 1983* at Bombay. Technical experts in the pharmaceutical field will present papers at the Lecture Series, in which senior representatives from Industry, Government, Research and Educational Organisations are expected to participate. Dr Nitya Nand, Director, Central Drug Research Institute, Lucknow will be the Chairman for the Lecture Series.

Further details regarding participation could be had from the Deputy Secretary, Indian Chemical Manufacturers Association, Sir Vithaldas Chambers, 16 Bombay Samachar Marg, Bombay 400023.