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A Comparison Of Various Measures Of Sensitivity Of M. Tuberculosis To Pyrazinamide*

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Information on the **in vitro** sensitivity of **Mycobacterium tuberculosis** to pyrazinamide is limited, mainly because of the difficulty in choosing an appropriate pH for the medium employed. A pyrazinamide sensitivity test, employing Lowenstein-Jensen medium acidified with hydrochloric acid to a preinspissation pH of 4.80-4.85, was developed at this Centre, and performed on cultures from tuberculous patients. Sensitivity was measured as the minimal inhibitory concentration (MIC) of pyrazinamide for various sires of the inoculum, and as proportions of the bacterial population resistant to various concentrations of the drug. For each measure, the findings in patients with no history of previous chemotherapy with pyrazinamide were compared with those obtained at 4-12 months after the start of daily treatment with pyrazinamide ; the definition of resistance was chosen such that it discriminated efficiently between the two populations, and classified only a small proportion of the former population as resistant. Four definitions of resistance were chosen–an MIC of 200 µg/ml or more employing an inoculum containing approximately 0.4 mg of bacilli per ml and a 10-colony end-point, and proportions of 20 % or more on 25 µg/ml, 5 % or more on 50 µg/ml, and 1% or more on 100 µg/ml. The four definitions were of similar efficiency. Employing them, it was found that wild strains with consistent resistance to pyrazinamide were rare in Madras patients ; also, in patients who received daily pyrazinamide, resistance emerged (if at all) at 4-6 months after the start of treatment.

Introduction

Pyrazinamide was first reported to have antituberculosis activity in the mouse (Kushner **et al** 1952, Malone **et al** 1952), but to be devoid of **in vitro** activity against **Mycobacterium tuberculosis** (Tarshis and Weed 1953, Schwartz and Moyer 1954, Steenken and Wolinsky 1954). Later studies revealed, however, that the drug did exert activity **in vitro,** but only in acid medium of pH 5.0-5.5 (McDermott and Tompsett 1954). Several acid media have since been used for pyrazinamide sensitivity tests (Canetti, Rist and Grosset 1963, Marks 1964, Stottmeier, Beam and Kubica 1967), but it has always been difficult to choose an appropriate pH for the medium such that pyrazinamide is active and as few as possible of the test strains are inhibited solely by the acidity of the medium. In consequence, there is limited information on the **in vitro** sensitivity

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of **M. tuberculosis** to pyrazinamide and on the emergence of pyrazinamide resistance during treatment with the drug.

In a preliminary study at this Centre, fluid acid media were found to be unsatisfactory since many strains of tubercle bacilli failed to grow in them. Three solid media– Steenken and Smith's acid medium (United States Veterans Administration 1960) Lowenstein-Jensen medium acidified with hydrochloric acid and Lowenstein-Jensen medium acidified with citric acid were, therefore, compared for their ability to sustain the growth of tubercle bacilli ; all three media had a pre-inspissation pH of 5.1 and a post-inspissation pH of 5.4-5.5. It was found that the Lowenstein-Jensen medium acidified with hydrochloric acid yielded the most satisfactory growth of tubercle bacilli. Furthermore, this medium, at its normal pH, is employed for all other drug sensitivity tests at this Centre. Therefore, further experimentation was restricted to the Lowenstein-Jensen medium acidified with hydrochloric acid. A comparative study of this medium at three pH levels, namely 4.80, 4.95 and 5.10 showed that the pH of 4.80 was the best, in terms of demonstrating the activity of the drug and sustaining the growth of tubercle bacilli. For all subsequent tests the medium employed had a pre-inspissation pH of 4.80-4.85.

The present report is based on the findings of pyrazinamide sensitivity tests undertaken at this Centre since 1963, and compares the relative efficiency of various measures of sensitivity (minimal inhibitory concentrations, proportions resistant) in detecting resistance to pyrazinamide ; an interim report was presented earlier (Tripathy 1966).

Material and Methods

Strains : The following cultures of M. tuberculosis were tested :

Wild (W) cultures : A pair of cultures, obtained before the start of chemotherapy, from each of 222 newly-diagnosed tuberculous patients admitted to a chemotherapy study at this Centre. All the cultures were sensitive to isoniazid and streptomycin according to standard definitions (Tuberculosis Chemotherapy Centre Madras 1959). For all analyses other than the one on variation between duplicate cultures, only the results on the first culture from each patient have been considered.

Failure (F) cultures : Cultures from patients who were treatment failures of one or more regimens not including pyrazinamide, at this Centre. Over a period of one year, 51 such cultures were tested from the same number of patients, and of these, 34 were resistant to isoniazid.

Probably resistant (PR) cultures : Cultures isolated from patients during treatment (1-12 months) with regimens containing pyrazinamide in a single daily dose of 1.0 to 1.5 g. In all, 154 cultures were obtained from 55 such patients (all of them were treatment failures of one or more regimens at this Centre), and tested over the same period as the F cultures.

British cultures : A sample of 25 cultures, obtained before the start of chemotherapy, from the same number of British patients with newly-diagnosed pulmonary

tuberculosis. The sample was obtained by random selection from cultures sensitive to isoniazid and streptomycin (according to standard definitions) in a national survey of the prevalence of drug resistance in Britain (Miller **et al** 1966).

H37Rv : The sensitivity of the standard strain, H37Rv, was determined in each batch of pyrazinamide medium, the total number of tests was 86.

Medium and drug concentrations : Acidified Lowenstein-Jensen medium with a pre-inspissation pH of 4.80-4.85 was employed. This was prepared by mixing the basic ingredients of Lowenstein-Jensen medium (Cruickshank 1965), and adding approximately 20 ml of 2N hydrochloric acid per litre of the medium ; the pH was then adjusted employing a pH meter with a glass electrode.

The concentrations of pyrazinamide employed were 12.5, 25, 50 and 100 µg/ml. Both the drug-containing medium and the drug-free (control) medium were distributed in 7 ml quantities in 1 oz screw-capped Universal containers and inspissated at 85°C for 50 min. The pH of the medium after inspissation ranged from 5.10 to 5.20.

Lowenstein-Jensen medium at its normal pH (6.8 to 7.0) was also employed, in order to determine the effect of acidification of the medium on the growth of the strains.

Sensitivity tests : The sensitivity tests were of the indirect type and were undertaken on the primary isolates in the case of the W, F and PR strains, and on subcultures in the case of the H37Rv and British strains. Approximately 4 mg (moist weight) of growth, as judged by eye, was taken with a 22-SWG nichrome loop and placed in a 4 oz screw-capped bottle containing 12 3-mm diameter glass beads and 0.2 ml distilled water. After shaking the bottle for 1 min, 0.8 ml distilled water was added to obtain a suspension containing approximately 4 mg of bacilli per ml (S_1). Four successive 10-fold dilutions (S_2, S_3, S_4 and S_5) were then prepared by adding appropriate amounts of distilled water. One loopful (27 SWG nichrome, 3-mm external diameter) from each of the suspensions, S_1, S_2, S_3 and S_4 inoculated on to slopes containing pyrazinamide 12.5, 25, 50 and 100 µg/ml (Table I). As controls, one drug-free acidified Lowenstein-Jensen slope was inoculated with the suspension S_1, one with the suspension S_2 and 2 each with the suspensions S_3, S_4 and S_5. Drug-free slopes of normal Lowenstein-Jensen medium were also set up in parallel as further controls.

Particular care was taken not to dig the loop below the surface of the soft acid medium, and not to inoculate into the water of condensation or on to the medium immediately adjoining the glass surface. The slopes were incubated at 37°C and examined 40 days later.

The minimal concentrations of the drug inhibiting growth (MICs) on slopes inoculated with suspensions S_1, S_2 and S_3 were determined. For this purpose, growth was defined as the appearance of 20 or more colonies on slopes inoculated with the S_1 suspension 10 or more colonies with the S_2 suspension and 5 or more colonies with the S_3 suspensions (If there was growth on the 100 µg/ml pyrazinamide slope–the highest concentration employed in this study–the MIC was regarded as 200 µg/ml or more).

Table I. An example of the findings of the pyrazinamide sensitivity test*

Suspension	Normal medium		Acidified medium (pre-inspissation pH 4·80-4·85)					End-point	MIC (μg/ml)
					Concentration of pyrazinamide (μg/ml)				
	Drug-free		Drug-free		12·5	25	50	100	
Standard (S_1)	3+		3+		2+	2+	**13**	**4**	20-colony	50
1 in 10 (S_2)	3+		3+		2+	**19**	3	0	10-colony	50
1 in 10^3 (S_3)	2+	2+	2+	2+	**17**	4	0	0	5-colony	25
1 in 10^3 (S_4)	**38**	**32**	**29**	**31**	1	0	0	0		
1 in 10^4 (S_5)	5	1	2	4						
Proportion resistant (%)					*5·7*	*0·63*	*0·04*	*0·01*		

*3+ indicates confluent growth, 2+ indicates more than 100 colonies and the number of colonies is recorded if it is 100 or less. Figures in bold type are counts selected for calculating the proportions resistant (see below)

Next, the proportions of the bacterial population resistant to different concentrations of pyrazinamide (12.5, 25, 50 and 100 μg/ml) were determined. For this purpose, the number of colonies on the drug-containing slope inoculated with the S_1 suspension was estimated, and expressed as a percentage of the estimated number of colonies on the acidified drug-free S_1 slope. The counts selected for estimation purposes were, in the order of preference, 20-70, 5-19 and more than 70 colonies on the drug-free slopes (the mean count was considered for the S_3, S_4 and S_5 suspensions), and 5-100, 0-4 and more than 100 colonies on the drug-containing slopes. If more than one suspension yielded satisfactory counts with the drug-containing medium, the count on the suspension closest to the one selected for estimating the bacterial population on the drug-free S_1 slope was chosen. When the satisfactory count was zero with the drug-containing medium, the proportion resistant was calculated assuming that one colony grew on the heaviest of the suspensions with the zero colony count (usually S_1) and the result expressed as less than this proportion. When classifying individual cultures as sensitive or resistant, such a result was excluded from the analysis if the proportion was higher than the critical proportion signifying resistance.

An example of the findings of the pyrazinamide sensitivity test is set out in Table I, together with the various measures of sensitivity derived from them.

Results

Growth on acidified drug-free medium : Of 538 cultures that grew on normal drug-free medium, only 4 failed to grow on acidified drug-free medium (Table II). Considering the remaining 534 cultures, the growth on the acidified medium was less than 50% of the growth on the normal medium for 147 (28 %). Finally, the mean viable

count in the acidified medium (1.2 x 10^4) was appreciably less than that in the normal medium (2.0 x 10^4).

Table II. Comparison of growth on normal and acidified drug-free Lowenstein-Jensen medium

Type of culture	Cultures with no growth on acidified medium	Cultures with growth on acidified medium						
		Total	Growth on acidified medium expressed as a percentage of growth on normal medium				Mean viable count	
			Less than 10	10–	50–	100 or more	Acidified medium	Normal medium
Madras W	0	222	17	26	74	105	$1{\cdot}8\times10^4$	$2{\cdot}5\times10^4$
Madras F	2	49	7	4	15	23	$1{\cdot}1\times10^4$	$1{\cdot}7\times10^4$
Madras PR	0	154	22	26	40	66	$1{\cdot}0\times10^4$	$1{\cdot}8\times10^4$
British	2	23	7	4	10	2	$0{\cdot}4\times10^4$	$1{\cdot}8\times10^4$
H37Rv	0	86	4	30	38	14	$0{\cdot}8\times10^4$	$1{\cdot}6\times10^4$
All cultures	4	534	57	90	177	210	$1{\cdot}2\times10^4$	$2{\cdot}0\times10^4$

Considering pre-treatment strains, there was evidence that British cultures grew less well than Madras 'cultures, especially in acidified medium. Thus, the mean viable count in normal medium was 1.8 x 10^4 for the British and 2.5 x 10^4 for the Madras cultures, and the corresponding mean counts in acidified medium were 0.4 x 10^4 and 1.8 x 10^4, respectively. Lastly, the growth on the acidified medium was 50 % or more of the growth on the normal medium for 12 (48 %) of 25 British cultures as compared with 179 (81%) of 222 Madras cultures, a highly significant difference ($P < 0.001$).

Sensitivity, expressed as an MIC, of different types of cultures : It will be recalled that the W, F and British cultures were all isolated from patients who had no history of previous chemotherapy with pyrazinamide, and that the PR cultures were obtained from patients at 1-12 months after the start of treatment with daily pyrazinamide. Therefore, the former group would have consisted predominantly of pyrazinamide-sensitive cultures while the latter is likely to have included an appreciable proportion of pyrazinamide-resistant cultures.

Table III sets out the cumulative distributions of various types of cultures accord, ing to the S_1-MIC, the S_2-MIC and the S_3-MIC. The total number of results available is slightly smaller than the number of tests set up, partly because of contamination but mainly due to the growth on the control slopes being insufficient for determining the MIC. In all, the S_1-MIC could not be determined for 8 (1.5 %) of 534 cultures tested, the S_2-MIC for 19(3.6 %) and the S_3-MIC for 41 (7.7 %) cultures.

In general, the W cultures and F cultures had similar distributions. The amalgamated group of cultures (W +F), however, was less sensitive than the British cultures and the strain H37Rv. For instance, 83 % of 268 W+F cultures had an S_1-MIC of

Table III. Cumulative distribution of cultures according to minimal inhibitory concentrations at 40 days

Suspension and end-point	MIC (µg/ml)	No chemotherapy with pyrazinamide					Duration (months) of chemotherapy with pyrazinamide (PR cultures)				Measure of discrimination (b—a)
		H37Rv (%)	British (%)	W (%)	F (%)	W+F (%) (a)	1—3 (%)	4—6 (%)	7—12 (%)	4—12 (%) (b)	
S_1	25 or more	62	57	85	77	83	74	86	94	90	7
20—colony	50 or more	39	24	58	50	57	49	76	82	79	22
	100 or more	25	14	36	35	36	40	69	65	67	31
	200 or more	14	10	20	17	19	26	59	55	57	38
	No. of cultures	84	21	220	48	268	53	51	49	100	
S_2	25 or more	38	24	68	65	68	58	85	79	82	14
10—colony	50 or more	16	14	33	37	34	31	69	62	66	32
	100 or more	10	10	16	15	16	23	54	52	53	37
	200 or more	4	5	9	2	8	8	52	44	48	40
	No. of cultures	81	21	219	46	265	52	48	48	96	
S_3	25 or more	19	20	51	45	50	37	81	70	76	26
	50 or more	11	13	14	20	15	18	64	49	57	42
5—colony	100 or more	8	13	8	9	9	10	53	47	50	41
	200 or more	5	0	3	0	2	8	40	42	41	39
	No. of cultures	80	15	213	44	257	51	47	43	90	

25 µg/ml or more as compared with 57 % of 21 British cultures ($P < 0.01$) and 62% of 84 tests on the strain H37Rv ($P< 0.01$).

Considering next the PR cultures, those isolated at 1-3 months were of the same order of sensitivity as the W+F cultures. However, PR cultures isolated at 4-6 months and 7-12 months were substantially less susceptible. For instance;48 % of 96 PR cultures isolated at 4-12 months had an S_2-MIC of 200 µg/ml or more as compared with 8% of 265 W+F cultures ($P< 10^{-8}$) and 8% of 52 PR cultures isolated at l-3 months ($P<10^{-5}$).

The findings in W+F cultures and PR cultures isolated at 4-12 months formed the basis for evolving definitions of pyrazinamide resistance in Madras Cultures.

Considerations involved in the choice of definitions of resistance: Two considerations were involved in the choice of definitions of resistance. These were (1) the definition should discriminate efficiently between the W+F cultures on the one hand and the PR 4-12 cultures on the other hand, and (2) the proportion of W+F cultures that are classified as resistant should be small, as pyrazinamide has hardly been used in Madras city.

Definitions of resistance based on MICs for different suspensions : Several definitions of resistance (Table III, column 2) were considered ; their ability to discriminate between the W+F cultures and the PR 4-12 cultures was measured by the difference between the two groups in the percentage of cultures classified as resistant (last column). The largest differences in percentages, indicating the best discrimination, were obtained by defining resistance as an S_1-MIC of 200 µg/ml or more, an S_2-MIC of 200 µg/ml or more or an S_3-MIC of 50 µg/ml or more. Further, the actual differences observed with the three definitions were approximately equal, being 38 %, 40% and. 42 %, respectively. However, as the S_1-MIC definition classified an unduly high proportion (nearly one-fifth) of the W+F cultures as resistant and as the S_3-MIC could not be determined for an appreciable proportion of the cultures (about 8 %), an S_2-MIC of 200 µg/ml or more was chosen as the most satisfactory MIC definition of resistance.

Definitions of resistance based on proportions resistant to different concentrations : The findings with the proportions resistant to 25 µg/ml, 50 µg/ml and 100 µg/ml pyrazinamide are presented in Table IV, As with the MICs, the W and F cultures had similar distributions, and the amalgamated group (W+F) was less sensitive than the British cultures and the strain H37Rv ; however, the differences were smaller than those obtained with the MICs. Again, PR cultures isolated at 4-6 months and 7-12 months had similar distributions, and the amalgamated group (PR 4-12 cultures) was substantially less sensitive than the W+F cultures.

As with the MICs, the findings in W+F cultures and PR 4-12 cultures formed the basis for evolving definitions of resistance. Several definitions of resistance (Table IV, column 2) were considered. As the discriminating ability of most of them was of the same order, the final choice was made by selecting that definition which yielded a 5 % prevalence of resistance in W+F cultures (it will be recalled that the corresponding proportion was 8 % with the S_2-MIC definition). The definitions thus chosen were *(a)* proportion of 20 % or more on 25 µg/ml, *(b)* proportion of 5 % or more on 50 µg/ml

Table IV. Cumulative distributions of cultures according to proportions resistant at 40 days

Concentration of pyrazinamide	Proportion resistant	No chemotherapy with pyrazinamide					Duration (months) of chemotherapy with pyrazinamide (PR cultures)				Measure of discrimination (b–a)
		H37RV (%)	British (%)	W (%)	F (%)	W + F (%) (a)	1–3 (%)	4–6 (%)	7–12 (%)	4–12 (%) (b)	
25 µg/ml	1% or more	15	10	25	27	25	28	71	64	67	42
	5% or more	2	5	11	8	10	17	55	56	55	45
	10% or more	0	0	8	8	8	11	51	52	51	43
	20% or more	0	0	5	4	5	8	43	48	46	41
	50% or more	0	0	1	4	1	6	33	44	39	38
	No. of cultures	85	21	220	48	268	53	51	50	101	
50 µg/ml	0·1% or more	32	38	37	36	37	40	78	68	73	36
	1% or more	5	5	12	15	12	17	52	52	52	40
	5% or more	1	0	5	2	5	9	48	48	48	43
	10% or more	0	0	3	2	3	6	46	48	47	44
	20% or more	0	0	1	0	1	6	38	46	42	41
	No. of cultures	82	21	219	47	266	53	so	so	loo	
100 µg/ml	0·1% or more	14	7	18	18	18	29	62	57	60	42
	0·5% or more	3	0	7	2	6	12	53	53	53	47
	1% or more	3	0	5	2	5	8	51	49	50	45
	5% or more	1	0	2	2	2	8	42	47	45	43
	10% or more	1	0	1	2	1	6	36	45	40	39
	No. of cultures	78	15	212	44	256	51	45	47	92	

and *(c)* proportion of 1% or more in 100 µg/ml. The proportion resistant to 100 µg/ml could not be determined **exactly** for about 8 % of the cultures ; however, most of these had values of **less than** 1% and could therefore be classified as sensitive.

The findings with 12.5 µg/ml pyrazinamide have not been tabulated here as definitions based on them did not discriminate very efficiently between the W+F cultures and PR 4-12 cultures ; thus, the maximum discrimination obtained was about 30 %, which is substantially less than the discrimination of 41-45 % obtained with the definitions based on the tidings with 25, 50 and 100 µg/ml pyrazinamide.

Efficiency of the four chosen definitions of resistance : Table V summarises the efficiency of the four chosen definitions of resistance. First, it will be noted that the percentage of W+F cultures classified as resistant is uniformly small, namely 5-8 % Secondly, the discriminating ability of the four definitions appears to be fairly similar ; thus, the difference between the W+F cultures and PR 4-12 cultures in the proportion of cultures classified as resistant was 40 % with the S_2-MIC definition and 41%, 43 % and 45% respectively, with the definitions based on the proportion resistant to 25 µg/ml, 50 µg/ml and 100 µg/ml pyrazinamide.

Table V. Efficiency of chosen definitions of resistance

Definition of resistance		Percentage classified as resistant		Measure of discrimination (b−a)
		W+F cultures* (a)	PR 4-12 cultures † (b)	
S_2—MIC (10-colony end-point)	**200 µg/ml or more**	**8**	**48**	**40**
Proportion resistant to 25 µg/ml	**20% or more**	**5**	**46**	**41**
Proportion resistant to 50 µg/ml	**5% or more**	**5**	**48**	**43**
Proportion resistant to 100 µg/ml	**1% or more**	**5**	**50**	**45**

*that is, cultures isolated from Madras patients with no history of treatment with pyrazinamide

†that is, cultures isolated from Madras patients at 4-12 months after the start of daily treatment with pyrazinamide

Extent of agreement between the findings with the four chosen definitions of resistance : Table VI studies the extent of agreement between the four chosen definitions of resistance, considered pair by pair, in the classification of PR cultures as sensitive or resistant. In general, the extent of agreement was of a high order, the proportion of cultures having the same classification by both definitions of a pair ranging from 92 % to 99 %. Furthermore, the disagreements appeared to be fairly evenly distributed. It may, therefore, be concluded that there is little to choose between the four definitions of resistance.

Association between the viable count on acidified drug-free medium and various measures of sensitivity : Table VII relates the results of pyrazinamide sensitivity tests in W+F cultures to the growth on the acidified drug-free medium. An S_2-MIC of 200 µg/ml or more (that is, a result classifying the culture as resistant) was obtained for 3 % of 75

cultures with a viable count of less than 10^4, 6% of 150 cultures with a viable count ranging between 10^4 and 10^5, and 22% of 40 cultures with a viable count of 10^5 or more, a highly significant trend ($P < 0.001$). Similarly, highly significant trends ($P < 0.001$) were obtained with S_2-MICs of 100 µg/ml or **more** and 50 µg/ml **or more.** It follows that if the S_2-MIC is employed as a measure of sensitivity (it will be recalled that definitions of resistance based on the S_1-MIC and the S_3-MIC were unsatisfactory), high viable counts on the acidified drug-free medium could result in over-estimation of resistance.

Table VI. Extent of agreement between the different definitions of resistance* in the classification of PR cultures† as sensitive or resistant

Measure of sensitivity		Total cultures	Same classification by measures (a) and (b)				Different classifications by measures (a) and (b)	
(a)	(b)		Sensitive	Resistant	Total No.	%	Resistant by (a), sensitive by (b)	Sensitive by (a), resistant by (b)
S_2–MIC	Proportion on 25 µg/ml	148	93	43	136	92	7	5
S_2–MIC	Proportion on 50 µg/ml	148	95	48	143	97	2	3
S_2–MIC	Proportion on 100 µg/ml	147	97	47	144	98	2	1
Proportion on 25 µg/ml	Proportion on 50 µg/ml	154	98	47	145	94	3	6
Proportion on 25 µg/ml	Proportion on 100 µg/ml	152	98	46	144	95	3	5
Proportion on 50 µg/ml	Proportion on 100 µg/ml	152	100	50	150	99	2	0

*The definitions were (1) S_2-MIC of 200 µg/ml or more, (2) growth of 20% or more on 25 µg/ml, (3) growth of 5% or more on 50 µg/ml and (4) growth of 1% or more on 100 µg/ml

†That is, cultures isolated from Madras patients at l-12 months after the start of daily treatment with pyrazinamide

In contrast, there was no evidence of an association between the viable count and the proportions resistant to 25 µg/ml (Table VII, right half), 50 µg/ml and 100 µg/ml (analyses not tabulated here).

Findings with duplicate cultures from the same patient : Table VIII examines the correlation between the S_2-MIC results of duplicate W cultures from the same patient. First, it will be noted that the same result was obtained with the duplicate culture for 162 (80 %) of 203 patients (this proportion is likely to be an over-estimate as three categories, namely MICs of 12.5, 25 and 50 µg/ml, have been amalgamated). Secondly, an MIC of 50 µg/ml or less was obtained with the second culture for 152 (90 %) of 169 patients who had an MIC of 50 µg/ml or less with the first culture, as compared with

only 17 (50 %) of 34 patients who had an MIC of 100 µg/ml or more with the first culture ($P<0.001$). This finding suggests that there were consistent differences between the patients in the sensitivity of their organisms to pyrazinamide. The findings with the proportion resistant to 100 µg/ml confirm this inference, (Table IX). Thus, of 156 patients who had a proportion of less than 0.1 % in the first culture, 131(87 %) had a proportion of less than 0.1 % in the second culture ; the corresponding values were 67% for 24 patients who had 0.1 % to 1% resistant bacilli in the first culture, and 50% for 10 patients who had 1% or more resistant bacilli in the first culture. The trend in these percentages is highly significant ($P<0.01$). Significant trends ($P< 10^{-5}$) were also obtained with the findings on 25 µg/ml and 50 µg/ml (analyses not tabulated here).

Table VII. Results in W+P cultures* related to growth on acidified drug-free medium

Viable count on acidified drug-free medium†	Total cultures	Percentage of cultures							
		S_2-MIC (µg/ml)				Proportion resistant to 25 µg/ml			
		25 or less	50	100	200 or more	Less than 1%	1 % —	5 % —	20% or more
Less than 10^4	75	84	11	3	3	72	17	3	8
10^4 —	150	61	23	11	6	74	15	7	3
10^5 or more	40	52	12	12	22	82	5	5	8

*that is, cultures isolated from Madras patients with no history of treatment with pyrazinamide
†Estimated for the slope inoculated with the standard suspension (S_1)

Table VIII. Correlation between results of duplicate W cultures* from the same patient (S_2-MIC).

MIC (µg/ml) of first culture	MIC (µg/ml) of second culture			Total patients
	50 or less	100	200 or more	
50 or less	152	12	5	169
100	8	5	2	15
200 or more	9	5	5	19
Total patients	169	22	12	203

*That is; cultures isolated from Madras patients with no history of previous chemotherapy

Of 19 patients whose first culture was classified as resistant by the S_2-MIC definition (namely, 200 µg/ml or more), only 5 had the second culture also classified as resistant by same definition. (Table VIII). The corresponding proportion was 4 of 10,

when resistance was defined as 1% or more growth on 100 µg/ml (Table IX). Thus, the proportion of patients who had both cultures resistant was small, namely 5 of 203 with the MIC definition and 4 of 190 with the proportion definition. It may, therefore, be concluded that wild strains with consistent resistance to pyrazinamide did not constitute more than 2-3 % of all wild strains.

Table IX. Correlation between results of duplicate W cultures* from the same patient
(Proportion resistant to 100 µg/ml)

Proportion resistant to 100 µg/ml in first culture	Proportion resistant to 100 µg/ml in second culture				Total patients
	Less than 0.1 %	0.1% – 1%	1% – 5%	5% or more	
Less than 0·1%	131	19	5	1	156
0·1% —	16	6	2	0	24
1% —	4	0	0	3	7
5% or more	1	1	1	0	3
Total patients	152	26	8	4	190

*That is, cultures isolated from Madras patients with no history of previous chemotherapy

Emergence of resistance during treatment with a daily regimen containing pyrazinamide : The pattern of month-by-month sensitivity test results was examined for 10 patients who repeatedly produced positive cultures during treatment at this centre with a daily regimen containing pyrazinamide (PR cultures). Two measures of sensitivity, namely, the S_2-MIC and the proportion to 100 µg/ml, were considered (data not tabulated here).

In six patients, resistance to pyrazinamide emerged at 4, 4, 5, 5, 5 and 7 months, respectively, by both criteria, namely, S_2-MTC of 200 µg/ml or more and 1 % or more growth on 100 µg/ml ; further, **all** subsequent cultures were resistant by both the definitions.

In one patient, all the cultures isolated at 1-11 months were sensitive by both definitions; at 12 months, however, the culture was resistant by the proportion definition (41%) but sensitive by the MIC definition (25 µg/ml). In the remaining three patients, there was no evidence, either from the MICs or from the proportions resistant, that resistance to pyrazinamide had emerged. None of the above four patients was very regular in taking drugs.

Discussion

Routine determinations of pyrazinamide sensitivity have proved to be difficult in many laboratories on account of the failure of a proportion of strains of **M. tuberculosis**

to grow in a medium whose pH is sufficiently acidic for the activity of the drug to be demonstrated. The acidified Lowenstein-Jensen medium used in the laboratory was, however, found to sustain the growth of all but 4 of the 538 strains tested. A great deal of care was exercised in the preparation of this medium. In particular, a pH meter with a glass electrode was used to adjust the pH. Next, the inoculum was evenly distributed over the surface of the soft acid medium, as experience showed that, on drug-containing slopes, a cluster of small colonies often developed in any area where the inoculum was locally dense, presumably because the concentration of bacterial pyrazinamidase in such areas was so high that it destroyed the pyrazinamide and made the medium more alkaline. Finally, the slopes were incubated at 37°C for 40 days ; even so, some strains produced minute colonies which were rather difficult to distinguish from the medium. In the light of these remarks, it may be concluded that pyrazinamide sensitivity tests should be undertaken only in specialized laboratories.

The results of the sensitivity test were expressed as minimal inhibitory concentrations (MICs) for various sizes of the inoculum and as proportions resistant to various concentrations of pyrazinamide. For each of these measures, the findings in a population consisting predominantly of sensitive cultures (obtained from patients with no history of chemotherapy with pyrazinamide) were compared with those in a population consisting of cultures obtained 4-12 months after the start of daily treatment with the drug; a definition of resistance was then chosen such that it discriminated efficiently between the two populations and, furthermore, classified only a small proportion of the former population (predominantly sensitive) as resistant.

Considering first the various MIC measures, the MIC (10-colony end-point) with a 1 in 10 dilution of the standard inoculum employed at this Centre provided the most satisfactory definition of resistance, namely 200 µg/ml or more. Next, there was little to choose between the proportion definitions, the optimal definitions being 20% or more on 25 µg/ml, 5 % or more on 50 µg/ml and 1% or more on 100 µg/ml. The definitions recommended by Canetti, Rist and Grosset (1963) are 50% or more on 20 µg/ml and 10 % or more on 100 µg/ml, employing a 40-day incubation period ; however, the pH of their Lowenstein-Jensen medium was 5.0 before inspissation, as compared to 4.80-4.85 in the present study. Novak, Feitova and Sytarova (1970) have suggested a proportion definition of 50% or more on 100 µg/ml, employing a 4-week incubation period and Lowenstein-Jensen medium with a pre-inspissation pH of 5.27.

The efficiency of the four Madras definitions of resistance was of the same order, each definition classifying as resistant 5-8 % of the predominantly sensitive group of cultures and 46-50 % of the group of cultures obtained 4-12 months after the start of daily treatment with pyrazinamide. Furthermore, highly satisfactory agreement, namely 92-99%, was obtained between pairs of definitions in the classification of **individual** cultures as sensitive or resistant. Nevertheless, there would appear to be a slight advantage in employing the proportion definitions as the findings were not influenced by the viable count on the drug-free acid medium, whereas larger viable counts were associated with higher MICs. This advantage is, however, offset by the practical

difficulties in undertaking a more complex test, involving serial dilutions of the inoculum and the counting of colonies which are sometimes minute.

In patients with no history of previous chemotherapy, a finding of pyrazinamide resistance in one culture was often not confirmed by the finding in a second culture ; this suggests, that a single resistant result pretreatment does not always reflect the true sensitivity of the strain. In a study of reserve regimens in East Africa (East African/ British Medical Research Council Pyrazinamide Investigation 1969), pyrazinamide sensitivity tests were set up in a very similar manner to those in the present study. No association was found between the pretreatment sensitivity of the strains, as measured by MICs or proportions resistant, and the response to treatment with regimens of streptomycin plus pyrazinamide. Therefore, in patients with no history of chemotherapy with pyrazinamide, a finding of pyrazinamide resistance in a **single** pretreatment culture should not be regarded as a contra-indication for treatment with this drug.

Considering next the cultures isolated from patients during daily treatment with pyrazinamide, resistance was found in 8-9 % of the cultures obtained in the first 3 months, 43-52 % of those obtained at 4-6 months, and 44-49 % of those obtained at 7-12 months (Tables III and IV). These findings indicate that resistance to pyrazinamide usually emerged between 4 and 6 months after the start of treatment. This was confirmed by an examination of the month-by-month sensitivity test results for 10 patients who were repeatedly culture-positive during treatment.

British cultures were appreciably more sensitive to pyrazinamide than Madras cultures, when sensitivity to the drug was measured as an MIC; however, when it was measured as the proportion resistant to different drug concentrations, the differences were not so appreciable: This may be explained by the fact that colony counts on drug-free acidified medium were substantially lower for the British cultures than for the Madras cultures, and the finding that low counts on drug-free acidified medium were associated with low MICs but had little effect on the proportions resistant. Finally, the difference between British and Madras strains in their ability to grow on drug-free acidified medium may be due to the fact that the former were maintained by repeated sub-cultivation on normal Lowenstein-Jensen medium while the latter were primary isolates ; it is also possible that the pH of 4.80-4.85, which was found to be the best for Madras strains, may not be optimal for all strains.

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