The Determination of the Most Probable Numbers of Streptomycin-fast Cells in Brucella Cultures and their Variability in Growing and Ageing Cultures

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SUMMARY: The method of most probable numbers (MPN) was employed in order to determine the number of resistant brucella cells at concentrations of streptomycin of 1, 10 and 100 $\mu\text{g./ml.}$ The examination showed agreement with the centrifugation method (plating of the sediment of at least 100 ml. of culture together with the required concentration of streptomycin) in a range up to 1000 resistant cells/100 ml. The method showed that there exist differences in the numbers of resistant cells in different strains, and that in cultures originating from the same colony variations occur. The general trend is a relative increase of the resistant cells with the age of the culture.

Methods for determining the number of streptomycin-resistant cells in bacterial cultures have been described by many authors. Klein & Kimmelman (1946) employed broth cultures of *Shigella dysenteriae*. Tenfold dilutions up to 10^{-6} were assayed in varying concentrations of streptomycin in melted agar, the test requiring at least 42 plates. In another experiment they distributed 40 ml. of broth culture into 400 broth tubes containing 1000 units streptomycin/ml. After 48 hr., growth appeared in 5 out of 400 tubes. Yegian & Vanderlinde (1948) incubated different sizes of inocula of *Mycobacterium ranae* on glycerol agar with varying quantities of streptomycin; one test required at least 120 plates. English & McCoy (1951) incubated 100 tubes containing 0.3 ml. broth culture of *Micrococcus pyogenes* and 200 units of streptomycin. One tube out of 100 showed visible growth after 24 hr. They estimated the number of bacteria incubated in 100 tubes at 67×10^8 and concluded, therefore, that one resistant cell was present in this total bacterial population. All these methods require large quantities of tubes, agar and plates, and are time and material consuming. We tried, therefore, to simplify this technique by the determination of the most probable number (MPN) of streptomycin resistant micro-organisms in a culture. This method was proposed by McCrady (1915) for the numerical interpretation of fermentation tube results. For our purposes, the determination of small numbers of streptomycin resistant cells in a large bacterial population, we used the tables published by Hoskins (1934), reprinted in *Standard Methods for the Examination of Water and Sewage* (1936). We expected that with this relatively simple technique it would be possible to detect the presence of streptomycin resistant organisms in brucella cultures at different stages of their incubation.

METHODS AND RESULTS

Preliminary experiments were made to determine the action of streptomycin on the whole bacterial population, and the time of the first visible growth of resistant bacteria at different levels of streptomycin. The methods employed were as follows:

Media. The liquid media contained, per litre, 30.0 g. dehydrated trypticase soy broth (Baltimore Biological Laboratories), 30.0 ml. glycerol and 1.0 ml. of a solution of aneurin containing 0.1 mg./ml.

Strains. Two strains of *Brucella melitensis*, M5 and M7, two strains of *Br. suis*, S89 and S6, and two strains of *Br. abortus*, A 19 and A 2308, were used. After several subcultures on the above medium 24 hr. cultures were used for the experiments described below.

Fig. 1. The changes in bacterial count within 8 hr. in broth containing 1.0 µg. streptomycin/ml.

Streptomycin. Streptomycin sulphate (Squibb) was used in all experiments.

Technique. All experiments were carried out in test-tubes of 20 mm. diameter. The total volume of liquid medium containing the streptomycin and the bacterial inoculum was 10.0 ml. The wide test-tubes containing this relatively small quantity of fluid were thoroughly agitated and then incubated at 37°. Small samples of tube contents were taken at intervals, serial dilutions prepared (1/40, 1/1600, 1/64,000, 1/2,560,000) and 1.0 ml. of each dilution poured to agar plates. At those stages of the experiments where the bacterial count was low the samples were diluted less, and finally the undiluted contents of the tube were poured into agar plates. Fig. 1 represents the number of micro-organisms/ml. found at different intervals within 8 hr. in contact with 1.0 µg. streptomycin/ml. Of the six strains examined five tended to grow, while one strain (A 2308) after an initial rise showed a definite decrease in the number of organisms, beginning at the fourth hour.

Fig. 2 shows the behaviour of the bacteria from the tenth hour after contact with 1.0 µg. streptomycin/ml. All strains show a gradual decrease of the

number of bacteria; two (M7 and A2308) were completely sterile after 24 hr.; the other four strains contained many living bacteria.

Fig. 3 shows the further behaviour of the four surviving strains. All of them showed on the following days in contact with streptomycin a steady increase of their bacterial count, strain M5 after a slight decrease continuing 2 days. This behaviour of the micro-organisms in the presence of streptomycin was modified under different conditions. With a greater inoculum the number of bacteria did not decrease below 10^6 /ml. and a S-shape curve resulted. When the concentration of streptomycin was $100\mu\text{g./ml.}$ the number of bacteria decreased after 4 hr. incubation.

Fig. 2. The changes in bacterial count within 24 hr. in broth containing $1.0\mu\text{g. streptomycin/ml.}$

These experiments showed that the rate of disappearance of the non-resistant bacteria and the time of the initial growth of the resistant bacteria depend upon the concentration of streptomycin and upon the number of organisms in the inoculum. It was necessary to decide whether the bacteria resistant to a given concentration of streptomycin were resistant only to this concentration or whether they had acquired complete resistance. In an experiment carried out with three different inocula of strain M5 exposed to $1.0\mu\text{g. streptomycin/ml.}$ for 24 hr. the following results were obtained:

Initial inocula/10 ml.	6.5×10^6	1.8×10^7	2.6×10^7
Surviving bacteria after 24 hr./10 ml.	5.8×10^8	1.5×10^6	6.9×10^5

The figures show that the percentage of surviving bacteria increases with the increase of inoculum size. About 100 single colonies were picked and transferred to plain agar, and the resulting cultures transferred to agars

containing 1.0, 10.0 and 100.0 μg . streptomycin/ml. The results obtained with the surviving bacteria from the three inocula were very similar:

Inoculum	...	...	1	2	3
Total colonies tested			31	33	32
Growing on 1.0 μg ./ml.			30	30	28
Growing on 10.0 and 100.0 μg ./ml.			1	1	1

Fig. 3. The changes in bacterial count 24–144 hr. in broth containing 1.0 μg . streptomycin/ml. Development of resistant micro-organisms.

This experiment showed that after 24 hr. the surviving bacteria were nearly all resistant to 1.0 μg . streptomycin/ml., but only about 3% were resistant to higher concentrations. All bacteria resistant to 10 μg ./ml. were resistant to 100.0 μg ./ml. Similar experiments were carried out with other strains exposed to different streptomycin concentrations. Most of the strains isolated at 1.0 μg ./ml. were resistant only against this concentration. Of 284 strains isolated at 1.0 μg ./ml. 17 were resistant against 10 or more μg ./ml. When a strain tolerated the tenfold concentration then it also tolerated all higher concentrations up to 1000 μg ./ml. On the other hand, all the strains

isolated from broth containing 10 and 100 μg . streptomycin/ml. tolerated all the higher concentrations up to 1000 μg ./ml.

In these brucella cultures there appear to be present mainly two streptomycin-resistant variants: (1) variants partially resistant to 1 μg ./ml. but not resistant to higher concentrations; (2) completely resistant variants resistant to 10 and 100 μg ./ml. and higher concentrations. The following experiments were, therefore, carried out at 1.0, 10.0 and 100.0 μg ./ml. to determine the MPN of the partially and completely resistant cells present in different cultures.

Determination of the MPN of organisms resistant to 1.0 μg . streptomycin/ml.

To determine the MPN, 24 hr. broth cultures were diluted in tenfold steps and from suitable dilutions 1 ml. volumes were added to tubes containing 9 ml. broth, and streptomycin added in the required concentration. After preliminary experiments to determine after a 5-day incubation period the range of inocula just sufficient to give resistant organisms, three inocula around this critical limit were selected and five broth tubes seeded with each inoculum. The inocula sizes differed by a factor of 10. The MPN was determined after incubation for 5 days for an inoculum ten times greater than the largest inoculum used in the experiment, by means of the tables of Hoskins (1984).

Table 1. *Determination of the most probable number (MPN) of brucella cells resistant to 1.0 μg . streptomycin/ml. in 24 hr. broth cultures*

Strain	Size of inocula			Positive results per 5 portions of each inoculum			MPN	No. of organisms per resistant cell
	1	2	3	1	2	3		
M5	1.8×10^7	1.8×10^6	1.8×10^5	5	0	0	$23/1.8 \times 10^8$	7.8×10^6
M7	3.5×10^7	3.5×10^6	3.5×10^5	1	0	0	$2/3.5 \times 10^8$	1.8×10^8
S39	1.2×10^7	1.2×10^6	1.2×10^5	4	0	0	$13/1.2 \times 10^8$	9.2×10^6
S6	2.6×10^7	2.6×10^6	2.6×10^5	5	0	0	$23/2.6 \times 10^8$	1.1×10^7
A19	1.3×10^7	1.3×10^6	1.3×10^5	5	0	0	$23/1.3 \times 10^8$	5.7×10^6
A2308	3.2×10^7	3.2×10^6	3.2×10^5	1	0	0	$2/3.2 \times 10^8$	1.6×10^8

Table 1 shows the results of a typical experiment. There exist marked differences between the other strains and strains M7 and A2308 which, in the experiment shown in Fig. 3, did not produce resistant organisms since the inoculum was too small. The results in Table 1 explain this previous result. The strains M7 and A2308 had only one resistant cell among at least 10^8 ; all the other strains had resistant cells in much smaller bacterial populations. The experiment shows that it is possible to determine the MPN of organisms resistant to a certain concentration of streptomycin when the following conditions are fulfilled: (1) the largest inoculum must be great enough to enable the appearance of resistant variants in at least one of five tubes; (2) the smallest inoculum must be small enough to enable resistant variants to appear in less than five tubes. When resistant organisms appear in all five

tubes inoculated with the smallest inoculum their number cannot of course be calculated.

Table 2. *Variation with time of the number of cells resistant to 1.0 µg. streptomycin/ml. in cultures originating from a single colony*

Strain	Age of culture (days)	MPN	No. of organisms per resistant cell
M5	1	$23/1.8 \times 10^8$	7.8×10^6
	3	$240/2.0 \times 10^9$	8.3×10^6
	17	$79/1.4 \times 10^8$	1.8×10^6
M7	1	$2/3.5 \times 10^8$	1.8×10^8
	3	$2/1.7 \times 10^8$	8.5×10^7
	17	$27/4.0 \times 10^8$	1.5×10^7
	25	$22/2.0 \times 10^8$	9.1×10^6
	30	$13/4.0 \times 10^8$	3.1×10^7
S39	1	$13/1.2 \times 10^8$	9.2×10^6
	3	$2/2.1 \times 10^7$	1.1×10^7
	17	$240/8.0 \times 10^6$	3.3×10^4
S6	1	$23/2.6 \times 10^8$	1.1×10^7
	3	$9/2.2 \times 10^7$	2.4×10^6
	25	$13/2.0 \times 10^7$	1.6×10^6
	30	$13/8.2 \times 10^7$	6.6×10^5
A19	1	$23/1.3 \times 10^8$	5.7×10^6
	3	$2/3.0 \times 10^8$	1.5×10^6
	17	$1600/6.0 \times 10^6$	3.8×10^3
A2308	1	$2/3.2 \times 10^8$	1.6×10^8
	3	$2/1.6 \times 10^8$	8.0×10^7
	25	$180/1.6 \times 10^8$	9.0×10^5

Table 2 shows that the MPN varies with age of culture. The general trend seems to be that in older more dissociated cultures the MPN of resistant variants increases. This increase of resistant cells was very marked in the cultures of the strains S39, A19 and A2308.

Determination of the MPN of resistant cells at 10 and 100 µg. streptomycin/ml.

Using different inoculum sizes at a constant concentration of streptomycin it was not possible to determine the minimal inoculum necessary to give resistant strains in streptomycin concentrations of 10 and 100 µg./ml. since resistant organisms appeared in many instances in smaller inocula, while relatively large inocula did not give resistant strains. A typical experiment is recorded in Table 3 which shows that, using only one tube for each inoculum, it is impossible to find a clear limit between inocula large enough and those too small to give rise to resistant variants. There exists, therefore, only the possibility of determining the MPN of resistant organisms in the total bacterial population. Since all the strains isolated at 10 µg./ml. were also resistant to all the higher concentrations all the following experiments were carried out at 100 µg./ml. To determine this number the method for the calculation of small numbers of coliform bacteria was used; 10.0, 1.0 and 0.1 ml. of 24 hr. broth cultures were inoculated into broth tubes containing

Table 3. *The appearance of resistant forms in cultures exposed to 10 and 100 µg. streptomycin/ml. for 10 days at 37°*

Streptomycin (µg./ml.)	Strain	No. of organisms inoculated/10 ml. (× 10 ⁶)						
		64	160	320	640	1600	3200	8000
10.0	M5	0	0	0	0	R	.	.
	M7	0	0	0	0	R	.	.
	S39	0	0	0	R	0	.	.
	S6	0	0	R	0	0	.	.
	A19	0	0	0	R	0	.	.
	A2308	0	0	0	0	0	.	.
100.0	M5	.	.	0	0	R	0	0
	M7	.	.	0	0	0	0	R
	S39	.	.	R	0	0	0	0
	S6	.	.	0	0	R	0	0
	A19	.	.	R	0	R	0	0
	A2308	.	.	R	0	R	0	0

0 = no appearance of resistant organisms.

R = resistant organisms appeared.

100 µg. streptomycin/ml. For the inoculation of 10.0 ml. of culture, tubes containing 10.0 ml. broth + 200 µg. streptomycin/ml. were used in order to bring the final concentration to 100 µg./ml. Five tubes were used for each size of inoculum. The results are summarized in Table 4 which shows that not all

Table 4. *The MPN of brucella cells resistant to 100 µg. streptomycin/ml. determined for 100 ml. of 24 hr. broth cultures*

Five replicate tubes were inoculated with varying amounts, to give three tenfold dilutions.

Strain examined	Volume (ml.)			MPN (resistant cells/100 ml.)	Total no. of organisms/ 100 ml.	No. of organisms/ resistant cell
	10.0	1.0	0.1			
	No. of positives in 5 portions					
M5	1	0	0	2.0	3.5×10^{10}	1.8×10^{10}
M7	1	1	0	4.0	4.5×10^{10}	1.1×10^{10}
S39	4	1	1	21.0	4.3×10^{10}	2.0×10^9
S6	3	1	0	11.0	4.5×10^{10}	4.1×10^9
A19	0	2	0	3.7	2.6×10^{10}	7.0×10^9
A2308	4	3	0	27.0	3.1×10^{10}	1.1×10^9

strains were equally able to produce completely resistant variants. Considerable quantitative differences existed and under the conditions of the experiment strain M5 was only once able to produce a resistant variant with an inoculum of 10.0 ml. Strain A19 also showed irregular behaviour; it produced resistant variants with an inoculum of 1.0 ml. in two tubes, but did not produce resistant variants in any of the 5 vol. of 10.0 ml. The ability of the strains M5 and A19 to produce resistant variants was therefore re-examined using larger volumes of broth cultures. Five volumes of 100 ml., 5 of 10 ml. and 5 of 1 ml. were used; concentration streptomycin 100 µg./ml. To the 100 and

10 ml. volumes equal volumes of broth + 200 μ g. streptomycin/ml. were added to bring the final concentration to 100 μ g./ml. This experiment is summarized in Table 5. It is interesting to note that the figure obtained in this experiment for the strain M5 was the same as obtained previously (cf. Table 4). The figure for A19 was about twice that obtained in the previous experiment. The MPN of cells resistant to 100 μ g./ml. remained fairly constant at the different periods of incubation, although in this case also the general trend seemed to show an increase of resistant cells with the age of culture (Table 6).

Table 5. *The most probable numbers of brucella cells resistant to 100 μ g. streptomycin/ml. determined for 1000 ml. of broth cultures of strains M5 and A19*

Five portions of each volume, 100, 10 and 1 ml., were seeded.

Strain	Volume (ml.)			MPN resistant organisms/ 1000 ml.	Total no. of organisms/ 1000 ml.	No. of organisms/ resistant cell
	100.0	10.0	1.0			
	No. of positives in 5 portions					
M5	4	1	1	21.0	3.8×10^{11}	1.8×10^{10}
A19	3	1	0	11.0	2.6×10^{11}	2.4×10^{10}

Table 6. *Variation in the number of cells resistant to 100 μ g. streptomycin/ml. in cultures originating from a single colony*

Strain	Age of culture (days)	MPN	No. of organisms/ resistant cell
M7	3	$4.0/6 \times 10^{10}$	1.5×10^{10}
	30	$10.0/4 \times 10^8$	4.0×10^7
A19	3	$4.0/3 \times 10^{10}$	7.5×10^9
	17	$23.0/12 \times 10^9$	5.2×10^8
A2308	3	$1.8/25 \times 10^9$	1.4×10^{10}
	17	$27.0/31 \times 10^8$	1.1×10^8
	30	$4.5/8 \times 10^7$	1.8×10^7

Agreement between the determination of the MPN with other methods

The figures obtained by the MPN method were compared with the results of direct observation of resistant cells when large quantities of culture were plated out in the presence of 100 μ g. streptomycin/ml. The method was compared with the centrifugation method in which the whole bacterial growth from 100 ml. of culture was collected on a refrigerated centrifuge and poured in plates containing 100 μ g. streptomycin/ml., and with a simple plating method in which only 2.0 ml. of culture were removed and plated with the same concentration of streptomycin. A comparison was made between the growth of a strain known to be resistant to 100 μ g. streptomycin/ml., starting from an inoculum of about 5 cells/100 ml., and the growth of the resistant cells surviving from a 48 hr. non-resistant culture to which 50 % (v/v) of fresh broth and 100 μ g. streptomycin/ml. were added.

Table 7 shows that the results of the MPN method agree with those obtained by the centrifugation method until the upper limit of 1600 cells/100 ml. of broth culture is reached. The direct plating method does not give reliable results so long as the number of resistant cells is lower than 100/100 ml. With

Table 7. *Streptomycin resistant cells appearing in a culture of a non-resistant strain exposed to 100 µg. streptomycin/ml., and from the culture of a resistant strain exposed to the same concentration of streptomycin*

Strain	Time (hr.)	No. of resistant bacteria determined by pouring plates with 100 µg. streptomycin/ml. and		Determination of MPN/100 ml. broth culture			
		2 ml. culture	the sediment of 100 ml. culture				MPN
				10.0	1.0	0.1	
S38 non-resistant	0	0	16	4	2	0	22
	10	0	23	5	1	0	33
	14	0	26	5	2	0	49
	18	2	59	5	1	0	33
	22	2	78	5	4	0	130
	34	22	340	5	5	3	920
	58	890	12,000	.	.	.	.
S39 resistant	0	0	5	3	0	0	7.8
	10	4	124	5	4	0	130
	14	14	278	5	5	3	920
	18	80	1,550	5	5	4	1,600
	22	265	5,560	5	5	5	.
	34	46,000*	960,000*	5	5	5	.

* By the aid of dilutions.

an increase of number of resistant organisms above 1000 the direct plating methods without or with suitable dilutions is the method of choice. The experiment presented in Table 7 also shows that in the non-resistant S39 strain, resistant bacteria were present in the culture at the time when streptomycin was added and that these bacteria multiplied slowly after a prolonged lag phase which lasted about 14 hr.

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