# A Comparison of the Growth Curves of the NQ Bacillus (Mycobacterium sp.) Derived by Photometric Turbidity, Microscopic Counting, and Viability in a Tube-Dilution-Series [1, 2]

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"In 1882 Ehrlich noted that the application of strong acids did not result in decolorization when tubercle bacilli, in contrast to other organisms, were stained with a solution of gentian violet in water saturated with aniline. The tubercle bacilli were thus said to be acid-fast. Acid-fastness has proved to be one of the most useful and significant of characteristics for separating tubercle bacilli and related organisms from all other types of bacteria." ([7]).

Many theories have been proposed to explain the acid-fast property of the mycobacteria, but no satisfactory explanation has yet been given. There does seem to be some basis of agreement among investigators of this phenomenon. "The one point on which there is complete agreement is that the integrity of the cellular structure must be maintained to preserve the acid-fast property." ([12]) It is agreed that the staining is cytoplasmic and that the cell walls or cell membranes are not stained but may, and most likely do, act as selective barriers to retain the dye in the presence of the acid decolorizer. The major factor in acid-fastness appears to be intracellular free dye and this dye responds to certain extracellular factors to precipitate ([12]) or to redistribute ([6]) and, in so doing produces microscopic morphologic aberrations that are not related to the actual cell structure. Yegian and Vanderlinde ([12]) have photographically documented this act of precipitation of the internal dye. He accomplished this by altering the Ziehl-Neelsen (ZN) staining character of the same cells from solid staining to "beaded" and back again to solid staining. He has also demonstrated, using a nigrosin negative staining technic that this stain is internal and actually concerns only a portion of the intact cell.

Berg ([1]) has shown the relationship between gentian violet and both mycolic and leprosinic acids to be stoichiometric. This indicates that a chemical aspect is also functional in the acid-fast stain. However, on a quantitative basis it is generally agreed that the chemical function is normally at such a low level that it is not a major determinant of the microscopic acid-fastness of an organism.

These investigations provide information on the nature of acid-fastness as a differential stain. They do not clarify the question of the distribution of acid-fast versus non-acid-fast cells within a given population of a single species of mycobacteria nor do they provide information on the relative staining proportions of a single cell. It is obvious ([6, 7, 12]) that any given cell is not always stained completely nor to the same extent on repetition. It is also obvious ([2]) that not all members of a single culture of mycobacteria are always acid-fast. Under these conditions it should be scientifically acceptable to question cell measurements and microscopic counts which are made on Ziehl-Neelsen stained cells. It is quite possible that the data, thus achieved, is indicating merely the length of distribution of the dye within the cell, on the one hand and,

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merely a measure of some unknown portion of the total population on the other.

These questions would not be pertinent if the total cell or the total population would be made evident by the ZN counterstain. They are not. This is confirmed by the necessity of Yegian and Vanderlinde [12] to resort to nigrosin staining to define the cell outline. Aqueous methylene blue or Loeffler's methylene blue when used as counterstains in a ZN procedure minus the basic fuchsin, do not stain mycobacterial cultures, with the exception of a few blue staining cells which are also obvious as blue staining cells in the complete ZN procedure [10]. With this procedure, the portion of the pure culture population that represents the acid-fast individuals is only evident as negatively stained bodies if the background of the smear contains a sufficient amount of protein. The cells are not stained blue internally. This characteristic opens up the possibility of three ZN classes of mycobacteria: cells stained red, cells stained blue and a number of cells that take neither carbolfuchsin nor counterstain. The latter group would be invisible in routine evaluation of microscopic counts of ZN stained mycobacteria.

Nyka [8] provided experimental and documentary evidence that such an effect does actually occur with *Mycobacterium tuberculosis*. He demonstrated that oxidation of tissues and smears with peroxide or periodate, prior to carbolfuchsin staining, made organisms microscopically visible where no organisms were apparent in adjacent tissue sections or in duplicate smears stained by routine ZN procedure. Accordingly, mycobacterial counting that included only the red stained bacilli would provide data that could be invalid for experimental conclusions that were based on the assumption that either the entire population or, constant proportions of the population were being investigated.

The present study was designed to investigate the existence of the unstained, microscopically invisible state in a cultivable species of mycobacteria and to determine whether the state can be associated with biologic activity. This question is directed ultimately to *Mycobacterium leprae*. Since *M. leprae* can not be cultured yet, it is not possible to investigate *M. leprae* directly. However, since acid-fastness is a genetic characteristic of the genus Mycobacteria, the demonstration of the existence of Ziehl-Neelsen unstained, viable organisms in one species of mycobacteria would, by definition, be evidence of the possibility for their existence in other species of the genus.

This research is based on the generally accepted axiom that biologic growth occurs as a logarithmic function when it is progressing without significant opposition. Any procedure to measure populations must portray this characteristic. If there is a divergence between two or more means of measuring the same population then an inconsistency is obvious. With respect to pure cultures of mycobacteria, there should be significant agreement between the growth curves of a single culture whether these curves are derived by turbidimetric measure, microscopic counts or by actual determination of numbers of viable cells in the population. Accepting this criterion, it is then assumed that the existence of a biologically active, non-ZN-staining mycobacterium would be demonstrable by an inconsistent relationship between a growth curve based on acid-fast counts and one based on numbers of viable units.

## MATERIALS AND METHODS

The procedure was very simply defined to follow mycobacterial cultures from the date of inoculation, over a period of 46 days, for increases in: (a) optical density, (b) microscopic counts of acid-fast stained standard aliquots, and (c) the number of viable cellular units per standard aliquot of culture.

The experimental organism was the NQ bacillus, a mycobacterium isolated via hamster ear and testis inoculation by Binford [3] from leprosy patients. The growth medium was Dubos medium at pH 6.8. This medium was slightly modified by exclusion of Tween 80 and incorporation of 20 per cent bovine serum. Fifteen screw cap culture tubes, 16 x 125 mm., containing 5.4 ml. of medium were inoculated with 0.6 ml. each of a 1 to 100 dilution of a pure 28 day old culture of NQ bacillus and incubation was at 30°C. The 15 tubes of experimental culture were read daily for turbidi-

ty directly in the culture tubes using a Coleman Jr. spectrophotometer at 525 nm., 16 mm. light path. The 15 readings were averaged and that tube which had a turbidity nearest to the daily means was selected to provide the sample aliquots for viability and microscopic counts.

Microscopic counts were made by the procedure of Shepard and McRae ([11]) as applied to experimental leprosy infections in mouse foot pads. At least two dilutions of each daily specimen were given blind to a technician whose primary task was the conduct of comparable counts on suspensions of infected mouse foot pad specimens. Three separate smears were counted for each of the dilutions. These counts were averaged and the mean of the values in the critical dilution was taken as the data for this investigation. If there was significant divergence between the three values of the critical dilution, a duplicate set of three smears of the critical dilution was counted to test for significant deviation. The critical dilution was that dilution which gave a good counting range in terms of bacteria per field; usually between five to 50. Occasional spot checks were made by blind recounts on randomly selected slides previously counted and stored.

Viability counts were made by a standard bacteriologic tube-dilution-series. In this experiment, increasing increment 10-fold dilutions were made directly into the experimental medium serving as a diluent. The dilutions ranged between $10^{-1}$ to $10^{-11}$ and usually at least six dilutions in this range were tested for Poisson distribution within the anticipated range of growth. At each of these six dilution levels, 10 tubes of the experimental medium were inoculated with 1.0 ml. of the dilution in question. The estimation of most probable numbers (MPN) was based on the table of values for Poisson distributions by Halvorson and Ziegler ([5]). This kind of a series of 10-fold dilutions, 10 tubes each dilution has a "factor for 95% confidence limits" of 2.32 according to Cochran ([4]).

All of the data derived by this procedure was indicative of Poisson distribution. A preliminary test for reproducibility was conducted with a single specimen inoculated into three replicate dilution series. The results were comparable and the data from each series was Poisson in nature and compared with each other well within the 95 per cent confidence limits.

## RESULTS

The photometric turbidity curves rose at a rate consistent with the anticipated growth for NQ bacillus in a serum medium. Figure 1 shows this curve over a period of 46 days. At 46 days, in this experiment, there was still some indication of increasing turbidity. Considerable experience with this control curve over several years has shown that the peak of this curve always occurs within this range of 0.3 optical density (OD) at about 30 to 40 days. If continued further there has always been a marked reduction in the rate of turbidity increase from this point but, nevertheless a constant slow increase did always occur up to about 0.35 OD over a period as long as six months.

The data of the acid-fast counts and viability are indicated also in Figure 1. However, the ordinate for these values differs from that used for turbidity. The turbidity curve was superimposed on the counts and viability curves to indicate rate relationships only. The optical density ordinate for turbidity has no relative point of reference with the ordinate of the Log No. of bacteria. Use of the zero optical density point at the 5 position on the Log No. scale was selected merely to give a minimum of interference with the other two curves. Accordingly, no relative significance can be attached to either height or cross-over points of the turbidity curve. The curves of viable units and acid-fast counts are on the same ordinate scale of Log No. and are relative to each other.

It can be seen that the acid-fast counts reached a peak of slightly more than $10^8$ bacteria per ml. at 10 days. After this there was some fluctuation with an apparently slightly increasing plateau being recorded during the 36 days of the plateau period. This plateau was drawn on the basis of 21 points. Every point fell on the lines of the erratic curve indicated in this figure. The lowest point on this erratic plateau occurred at 20 days. The microscopic counts on this day indicated a bacterial concentration of $8 \times 10^7$ bacteria per ml. The highest

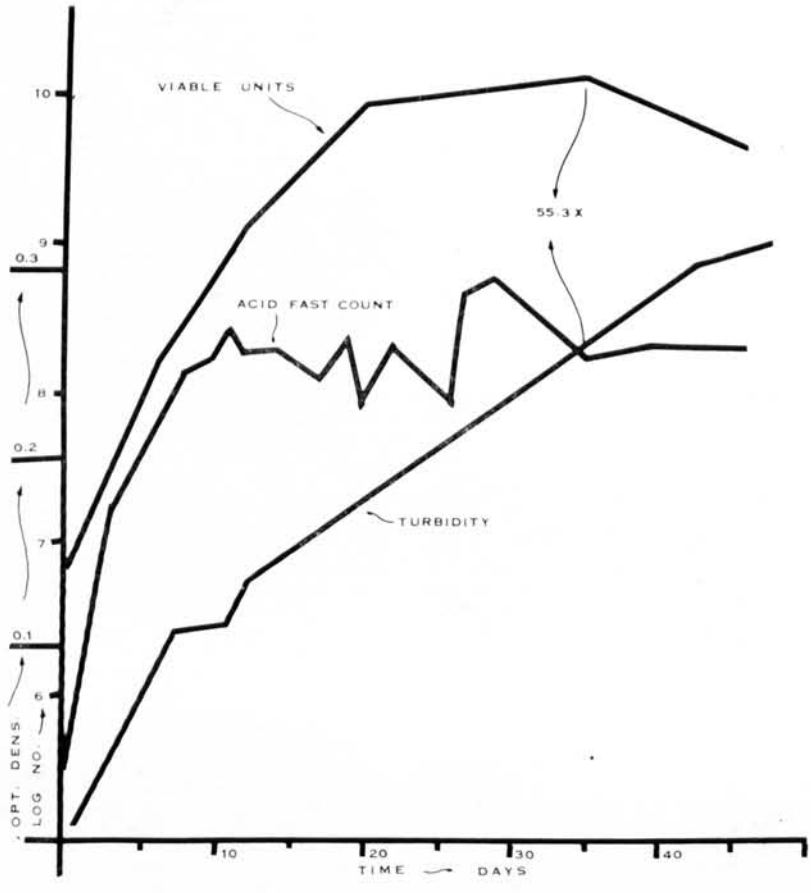


FIG. 1. The growth curves of the NQ bacillus based on turbidity, acid-fast counts and viability.

point on the plateau occurred at 30 days with a reading of 7 x $10^8$ bacteria per ml.

The viability curve was far more uniform than the acid-fast count curve but, was produced by a fewer number of points. There were 11 points on the entire curve. Of these points, only two at three days and 17 days, fell off of the curve as drawn. The viability point at three days actually coincided with the value for the microscopic count at 2 x $10^7$ bacteria per ml. At 17 days, the off-curve point actually occurred at 2 x $10^9$ bacteria per ml. The curve, as drawn, crosses the abscissa value of 17 days at an ordinate value of 6 x $10^9$ bacteria per ml. at this point.

Viability increased almost linearly on the log scale until the 20th day. At this point the values indicated a bacterial concentration of 9 x $10^9$ bacteria per ml. The six points from day 20 to day 46 are on the indicated curve with a maximum value of 1 x $10^{10}$ at day 35 and a minimum of 7 x $10^9$ at day 46.

## DISCUSSION

When Gram-stained organisms of the order Eubacteriales are studied for the relationship between microscopic counts and viability, a relationship within experimental error is nearly always evident. It is generally accepted that the microscopic counts are always somewhat higher than viability counts. This difference is attributed to stainable, microscopically visible cells which are either dead or do not reproduce under the experimental conditions.

In this investigation using Ziehl-Neelsen stained mycobacteria, the pattern was re-

versed. For some reason, viability continued to increase numerically beyond the level achieved by microscopic counts. Figure 1, shows that at day 35, there were 55 times more viable units than could be accounted for by microscopic counts. At 26 days viable units were 100 times more numerous than acid-fast cells and at 46 days the ratio was 23-fold. This reverse pattern, even disregarding degree, was very difficult to rationalize.

The mycobacteria are notorious for clumping and this could have been a factor to consider. However, in microscopic counting clumps are generally visualized. In the procedure used for this experiment, the count included an estimate of the number of bacilli in the clumps. The NQ bacillus is not a notorious clumper but clumps do occur, especially in the range of about five bacilli per clump. On the other hand, clumps would have influenced the tube-dilution series to an even greater extent than the counts. Consideration of clumps would have increased the viability values above the values reported here. The viability curve was reported on the assumption that a single cell was instrumental in initiating growth when, in point of fact, the growth in the terminal tubes could have resulted from a clump or clumps of cells.

The lowest point on the viability curve at 46 days and the equivalent point on the acid-fast count curve differed by 23-fold. If the viability curve at this point would have been reduced to the minimum level of the 95 per cent confidence limit and the count value increased by the 10 per cent possible error accepted for microscopic counting, the points would possibly concide. This acceptance of the extremes of possible experimental error at the closest points of agreement of the curves would still not reduce viability to the lower than acid-fast count value that could be expected so late in the growth curve.

This reverse pattern was not only evident in the peaks of the curves, it was also evident in the duration of the logarithmic increase phases of these two curves. While the microscopic count curve peaked at 11 days, the viability showed marked increases for nine days beyond this point. The reality of this increase seemed to be evident in the mechanics of the data plots. At day 3, viability and counts were equal. At day 6, viability was two times greater than counts. At day 12 the ratio was four times and at day 18 about ten times. The ratio of viability over microscopic counts increased with respect to age of the culture during the mid to late logarithmic phase of growth. It would be at this time that one should have anticipated the maximum staining intensity of the organisms.

It was interesting to note that, even though there was no relative point of reference for the curves of turbidity and microscopic counts, the turbidity curve showed increasing particle density of the culture long after the microscopic counts indicated that no new acid-fast cells were being reproduced. Stained smears and wet mounts, observed microscopically did not show any significant nonbacterial material that could account for this phenomenon. There was no evidence of medium serum protein or lipid participation in the turbidity. In fact, the countereffect of a reduction of the optical density due to clarification in the medium of the slight serum-hemaglobin color appeared to be more influential than the possibility of nonbacterial precipitation increasing the OD.

The paralleling of the viability and turbidity curves was far more apparent than the parallel relationship between counts and turbidity. There was an apparent break in the early stage of the turbidity curve at day ten. This preliminary plateau has occurred consistently in the work in this laboratory ([9]). The break in the curve appeared to be real. Each point on the turbidity curve was based on the means of daily readings of 15 tubes of culture. The early plateau was produced by three points over a period of four days. The rate of turbidity increase after the plateau was also obviously different from the rate before the plateau. A possible explanation for the nature of this data could exist in a hypothesis that mycobacteria which stain and those which do not stain have some *in situ* difference in light absorption properties. If this hypothesis is acceptable, then the initial rapid rate of turbidity increase could be related to the early rapid increase in acid-fast count. The early plateau was consistent

in time with the peak of acid-fast count and the slower subsequent rate of turbidity increase could have been primarily a function of the continued increase in numbers of the viable unstaining entity and the cessation of acid-fast increases which occurred beyond this point. The relationships between turbidity and acid-fastness and, turbidity and viability would actually never be direct because the turbidity curve would reflect the increasing cumulative absorption values of the mixed populations.

These two hypothetic cell types could be interconvertible. The data in Figure 1 for T = O was extrapolated from information obtained on the inoculum itself and not on the inoculated tubes. Viability at T = O is indicated as being $8 \times 10^6$ and the acid-fast count as $3 \times 10^5$. These figures are based on values of $8 \times 10^9$ and $3 \times 10^8$ respectively, obtained from the inoculum which was subsequently diluted 1:1,000 in the process of initiating this experiment. It can be seen from Figure 1 that these values for the 28 day old inoculum are in good agreement with the values reported here for the experimental cultures at 28 days.

At three days, the acid-fast counts and viabilities were almost identical. Counts showed $1.65 \times 10^7$ bacteria per ml. While the equivalent viability data, based on 10, 9 and 1 turbid tubes out of 10 each on the $10^6$, $10^7$ and $10^8$ dilutions, respectively, had an MPN of $1.97 \times 10^7$ bacteria per ml. ([5]). From this data, it can be seen that viability showed a moderate lag phase with an increase from $8 \times 10^6$ to $1.5 \times 10^7$ in three days. During this same period, however, the microscopic counts increased from $3 \times 10^5$ to $1.7 \times 10^7$. Again there appears to be some departure from the kind of results that would normally be anticipated in bacterial cultures. Microscopic counts showed no significant lap phase and this increase in acid-fastness occurred during the period of growth when bacteriologists generally anticipate the most difficulty in stain differentiation. This indicated the possibility that, during the first three days of incubation (lag phase) most of the unstaining bacilli in the inoculum became acid-fast capable in character. This could account for the apparently extremely rapid initial rate of growth, based on acid-fast count, of almost two logs in degree that occurred in the first three days after inoculation. This rate of growth would be consistent with a generation time value (G) of 6.2 hours for the NQ bacillus. That rate was too fast and, interconversion from nonstaining to acid-fast capable becomes a distinct possibility. This would suggest a cyclic phenomenon in the reproductive aspect of the mycobacteria.

This experiment reported data that was derived only over a period of 46 days and it appears that that data gave only the results of the early growth phase and stationary phase of the NQ bacillus. No significant death phase was evident. The data showed that viability was, quantitatively, always greater than could be accounted for by microscopic counts. The viability curve did appear to drop off in a death phase in the period between 35 to 46 days. This drop-off is most likely a reality. Subsequent studies on other cultures were done to test this possibility. Microscopic count and viability relationships were done in a similar manner on a six month old culture and on a five year old culture of NQ bacillus in the same medium as that used in this experiment. Both of these cultures had been maintained at 30°C for the entire age of the cultures. The microscopic counts of these cultures were quite subjective because of the granular, fragmented and beady nature of the acid-fast stained material. It was difficult to define just what should or should not be counted. It was estimated in these studies that viability was in the order of 50 per cent of the number of acid-fast cells at six months. The culture incubated for five years, surprisingly, still possessed viability but, it was less than 1 per cent of the estimate of the number of acid-fast bacilli. These results indicated that the condition of greater viability than stainability was a function of reproduction of the culture and, accordingly, the characteristic that makes a mycobacterium refractory to the Ziehl-Neelsen stain is most likely associated with physiologic youth.

The fact that viability counts were lower than acid-fast counts in older cultures tended to support the data in the younger cultures. The drop-off in viability was to be anticipated with aging. It showed that the

procedure was capable of demonstrating a condition of viability below microscopic counts. It also showed that the viability curve was not only acceptable as being demonstrative of a valid biologic growth curve but, in this respect, was more acceptable than that of the microscopic count which showed no significant evidence of a death phase and which failed to show any evidence for the continued increase in turbidity.

The viability curve as reported here indicated that the NQ bacillus, a mycobacterium, could show a biologic growth curve comparable to other microorganisms. This fact has never been satisfactorily evident in data based on microscopic counts, dry weights, nitrogen determinations, turbidities or, plate counts.


## SUMMARY

The Ziehl-Neelsen stain is primarily used in the differentiation of mycobacteria from other microorganisms. The mechanics of this stain are poorly understood but, the evidence in the literature indicates that acid-fastness is most likely due to retention and distribution of free dye within the cytoplasm. The degree of retention and the homogeneity of distribution of the dye is apparently variable, to some extent, as a result of extracellular factors. This uncertainty regarding the uniformity of the Ziehl-Neelsen stain would require that the validity of its use as an experimental parameter for intraspecies evaluations would be dependent on the growth curve relationships that exist between microscopic counts, and viability in the experimental systems. These relationships would be critical in any study where data based on observation of Ziehl-Neelsen stained mycobacteria would be extrapolated to conclusions concerning functions of viability.

The growth curves of a pure culture of the NQ bacillus (Mycobacterium sp) were derived by three procedures: turbidity, microscopic counts and viability by most probable numbers based on the Poisson distribution in a tube-dilution-series. Turbidity increases continued beyond 30 days. Microscopic acid-fast counts peaked at 11 days. Viability increased for 20 days. The three curves were not compatible as being representative of the same phenomenon. The viability curve was more representative of the accepted version of biologic reproduction than either of the other two curves. Contrary to the results normally anticipated in bacteriologic growth curves, the number of viable reproductive units was significantly greater than could be accounted for by the counting of acid-fast stained smears. These data suggested the possibility that, in the active reproductive phase of a mycobacterial culture, a significant proportion of the population remained unstained by the Ziehl-Neelsen procedure. This characteristic appeared to be related to the physiologic youth of the culture.



## RESUMEN

La tinción de Ziehl-Neelsen se utiliza primordialmente para la diferenciación de las microbacterias de otros microorganismos. Los mecanismos de esta tinción no están bien aclarados, pero la evidencia en la literatura indica que la ácido-resistencia se debe probablemente a la retención y distribución de colorante libre dentro del citoplasma. El grado de retención y la homogeneidad de distribución del colorante son aparentemente variables, en cierto grado, a consecuencia de factores extracelulares. Esta incertidumbre con respecto a la uniformidad de la tinción de Ziehl-Neelsen podría requerir que la validez de su utilización como un parámetro experimental para evaluaciones intraespecie fuera dependiente de las relaciones de curvas de crecimiento que existen entre los recuentos microscópicos y la viabilidad en los sistemas experimentales. Estas relaciones podrían ser críticas en cualquier estudio en el cual los datos obtenidos de la observación de microbacterias teñidas con Ziehl-Neelsen fueran extrapolados para llegar a conclusiones en relación a funciones de viabilidad.

Se derivaron lea curvas de crecimiento de un cultivo puro del bacilo NQ (especie Mycobacterium) utilizando tres procedimientos: turbidez, recuentos microscópicos y viabilidad, por medio de números de mayor probabilidad basados en la distribución de Poisson, en una serie de diluciones en tubos. Los aumentos de turbidez continuaron hasta más allá de los 30 días. Los recuentos microscópicos de ácido-resistencia tuvieron su punto máximo a los 11 días. La viabilidad aumentó durante 20 días. Las tres curvas no fueron compatibles como representación de un mismo fenómeno. La curva de viabilidad fué más representativa de la versión

aceptada de reproducción biológica que cualquiera de las otras dos curvas. Contrariamente a los resultados que se anticipan normalmente en las curvas de crecimiento, el número de unidades viables para reproducción fué significativamente mayor que lo que se traducía por medio del recuento de frotis teñidos para ácido-resistencia. Estos datos sugieren la posibilidad que, en la fase reproductiva activa de un cultivo de microbacterias, una proporción significativa de la población no sea teñida por el procedimiento de Ziehl-Neelsen. Esta característica parece estar relacionada con la juventud fisiológica del cultivo.

## RÉSUMÉ

La coloration de Ziehl-Neelsen est surtout utilisée pour permettre de distinguer les mycobactéries des autres microorganismes. Le mécanisme de ce procédé de coloration est mal compris; les donnóes qui peuvent être recueillies dans la litérature indiquent toutefois que l'acido-résistance est le plus probablement due à la rétention et à la distribution du colorant libre à l'intérieur du cytoplasme. Le degré de rétention et l'homogénéité de la distribution du colorant peuvent apparemment varier dans ine certaine mesure, par suite de facteurs extra-cellulaires. Le fait que la coloration par la technique de Ziehl-Neelsen ne soit pas uniforme entraíne une conséquence importante. Son utilisation comme paramètre expérimental pour l'évaluation des caractéristiques microbiologiques d'une espèce à l'autre n'est valable que si l'on tient compte du fait qu'elle est liée à la relation qui existe entre les courbes de croissance et les numérations microscopiques, ainsi qu'à la viabilité dans les systèmes expérimentaux. Ces relations pourraient présenter une importance critique dans toute étude, dont les résultats, basés sur l'observation de mycobactéries colorees par la méthode de Ziehl-Neelsen, seraient extrapolées en vue d'établir des conclusions quant aux possibilités de survie des organismes.

Les courbes de croissance d'une culture pure de bacilles NQ (espèce Mycobacterium) ont été obtenues par trois procédés: la turbidité, les numérations microscopiques, et les études de viabilité en se basant sur les chiffres les plus probables calculés en faisant l'hypothèse que le nonbre de ces bacilles, dans une série de tubes à dilution de plus en plus grande, suit une distribution de Poisson. L'accroissement de turbidité a persisté au-delá de 30 jours. Les numérations microscopiques de bacilles acido-résistants étaient les plus élevés au onziéme jour. La viabilité a augmenté pendant 20 jours. Les différences notées entre les trois courbes be permettent pas de les considérer comme représentant le même phénomène. La courbe de viabilité suggérait, davantage que ne le faisaient les autres courbes, la théorie aujourd'hui acceptée d'une reproduction biologique. Contrairement aux résultats auxquels on pourrait s'attendre en examinant les courbes de croissance des bacilles, le nombre d'unités viables et capables de reproduction était significativement plus élevé qú il néut été possible de prédire par la simple énumération des bacilles acido-résistants sur frottis colorés. Ces données suggèrent que, lorsqu'on procède à la coloration des bacilles dans une culture de mycobactéries en phase de reproduction active, une proportion significative de la population bactérienne échappe à la coloration par la technique de Ziehl-Neelsen. Il semble que cette caractéristique soit liée à la jeunesse physiologique de la culture.

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