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ORIGINAL ARTICLE

Effect of temperature on storage of ethionamide during susceptibility testing

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ABSTRACT

Objective: Ethionamide being thermolabile in nature, effect of temperature on the drug and its stability in liquid and solid media during susceptibility testing procedures was assessed to understand the inconsistency in DST formats.

Methods: Working solution of ethionamide and Lowenstein- Jensen (LJ) media incorporated with ethionamide were preincubated at 4°C and 37°C prior to DST methods was incubated till 4 weeks at different temperatures and utilized for DST in MGIT 960.

Results: Degradation of ethionamide working solution was observed at 37°C after 3 weeks of incubation. Ethionamide incorporated LJ media can be stored without compromise on susceptibility up to 5 weeks. But, a week of prior incubation at 37°C has deleterious effect on the DST profile. Ethionamide was found to degrade at 37°C after different time points when stored as solution or as LJ media.

Conclusion: Use of DST formats that provide results within 2 weeks can be recommended for ethionamide susceptibility testing. *J Microbiol Infect Dis 2013; 3(3): 128-132*

Key words: Degradation; Ethionamide; LJ medium; MGIT 960; *Mycobacterium tuberculosis*.

Ethionamide depolama sıcaklığının duyarlılık testine etkisi

ÖZET

Amaç: Ethionamide ısıya duyarlı yapısı nedeniyle, ilaç duyarlılık testinde (DST) ısının etkisini ve sıvı veya katı ortamdaki dayanıklılık durumunun araştırılması amaçlanmıştır. Yöntemler: Ethionamide çalışma solüsyonu ve Lowenstein-Jensen (LJ) besiyeri hazırlanarak 4°C ve 37°C bekletildi ve ardından DST yöntemiyle 4 haftaya kadar MGIT 960 besiyeri ile test edildi.

Bulgular: Ethionamide çalışma solusyonunda 37°C'de 3 haftalık inkübasyonun ardından bozunma olduğu gözlenmiştir. Ethionamide LJ ortamında beş haftaya kadar etkinliğinde bozulma olmadan saklanabilir. Fakat 37°C de bir hafta daha bekletilmesi ilaç duyarlılık profiline zararlı etkili olabilir. Ethionamide 37°C bekletilmesiyle farklı zaman aralıklarında sıvı ya da LJ ortamında bekletilmesiyle etkinliğinde değişmeler olduğu saptandı.

Sonuç: DST ortamında ethionamide için iki haftaya kadar olan testlerin değerlendirilmesi önerilir.

Anahtar kelimeler: Bozunma, Etionamide, LJ besiyeri, MGIT 960, *Mycobacterium tuberculosis*

INTRODUCTION

Ethionamide (ETH) though used in the treatment of multi drug resistant tuberculosis (MDR-TB) patients in the country for more than a decade; ambiguity in laboratory susceptibility results stills remains.[1] Drug susceptibility testing (DST) for ETH pose a challenge as there arises inconsistency between testing formats for reasons unclear; mode of action, interpretation of resistance and potency of drug in the medium.[2] Potency of the drug is a measure of purity of the drug in a formulation or in the complex used to stabilize the drug.[3] On the other hand; potency also indicates effectiveness of the drug during testing procedures. If potency of the drug is maintained till completion of the test, the interpretation is considered valid and reproducible.[4] Effort is underway to understand probable reasons for discrepancy within phenotypic testing methods. Several studies have dealt upon degradation of anti-tuberculosis drugs.[5-7] Several anti-tuberculosis drugs except ka-

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namycin and capreomycin was found to deteriorate even as early as 1 week and ethionamide after 2 weeks of incubation at 37°C.[8] Degradation of drugs during combined therapy was reported especially in rifampicin when used with isoniazid. Isoniazid acts as catalyst triggering the degradation of rifampicin in acidic medium.[5] Recovery of drug was also measured when mixed with food and juices in order to assess the effect of food components in maintaining stability of the drug.[7] Loss in potency was indicated in the data sheet of certain drugs such as streptomycin dihydro-sulfate wherein around 50% loss is observed with varying pH and temperature. But the drug as solution is stable when stored at -20°C for more than a year.[9] Therefore, effect of temperature on storage of ethionamide in solution and in combination with egg based solid Lowenstein- Jensen (LJ) media and was undertaken to determine a probable cause for discrepancy between susceptibility testing formats.

METHODS

Effect of temperature on ethionamide used in liquid culture systems (MGIT960)

Stock concentration of 10,000µg/ml of ethionamide drug was prepared by dissolving 10mg of ETH powder (Sigma, USA) in 10ml of triethylene glycol (Merck). Working concentrations (2.5, 5.0, 7.5 and 10µg/ml) were prepared from stock by diluting with sterile distilled water. One microliter aliquots of each concentration were stored at -80°C, 4°C and 37°C for a maximum of 4 weeks and tested once weekly. Drug susceptibility testing was performed weekly once in MGIT 960 system following manufacturer's instructions for 5 strains inclusive of $H_{37}Rv$, 3 strains sensitive and one strain resistant to ETH.[10] Susceptibility results were recorded once the instrument provided a printed report. To minimize clonal variation, strains used in the experiment were sub cultured from primary MGIT 960 positive tube into fresh tube and used weekly.

Effect of temperature on ethionamide drug used in solid LJ media

Drug susceptibility testing of *Mycobacterium tuberculosis* isolates for ETH was performed according to standard procedure.[11-12] Strains used in MGIT 960 were used in solid LJ medium. Strains were sub-cultured from original slope and one slope of each strain was tested every week for DST. Drug media at the specified concentrations of ETH were prepared in a single lot to restrict batch variation. Prepared media was pre-incubated at two different temperatures viz. 4°C and 37°C for a minimum of 1 week to a maximum of 6 weeks before performing DST procedures. Susceptibility testing was done once weekly by proportion sensitivity testing (PST) and conventional minimum inhibitory concentration (MIC) methods.

Conventional MIC method was performed using 4mg moist weight of culture, whereas for PST method, 1 mg of culture with three serial decreasing 1 in 10 fold dilutions were used. Briefly, one- third loopful of 2-3 weeks old culture on LJ media was suspended in 1ml of sterile distilled water and vortexed to obtain an even suspension. Coarse particles or clumps in the suspension were allowed to settle at room temperature. Ten microliters of the suspension was inoculated onto drug containing and drug free LJ medium. Concentrations of ETH viz. 20, 28.5, 40, 57, 80, 114 as in the conventional procedure and an additional concentration of 156 µg/ml were used. Tenfold dilutions from 1 mg/ml concentration were prepared by adding 0.2 ml to 1.8 ml sterile distilled water (S1, 10-1). Two further serial dilutions 10-2 (S2) and 10-3 (S3) were prepared in the similar manner. Ten microliters from the above dilutions were inoculated onto drug free and drug containing (at 40ug/ml of ETH) media. Results were read after 28 days and 42 days of incubation at 37°C for MIC and PST methods respectively.

Interpretation of conventional MIC method: Isolates with ≥20 colony counts (1+ grading) were considered resistant to particular drug concentration of ETH. Break point MIC value for defining resistance in conventional MIC method presently used was ≥114 µg/ml and value less than that was considered susceptible.

Interpretation of PST method: World Health Organization (WHO) recommended concentration of 40 µg/ml was used for ETH in PST method. Isolates with more than 1% of colony forming units in drug containing LJ slopes in comparison with drug free LJ slopes were considered as resistant. Isolates with values less than 1% criteria defining resistance were considered as susceptible. As a modification, we considered isolates with PST values between 0.9% and 1.1% as "borderline".

RESULTS

Effect of temperature on ethionamide drug used in liquid culture systems (MGIT960)

Results of the study indicated that there is no marked difference in susceptibility patterns of the strains tested using ETH stored under -80°C and 4°C (Table 1). There was no interchange between the resistant or susceptibility pattern among the

strains though the drug was stored for 4 weeks at the specified temperature. To contrast, when ETH stored at 37°C was used for susceptibility testing, no appreciable change in pattern was observed until week 3. When pre-incubated (at 37°C for 4 weeks) drug was used, strains that were classified as susceptible to ETH demonstrated resistant phenotype. The single strain that remained susceptible in MGIT 960 till the end time point showed similar profile in DST by MIC and PST methods on LJ medium. However, difference in susceptibility was not noted among resistant strains at both incubation temperatures.

Table 1. Effect of temperature on ethionamide (ETH) drug used in liquid culture systems (MGIT960)

Strain / ETH Susceptibility Pattern	Weeks	Susceptibility results obtained for ETH pre- incubated at specified temperatures		
		-80°C	4°C	37°C
$H_{37}Rv$ / standard susceptible strain	Wk1	Sens	Sens	Sens
	Wk2	Sens	Sens	Sens
	Wk3	Sens	Sens	Sens
	Wk4	Sens	Sens	**Res**
Strain1 / susceptible	Wk1	Sens	Sens	Sens
	Wk2	Sens	Sens	Sens
	Wk3	Sens	Sens	Sens
	Wk4	Sens	Sens	Sens
Strain 2 / susceptible	Wk1	Sens	Sens	Sens
	Wk2	Sens	Sens	Sens
	Wk3	Sens	Sens	Sens
	Wk4	Sens	Sens	**Res**
Strain 3 / susceptible	Wk1	Sens	Sens	Sens
	Wk2	Sens	Sens	Sens
	Wk3	Sens	Sens	Sens
	Wk4	Sens	Sens	**Res**
Strain 4 / Resistant	Wk1	Res	Res	Res
	Wk2	Res	Res	Res
	Wk3	Res	Res	Res
	Wk4	Res	Res	Res

Sens – Sensitive; Res – Resistant; Wk- Week

Effect of temperature (4°C) on ethionamide used in solid culture

Solid LJ media containing ETH can be stored up to 4 weeks and conventional MIC method can be performed without any variation in susceptibility pattern of the strains (Table 1). $H_{37}Rv$ and sensitive strains showed susceptible phenotype till 4 weeks of incubation at 4°C. Resistant strains showed resistant phenotype throughout the time period and also served as control for the experiment. Of the 3 susceptible strains, 2 remained the same when LJ media incubated till 6 weeks was used. The single sensitive strain and $H_{37}Rv$ exhibited resistance after 4 weeks of incubation.

Table 2. Susceptibility results for ETH using LJ media stored at different temperatures

Strain / ETH Susceptibility Pattern	Weeks	37°C		4°C	
		MIC (µg/ml)	PST (40 µg/ml)	MIC (µg/ml)	PST (40 µg/ml)
$H_{37}Rv$ / standard susceptible strain	Wk1	80	0	40	0
	Wk2	156	20	57	0
	Wk3	156	100	40	0
	Wk4	>156	25	57	0
	Wk5	>156	100	114	22
	Wk6	>156	25	80	25
Strain1 / susceptible	Wk1	114	6.6	80	0
	Wk2	156	10	<20	0
	Wk3	>156	66.6	57	2.3#
	Wk4	>156.	13.5	40	0
	Wk5	>156	17	80	0
	Wk6	>156	192	80	11.7
Strain 2 / susceptible	Wk1	57	1.3	28.5	0
	Wk2	156	350	40	2*
	Wk3	>156	50	40	1.11*
	Wk4	>156	137.5	57	3.3*
	Wk5	>156	93.7	40	0
	Wk6	>156	41.6	57	0.4
Strain 3 / susceptible	Wk1	40	0	28.5	0
	Wk2	80	297	28.5	0
	Wk3	>156	46.1	<20	0
	Wk4	>156	171.4	28.5	0
	Wk5	>156	140	28.5	2.5
	Wk6	>156	47	40	1
Strain 4 / resistant	Wk1	>156	133	>156	133
	Wk2	>156	116	>156	71.4
	Wk3	>156	83.3	>156	25
	Wk4	>156	20	>156	83.3
	Wk5	>156	13.3	>156	140
	Wk6	>156	112	>156	85

ETH=ethionamide; MIC=minimum inhibitory concentration method- represented as the drug concentration that inhibited 99% of the bacteria; PST=proportion sensitivity testing method- represented at the percentage of proportion of bacteria resistant to the drug tested at their critical concentration determining resistance. The strain is

considered resistant if the proportion percentage is more than the defined criteria i.e., 1% for ETH at 40 µg/ml; Wk=week; #=Smooth morphology was observed; *=unexplained phenomenon, could possibly due to technical fault.

Referring to PST method, media can be stored effectively for a maximum of upto 5 weeks as there was no change in susceptibility pattern of strains tested. Resistant strain remained resistant throughout the testing period. Susceptible strains showed sensitive phenotype till week 4 but indicated resistant phenotype at week 5. One of the three susceptible strains remained sensitive till week 5.

Effect of temperature (37°C) on ethionamide drug used in solid culture

Ethionamide drug media can be stored for one week at 37°C without any compromise on the susceptibility pattern of the drug in MIC and PST methods. Change in drug susceptibility pattern was observed in media stored for two weeks in all the strains tested except a single susceptible strain that exhibited sensitive phenotype. Resistant strains had no change in their phenotype during entire time point. Of the four susceptible strains, two were resistant and two were susceptible by MIC method. Laboratory standard strain $H_{37}Rv$ showed borderline resistance as the numbers of CFUs were just more than the criteria fixed. But in PST it was depicted as susceptible. Therefore, the inconsistency may probably due to effect of higher inoculum used in MIC method than in PST method (Table 2).

DISCUSSION

Ethionamide is a thermolabile drug. Hence, it is advised to use drug incorporated media judiciously as soon as it is prepared and storage might deteriorate the drug activity.[13] Ethionamide has been indicated to exhibit cross resistance with isoniazid.[14-16] Therefore, reliability of the ETH susceptibility testing is the need of the hour. Ability of the drug to maintain its potency till completion of the test is essential for precise interpretation of susceptibility.[3,4] Major implications can be deduced from the present study. Primarily, the role of temperature as one of the major defining factor in susceptibility testing and its effect on the drug as well as on drug incorporated media was studied. Another is the stability of the drug during the testing procedures.

Present study gains importance as it attempts to understand the reasons for uncertainty in phenotypic tests employed for ethionamide. Role of drug deterioration during the testing procedures as a cause for such discrepancies was determined. Earlier report on the stability of ethionamide was based on agar based medium.[8] This is probably the first study on stability of ethionamide in LJ medium which is preferably used in resource limited setting and in automated liquid culture systems such as MGIT 960. Stability in MGIT 960 was assessed because the cut off MIC is much below the therapeutic index of the drug and reliable results can be obtained for the drug (data not shown). Interpretation of degradation can be monitored easily by using the standardized method routinely followed in the laboratory. Morphology of the colony was used earlier to grade degradation.[8] Therefore, shift in MIC values and susceptibility pattern was considered as a marker for drug degradation.

In accordance with previous report, loss of drug activity was found negligible as there was no change/ shift in the susceptibility pattern of the strains at -80°C and 4°C till one month of incubation whereas it was found to degrade after 3 weeks upon incubation at 37°C.[8] This was supported by susceptibility profile of the panel strains where in resistant phenotype was observed at 37°C after 3 weeks in known susceptible strains except one strain that remained susceptible till the end. This strain unlike the other susceptible strains in the panel could have had more number of susceptible population that are well below the therapeutic index of the drug and least number of borderline and/ resistant clones that lead to erroneous results. The present study reiterates the fact put forth by Griffith and Bodily that ethionamide degrades at 37°C and the same has deleterious effect on susceptibility pattern of the strains.[8] One more advantage is the use of automated (MGIT 960) method employed to assess effect of temperature on the drug. Problem of drug degradation does not affect the results obtained in MGIT 960 as the entire susceptibility testing algorithm is completed within 13 days.[10] Hence results obtained are due to the degradation of drug owing to prior incubation and not because of the adverse exposure during DST procedures. It is therefore recommended to use the drug if stored for more than 3-7°C within a week to obtain reliable results. There is a chance for loss of activity in drug incorporated media during conventional MIC and PST procedures that warrant 4-6 weeks of incubation at 37°C before interpretation.

Another approach was to determine the stability of ethionamide in solid LJ medium. This was performed to understand whether the discrepancy in ETH susceptibility is due to degradation of drug in medium. Use of long term stored media in DST

procedures may induce false resistance due to degradation of drugs.[4] The results indicates that till one month the media can be stored and used (if required) without shift in the susceptibility pattern.

As expected, storage at 37°C even after one week induces major change in the susceptibility pattern. It was evidenced by shift in MIC value for clinical and laboratory susceptible strains. Even one week prior incubation at 37°C had deleterious effect on the pattern. It is also possible that due to loss of activity of ETH the persistors might indicate a false resistance. Observation made from these results implies that there is deterioration of the drug after 5 weeks of incubation at 37°C; i.e., 1 week pre-incubation and 4 weeks incubation for MIC method. Finalization of conventional MIC is performed after 4 weeks of incubation. Hence, there is no issue of drug deterioration during this time point, but problem of drug deterioration arises in PST method where finalization of the results is performed after 6 weeks of incubation. Therefore, it can be inferred that conventional MIC method can be considered for ethionamide DST, but there may be discrepancy in PST method. Thus, any strain with susceptible phenotype in PST can be considered true susceptible, but if resistant, there can be chances of false resistance due to inactivation of the drug due to prolonged incubation. These results provide additional support / indication for previous observation and hypothesis stated by Mitchison that MIC method is better than PST method in susceptibility testing procedures.[17]

Advantages of the study are: the use of routinely employed LJ medium, automated liquid culture system MGIT 960 for assessing activity of the drug and use of *M. tuberculosis* strains resistant and susceptible to ethionamide. Zone of inhibition or vertical diffusion studies not very useful in quantitatively determining the loss of drug activity.[8] One limitation is that validation by quantitative measurement of loss of drug activity by spectrophotometric method. Binding of ethionamide to egg proteins could not be ruled out as a factor for inconsistency in DST procedures employing LJ medium until confirmed.[18]

To conclude, results of both broth cultures and solid culture indicate that it is recommended to store the drug as solution or as media at 4°C for maximum of 1 month prior to use. Effect of temperature on loss of drug activity/degradation during incubation needs to be further confirmed with analytical methodology. Susceptibility procedures using liquid culture formats like MGIT 960 and LRP assay that delivers results with in two weeks' time are probably ideal and reliable for ethionamide.

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