HIGH ETHIONAMIDE RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* STRAINS ISOLATED IN KENYA.

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SUMMARY

Background: Increasing development of tuberculosis (TB) resistance to the currently available drugs including second-line anti-TB drugs that are being used for treatment of Multi-Drug Resistant TB (MDR-TB) patients has frustrated efforts to control TB worldwide. Ethionamide (Eth) is one of the drugs used in the regimen for treatment of these patients.
Objective: To determine level of Ethionamide resistance among second-line anti-tuberculosis drugs in *Mycobacterium tuberculosis* (MTB) strains isolated in Kenya.
Design: A retrospective lab-based study involving archived strains from previous studies carried out at the Centre for Respiratory Diseases Research (CRDR), Kenya Medical Research Institute (KEMRI) from 2002 to 2007.
Setting: Centre for Respiratory Diseases Research (CRDR), Kenya Medical Research Institute (KEMRI).
Methods: A total of 216 MTB strains with pre-determined first-line drug susceptibility testing (DST) results were used including 78 first-line resistant to individual and combined drugs, and 138 susceptible to streptomycin, rifampicin, isoniazid and ethambutol. The strains were subjected to DST to ethionamide among other second-line.
Results: Thirty two [32/216 (14.8%)] strains showed resistance to second-line drugs. Resistance to Eth was the highest [18/32 (56.3%)] including co-resistance with isoniazid [8/18 (44.4%)]. Nine [9/18 (50%)] strains were fully resistant and 9 [9/18 (50%)] were intermediate resistant to Eth.
Conclusion: Unexplainable high levels of Eth resistance is a cause for concern. This will impact negatively on the outcome of management of MDR-TB especially in Kenya where the use of this drug is almost mandatory. Close monitoring of Eth before initiating individual patient management may be necessary.
Key words: Ethionamide, Resistant, MDR-TB.

[Afr J Health Sci. 2012; 20:37-41]

Introduction

Tuberculosis (TB) is far from being controlled. Despite the fact that several reasons could be attributed to this, a significant contributing factor maybe the development of resistance to the currently available drugs due to the successful adaptation of the pathogen to these drugs.

Anti-TB drugs are a two-edged sword. While they destroy pathogenic *Mycobacterium tuberculosis* (MTB) they also select for drug resistant MTB against which those drugs are then ineffective[1]. Drug resistant TB is a state where MTB organisms are resistant to antimicrobials agents at the levels attainable in blood and tissue[2].

Infection with Multi-Drug Resistant tuberculosis (MDR-TB) complicates TB treatment by necessitating the selection of substitute drugs, collectively referred to as second-line drugs, to replace the ineffective first-line drugs. Second-line anti-TB drugs are being used for treatment of MDR-TB patients. Ethionamide (Eth) one of the most frequently used and efficacious second-line drugs is used in treatment of MDR-TB with a combination of the following drugs; an injectable anti-TB aminoglycoside (e.g., amikacin, capreomycin, Kanamycin), a fluoroquinolone (e.g., gatifloxacin, ciprofloxacin, ofloxacin), ethambutol, pyrazinamide, isoniazid, rifampicin, cycloserine and para-amino salicylic acid. Eth is never and should never be used alone[3].

Eth is an efficacious, relatively non-toxic and cheap, and easily available drug, which has been in use since the 1960s. When used within the range of 0.5 to 5.0 mg/ml in vitro it leads to a loss of acid fastness in MTB by inhibiting mycolic acid biosynthesis[4]. Eth is a structural analog of isoniazid[5, 6]. Both compounds

are known to inhibit mycolic acid biosynthesis[7]. Low-level isoniazid-resistant strains frequently display low-level Eth resistance, while high-level isoniazid-resistant strains typically remain Eth susceptible[8]. The structural similarity and existence of cross-resistant phenotypes suggested that these two drugs share a common molecular target[9].

In Kenya Eth is used in the treatment of MDR-TB patients according to World Health Organization (WHO) standards. Patients diagnosed with MDR-TB based on DST results receive a standardised second-line regimen consisting of ofloxacin, cycloserine, Eth and amikacin. Ethambutol and pyrazinamide are also added to the regimen based on DST results[10, 11]. Treatment of MDR-TB takes months to years and must be done on the basis of susceptibility testing, it is impossible to treat such patients without this information. If treating a patient with suspected MDR-TB, the patient should be started on streptomycin, isoniazid, rifampicin, ethambutol, pyrazinamide, moxifloxacin and cycloserine pending the result of laboratory susceptibilty testing[12].

Materials and Methods

Samples: A total of 216 MTB strains were used in this study. The strains were obtained from the archives of previous studies at the Centre for Respiratory Diseases Research (CRDR), Kenya Medical Research Institute (KEMRI). They included both first-line drug resistant and first-line susceptible strains. All the first-line resistant strains obtained during the period between 2002 and 2007 available were taken and randomly selected the first-line susceptible strains.

Table 1: Type of first-line strains used in the study.

First-line resistant strains (78)	First-line susceptible strains
Isoniazid related resistant strains (73)	138
Rifampicin related resistant strains (25)	
Streptomycin related resistant strains (24)	
Ethambutol related resistant strains (11)	
Multi-drug resistant strains (25)	

Laboratory procedures: The strains were first subcultured on fresh Lowenstein Jensen (LJ) media and incubated until there was growth to obtain fresh strains with confluent growth. Drug susceptibility testing (DST) was performed on LJ media using the resistant ratio (RR) method. The drug used was Eth among other second-line drugs[13].

Drug containing LJ media were made by adding appropriate amounts of drugs aseptically to LJ media before inspissation. First a stock solution were prepared and the drug solution sterilised using a membrane filter with a size of 0.45µm to maintain aseptic conditions. Appropriate working concentrations of each drug were made using sterile distilled water and then added aseptically to the LJ media. Five ml of the media was then dispensed into sterile 1 ounce universal bottles and then inspissated for 1 hour at a temperature of 85°C[13].

RR method was used for Eth. Dilutions of the drug was made and incorporated into the LJ media. The final drug concentrations that was used for Eth was 10-160 µg/ml doubling dilutions[13].

Each bacterial suspension was prepared by adding approximately 4 mg moist weight of the test sample of the bacterial mass visualized as 2/3 loopful of a 3mm internal diameter, 24 standard wire gauge wire loop into 1 ml of sterile distilled water in a 7 ml bijou bottle containing three 3mm glass beads. This suspension was vortexed for 30 seconds to produce a uniform suspension of 1.0 McFarland turbidity standard (10^7 CFU/ml). A standardized inoculum of 0.1 ml of the bacterial suspension was inoculated onto drug-free and drug containing LJ media using a loopful of a 3 mm diameter, 27 standard wire gauge wire loop[13]. These cultures were then incubated at a temperature of 37°C for four weeks with weekly observations for growth.

Interpretation of results: In the RR method growth of less than 20 colonies on the media containing the lowest concentration of the drug was taken as the end point. The results were recorded as RR, defined as the minimum inhibitory concentration (MIC) of the test organism divided by MIC of the H37Rv control strain. A RR of 2 or less showed susceptibility, a RR of between 3 and 5 showed intermediate resistance and a RR of 6 and above showed resistance. The results for RR were interpreted as either fully resistant, intermediate resistant or susceptible[13].

Quality control and safety: All manipulations were done in a class II biosafety under sterile conditions. Control strain MTB H37Rv which is susceptible to all the drugs was included in each new batch of media. Control strains of known resistance patterns for each drug tested were also included. LJ media was prepared using fresh eggs (less than seven days old) and sterility check was carried out on all batches of media by incubating a few slopes of the LJ media randomly at 37°C for at least five days and checked

daily to ensure that there was no contamination. All batches of media for DST were stored at 4°C for not longer than four weeks from date of preparation. Preparation of suspensions from each strain was done using individual sterile wire loops per inoculation to avoid cross-contamination between the strains[14].

Ethical considerations: This study was cleared by both the Kenya Medical Research Institute (KEMRI) Scientific Steering Committee (SSC) and the National Ethical Review Committee (ERC).

Data analysis: Using S.P.S.S. computer data analysis programme, analysis of data was done using chi-square to compare resistance and susceptibility among the drugs and to compare resistance and susceptibility between the first line susceptible and resistant strains[15]. The data was presented inform of tables.

Results

Of the 216 strains tested, 198 (91.7%) were sensitive and 18 (8.3%) were resistant to Eth. A total of 32 [32/216 (14.8%)] strains showed resistance to second-line drugs. Of these, nine (47.4%) were fully resistant and nine (69.2%) were intermediate resistant to Eth (Table 2 and 3).

Table 2: Susceptibility pattern of the 216 *Mycobacterium tuberculosis* strains to ethionamide.

Second-line anti-TB drugs	Sensitive n (%)	Fully resistant n (%)	Intermediate resistant n (%)	Total n (%)
Eth	198 (91.67)	9 (4.17)	9 (4.17)	216 (100)

Of the 32 resistant strains a total of 18 (56.3%) isolates were resistant to Eth. Eth showed the highest number of resistance with seven (41.2%) strains being fully resistant to Eth alone and two strains being resistant to Eth and two other second-line drugs (Table 3).

Table 3: Specific resistant levels in Ethionamide

Type of strain	Numbers of strains (n)
Mono-resistant to Eth	7
Fully resistant to Gatifloxacin and Eth	1
Fully resistant to Eth and Cycloserine	1
Intermediate resistant to Eth	9
Total Resistant	**18**

Of the 25 rifampicin resistant strains tested, 23 (92%) were found to be sensitive and two (8%) were fully resistant to Eth. Of the 73 isoniazid resistant strains, 65 (89.1%) were sensitive, six (8.2%) fully resistant and two (2.7%) strains showed intermediate resistance to Eth. Of the 11 ethambutol resistant strains, 10 (90.9%) were sensitive to and 1 (9.1%) fully resistant to Eth. Of the 24 streptomycin resistant strains, 22 (91.7%) were sensitive and two (8.3%) were fully resistant to Eth (Table 5).

Table 5: Susceptibility profile of the 25 MDR–TB strains to Ethionamide

Second-line anti-TB drugs	Sensitive n (%)	Fully resistant n (%)	Total n (%)
Eth	23 (92)	2 (8)	25 (100)

Out of the 25 MDR-TB strains tested, two (8%) were fully resistant to Eth and 23 (92%) were sensitive to it (Table 6).

Table 4: Susceptibility profile of Ethionamide to first-line resistant strains

First-line anti-TB drugs resistant strains (n)	Sensitive N (%)	Total resistant strains n (%)	Fully resistant n (%)	Intermediate resistant n (%)	P-value
Rifampicin (25)	23 (92)	2 (8)	2 (8)	0 (0)	0.337
Isoniazid (73)	65 (89.1)	8 (11)	6 (8.2)	2 (2.7)	0.083
Ethambutol (11)	10 (90.9)	1 (9.1)	1 (9.1)	0 (0)	0.838
Streptomycin (24)	22 (91.7)	2 (8.3)	2 (8.3)	0 (0)	0.325

Discussion

The findings of this study indicate that second-line drug resistant TB has not only developed but is also high in Eth. It was also established that MTB has developed further resistance to anti-TB drugs over the

years since drug resistant TB was first reported in Kenya. Furthermore MDR-TB was first isolated and documented in Kenya in the year 2002[16].

Resistance to second-line drugs is associated with worse treatment outcomes than MDR-TB since an inadequate or poorly administered treatment regimen allows a drug-resistant strain to become the dominant strain in a patient infected with TB. Short-course chemotherapy for patients infected with drug-resistant strains may create even more resistance to the drugs in use. On-going transmission of established drug-resistant strains in a population is also a significant source of new drug-resistant cases[17]. Based on this study, Eth is the drug with the highest resistance at 41.2% compared to the other SLDs tested. This high Eth resistance may hamper treatment of MDR-TB patients in Kenya since Eth is one of the key drugs used in the regimen of treatment of MDR-TB.

In a study done by Heym *et al,* 16 out of 36 isoniazid resistant strains were found to be also resistant to Eth[18]. In this study 8 strains found to be resistant to both Eth and isoniazid. This is the first time in Kenya that isoniazid and Eth resistance association has been documented. Eth, like isoniazid is considered an inhibitor of mycolic acid synthesis but much less active against MTB[3]. Studies have shown that for certain strains, a low level of isoniazid resistance is correlated with co-acquisition of Eth resistance, suggesting that both share a common molecular target most likely involving the *mab-inhA* genes that are involved in resistance[9].

Relationship between isoniazid and Eth could be because resistance genes encode information on a variety of mechanisms that microorganisms use to withstand the inhibitory effects of specific antimicrobials. These mechanisms can confer resistance to other antimicrobials of the same class and sometimes to several different antimicrobial classes[19]. In this study, isoniazid resistant strains showed high resistance to Eth indicating that the two drugs may be correlated in resistance. It is known that high level resistance to isoniazid is associated with mutations to catalase peroxidase gene, *kat*G[20], and that cross-resistance to isoniazid and ethionamide results from mutations in the *inhA* gene[9].

Conclusions

The level of resistance to Eth was high, and continued failure to improve TB control will impact negatively on the outcome of management of MDR-TB especially in Kenya where the use of this drug is almost mandatory. Close monitoring of Eth before embarking on individual patient management is necessary. With the presence of second-line anti-TB drug resistance strains in Kenya, control of TB will rely on quality assured and nationally recommended treatment regimen administered under strict supervision as part of the Directly Observed Therapy Short Course Plus (DOTS-Plus) programme.

Recommendations

Since this study showed a trend towards the co-relationship between isoniazid and Eth resistance, further studies should be carried out to show this relationship. All isoniazid resistant strains should also be tested for Eth resistance. Close monitoring of Eth resistance before initiating individual patient management may be necessary.

Acknowledgements

The authors would like to thank; CRDR TB laboratory staff for their technical assistance, Mr. Moses Mwangi for his statistical assistance and Dr. Evans Amukoye for providing conducive environment to conduct this work.

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