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Fluorescein diacetate and ethidium bromide staining to determine the viability of *Mycobacterium smegmatis* and *Escherichia coli*

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Summary The ability of the fluorescein diacetate and ethidium bromide fluorescent staining method to assess the percentage of viable bacterial cells in suspension was compared with the plate counting method. *Mycobacterium smegmatis* and *Escherichia coli* bacterial cell suspensions were incubated at 60°C. At different time intervals samples were taken and the percentage of viable cells in each sample was assessed by the fluorescent staining method and compared with the plate counting method. The fluorescent staining method showed a positive correlation with the plate counting method. However, the viable counts by the plate counting method were lower than the staining method when incubated at 60°C, indicating a lag period in the decay of enzymes after bacterial death. Hence, the fluorescent staining technique can be used to assess the trend of bacterial death rather than to assess the exact number of viable bacilli.

Introduction

Fluorescein diacetate (FDA) was used to determine the viability of mammalian cells.[1] It is a nonpolar, nonfluorescent fatty acid ester and passes readily into living cells where it is hydrolysed by esterases to yield polar, fluorescent fluorescein, if the membrane integrity is intact. The fluorescein rapidly accumulates inside the cells resulting in green-stained cells when viewed under fluorescent microscope.

Ethidium bromide (EB) enters cells whose cell membrane integrity is altered and intercalates with double stranded nucleic acid to form a red-orange fluorescent complex.[2]

Both FDA and EB were combined into a single assay and used to measure the viability of *M. smegmatis* and *M. phlei*.[3] It has been suggested th t FDA:EB staining procedure can be used to assess the viability of *M. leprae*.[4–6] In the present study, the FDA:EB

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staining procedure is compared with the time honoured plate counting method to assess the percentage of viable *M. smegmatis* and *Esch. coli* cells in suspension.

Materials and methods

Kvach & Veras's (1982) procedure was adopted to prepare single cell *M. smegmatis* suspension.[3] The single cell suspension was placed in a bath of water at 60°C. At 0 min, 20 min and 50 min, samples were taken and used for FDA:EB staining, plate counting (serially diluted suspensions were plated on Dubos agar medium, incubated at 37°C and colonies counted after 72 h) and calculating the total number of cells. (Ten micro litres of the serially diluted single cell suspensions were smeared in a fixed area on a microscopic slide and stained using the Ziehl–Neelsen method. The number of bacilli in the circled areas were counted and from this the total number of bacilli in the undiluted suspension was calculated.)[7]

The percentage of viable cells using the plate counting method was calculated by the following formula:

$$\text{Percentage of viable cells by plate counting method} = \frac{\text{Coloney count/ml, in undiluted cell suspension}}{\text{Total number of cells/ml, in undiluted sample}} \times 100.$$

Esch. coli was grown in nutrient broth at 37°C. The cell suspension was kept in a bath of water at 60°C. Samples were taken at 0 min, 30 min and 60 min and used for FDA:EB staining, calculating the number of cells (as described earlier except that a simple staining with dilute carbol fuchsin was done instead of acid-fast staining) and plate counting (serially diluted suspensions were plated on nutrient agar plates and colonies were counted after overnight incubation at 37°C).

FDA:EB working solution was prepared as per the procedure of Kvach & Veras.[3] A working solution of 0·5 ml of FDA:EB was added to 1·0 ml of *Esch. coli*/*M. smegmatis* single cell suspension and incubated for 10 min at room temperature. A wet mount was prepared and viewed under a dark field fluorescent microscope. B223 as the primary filter and G247 as the secondary filter were used. A total number of 200 red and green cells were counted. Green cells were considered as live and red cells as dead. Percentage viability by this procedure was calculated using the formula mentioned below:

$$\text{Percentage of viable cells by staining procedure} = \frac{\text{No. of green cells}}{\text{Total No. of cells counted}} \times 100.$$

Results

M. smegmatis incubated at 37°C for 48 hr showed $90 \pm 5\%$ (mean $\pm$ standard deviation of three experiments) green cells by staining and $83 \pm 10\%$ viable cells by plate counting. There were $20 \pm 7\%$ and $5 \pm 2\%$ green cells after heating for 20 min and 50 min respectively at 60°C. The plate counting showed $0{\cdot}001 \pm 001\%$ and 0% viable cells after heating at 60°C for 20 min and 50 min respectively (Figure 1).

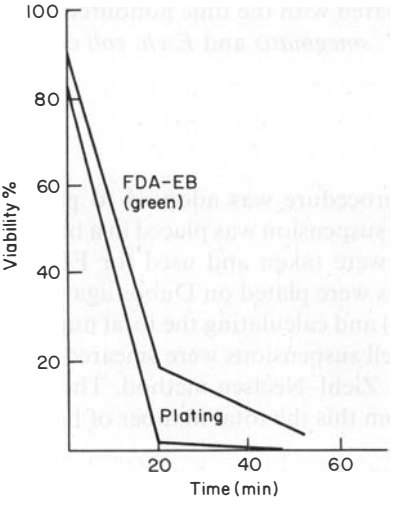

Figure 1. Percentage of viable *M. smegmatis* cells (at 60°C) detected by FDA:EB staining (green cells) and the plate counting method.

Esch. coli grown overnight showed 90 ± 6% green cells by staining and 85 ± 7% viable cells by plate counting. After heating at 60°C for 30 min, 46 ± 5% green cells were seen by staining while the plate counting showed 0·003 ± 0·002% viable cells. After 60 min at 60°C, there were 20 ± 7% green cells whereas no bacterial colony (0%) was seen in the plate (Figure 2).

Autoclaved samples of both *Esch. coli* and *M. smegmatis* showed 0% green cells.

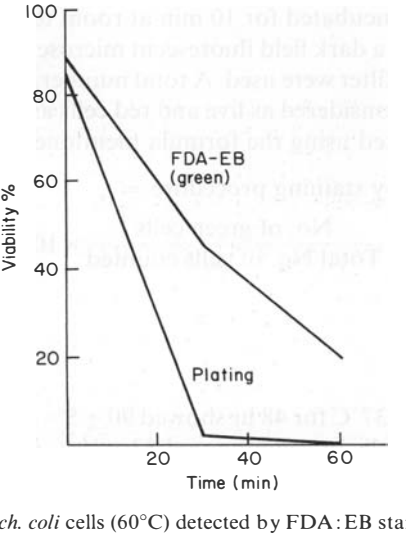

Figure 2. Percentage of viable *Esch. coli* cells (60°C) detected by FDA:EB staining (green cells) and the plate counting method.

Discussion

In *M. smegmatis*, the percentage of green cells decreased when they were heated for longer duration (90%, 20% and 5% at 0 min, 20 min, and 50 min respectively at 60°C). This corresponds with the decrease in percentage of viable cells seen in plate counting (83%, 0·001% and 0% at 0 min, 20 min, and 50 min respectively at 60°C).

Similarly, the percentage of green *Esch. coli* cells decreased with increase in duration of heating (90%, 46% and 20% at 0 min, 30 min and 60 min respectively at 60°C). This corresponds positively with the decreasing percentage of viable cells seen in plate counting (85%, 0·003% and 0% at 0 min, 30 min and 60 min respectively 60°C). Thus the decreasing percentage of green cells correspond with decrease in viability (as seen from plate counting). Yet the correlation is not absolute. The percentage of green cells is always higher (7–20% and 5–45% in *M. smegmatis* and *Esch. coli* respectively) than that expected by plate counting. A similar observation has been made by others.[3,8]

Kvach & Veras have suggested that the higher percentage of green cells as compared to colony forming units could be due to bacterial cell clumping and adherence of some bacteria to the L-shaped glass rod used to spread the suspension on plates.[3]

In our study, the bacterial suspensions were spread over the plate by simple tilting and rotation of plates and we did not use a L-shaped glass rod. When the bacterial suspensions were autoclaved (121°C for 15 min) all the cells became red and not even a single green cell was seen. But at a lower temperature (60°C) more green cells than expected were seen. Thus the persistance of some green cells is probably related to the inactivation of enzyme rather than clumping or adherence of bacteria to the L-shaped glass rod.

Our study clearly shows that the enzyme responsible for the fluorescent staining remains active for sometime after the death of bacilli and supports the similar view expressed by Katoch *et al.*[9] The fluorescent technique thus appears to be useful mainly in assessing the reduction of viability rather than true quantitation of viable cells. This needs to be kept in mind when assessing the viability of mycobacteria from clinical samples.

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La coloration au diacetate de fluorescéine et bromure d'éthidium pour determiner la viabilité de *Mycobacterium smegmatis* et d'*Escherischia coli*

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Sommaire La capacité de la méthode de coloration au diacetate de fluorescéine et bromure d'éthidium pour estimer le pourcentage de céllules bactériennes viables en suspension a été comparée avec la méthode de compte des colonies sur plaque. Des suspensions de céllules de *Mycobacterium smegmatis* et d'*Escherischia coli* ont été incubées à 60°C. Aux différents intervalles de temps des échantillons ont été pris et le pourcentage de céllules viables estimé sur chaque échantillon par la méthode de coloration fluorescente. Le résultat obtenu a été comparé à la compte des colonies sur plaque. La méthode de coloration fluorescente a montré une corrélation positive avec la méthode de compte sur plaque. Néanmoins, la valeur obtenue pour la recompte des colonies viables sur plaque a été moins élevée que chez la méthode de coloration après incubation à 60°C, ce qui indique un décalage entre la morte des bactéries et la détérioration des enzymes. D'ou la méthode de coloration fluorescente peut être utilisée pour estimer les tendances dans le dépérissement des bactéries plutôt que pour le calcul exacte du nombre de bacilles viables.

Coloración a base de diacetato de fluoresceíne y bromuro de etidio para determinar la viabilidad de *Mycobacterium smegmatis* y de *Escherichia coli*

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Resumen Se comparó el método de coloración a base de diacetato de fluoresceína y bromuro de etidio para calcular el porcentaje de células bacterianas viables en suspensión con el método de cuenta de colonias sobre placas. Se incubaron a 60°C suspensiones de células bacterianas de *Mycobacterium smegmatis* y de *Escherichia coli*. Se tomaron muestras a intervalos de tiempo distintos, se calculó el porcentaje de células viables en cada muestra por el método de colorantes fluorescentes y se comparó el resultado al obtenido por el método de cuenta de colonias sobre placas. Se halló una correlación positiva entre los resultados obtenidos por ambos métados. No obstante, la cuenta de células viables por el método de cuenta de colonias sobre places resultó inferior a la cuenta obtenida por medio del método de colorantes (llevándose la incubación a cabo a 60°C). Esto sugiere que existe un periodo de retraso tras la muerte de las bacterias hasta iniciarse el deterioro de las enzimas. Por lo tanto, el método de coloración fluorescente puede utilizarse más bien para evaluar las tendencias del decaimiento de las bacterias que para calcular el número exacto de bacilos viables.