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Research Article
Effect on *Mycobacterium tuberculosis* Clinical Isolates Susceptibility Against First and Second-line Drugs Using MGIT 960

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Abstract

Background and Objective: With the expanding knowledge about efflux pumps contribution to the resistance mechanism of *Mycobacterium tuberculosis* has increased the attention to efflux inhibitors usage as adjuvants in tuberculosis therapy. Therefore, this study examined the effect and interaction between efflux inhibitor towards first (isoniazid, streptomycin, rifampicin, ethambutol) and second-line (kanamycin, ofloxacin, capreomycin, moxifloxacin) anti-tuberculosis drugs in *M. tuberculosis* susceptibility. **Materials and Methods:** Sixty-five *M. tuberculosis* isolates collected from sputum samples of tuberculosis patients in Makassar and exposed to anti-TB drugs at critical concentration in the presence or absence of verapamil (40 μg mL^{-1}) using drug susceptibility test (DST) proportion method in Mycobacterium Growth Indicator Tube (MGIT) 960 system. **Results:** About 14 isolates (21.54%) were mono-resistant, 20 isolates were MDR-TB (30.67%), 20 isolates (30.67%) were Pre XDR-TB and 7 isolates (10.77%) were XDR-TB. There were 8 drugs that were tested but only 6 drugs showed a decrease of mean growth unit in STR, INH, RIF, CAP, OFX and MOXI after the addition of efflux pump inhibitor (synergy observed). The overall effect of verapamil towards all groups of drugs tested showed p-value of 0.001 ($p<0.05$). **Conclusion:** It was concluded that the addition of verapamil plays a significant role in restoring the susceptibility of *M. tuberculosis* isolates.

Key words: Efflux pumps, tuberculosis therapy, verapamil, anti-tuberculosis, drug susceptibility test, *Mycobacterium tuberculosis*, efflux inhibitors

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mycobacterium tuberculosis is the leading cause of global tuberculosis (TB) infection[1]. The emergence of *M. tuberculosis* strains bacteria that are resistant to first-line (called Multi Drug Resistant/MDR) and second-line drugs (called Extensive Drug Resistant/XDR) lessen the alternative of antibiotic use to treat TB disease[2-7]. Bacterial resistance to antibiotics is not caused by a single mechanism but is a combination of genetic factors such as spontaneous mutations of target genes and the transfer of genetic elements and intrinsic factors such as changes in permeability of bacterial cell barriers, presence of porins and activation of efflux pump systems[8-14].

In physiological conditions, efflux pump generates an important contribution to the phenotype of low level drug resistance due to the function of the protein efflux pump transporter which limited the intracellular drug concentration[15-17]. Some components are known to have a potential to inhibit the efflux pump system but the working mechanism of the inhibitor components in the cell is not yet clear[3,16-18-25].

Verapamil is an effective efflux pump inhibitor that possesses the potential to be used as an adjuvant in TB treatment therapy. A number of *in vitro* and *in vivo* studies related to the use of verapamil as an efflux inhibitor at various verapamil concentrations toward certain drugs have been carried out[26-31]. It requires a high concentration of verapamil in order to provide bactericidal effects on *M. tuberculosis* but it can be toxic to mammalian cell membranes[30]. *In vivo* studies showed that the use of verapamil combined with certain antibiotics in mice infected with *M. tuberculosis* showed a significant reduction in the number of TB bacilli[24]. However, the combination of verapamil effects and antibiotic regimens at critical concentrations recommended by the World Health Organization (WHO) but still used in the current TB patients treatment, is not very clear. The drug susceptibility testing (DST) method based on MGIT (Mycobacterium growth indicator tube) liquid medium was used to shorten the reporting time of results. There have been many diagnostic studies using the DST MGIT method[21,32,33], which aimed to compare conventional DST using solid medium and DST MGIT. This study aimed to evaluate the effect and interaction between verapamil at certain concentration towards first (isoniazid, streptomycin, rifampicin, ethambutol) and second-line (kanamycin, ofloxacin, capreomycin, moxifloxacin) anti-tuberculosis drugs on *M. tuberculosis* susceptibility.

MATERIALS AND METHODS

Clinical isolates: This study was carried out in the Tuberculosis Laboratory of Balai Besar Laboratorium Kesehatan (BBLK) Makassar Indonesia, between January until November, 2018. Mycobacterial isolates in MGIT tubes were screened according to microscopic and macroscopic characteristics, in order to differentiate *M. tuberculosis* from non-tuberculous mycobacteria. *M. tuberculosis* identification with MPT64 antigen (Standard Diagnostic Inc., Abbot, USA) detection according to manufacturer instruction. This study included 1 tube of MGIT with p-nitrobenzoic acid (PNB) (Sigma-Aldrich, St. Louis, MO, USA) in a final concentration of 500 μg mL^{-1} for each isolate. The PNB tubes were inoculated the same way as the drug containing tubes[34].

Drug susceptibility testing (DST) proportion method on BACTEC MGIT 960: Sub-cultures were prepared by inoculating 0.5 mL of previously prepared *M. tuberculosis* stock-culture in MGIT 960 media supplemented with 800 μL of OADC (Becton, Dickinson and Company, Sparks, USA). These sub-cultures were then incubated at 37°C in the MGIT 960 instrument until a positive growth reading was obtained. The growth control (GC) tubes were prepared by inoculating 0.5 mL of the 1:100 diluted sub-cultures into 800 μL OADC enriched MGIT tubes. Subsequently, 500 μL of the undiluted positive sub-cultures were inoculated into MGIT tubes enriched with 800 μL OADC and 100 μL of the anti-TB drug specific concentration (Streptomycin 1,00 μg mL^{-1} (STR), Isoniazid 0,10 μg mL^{-1} (INH), Rifampicin 1,00 μg mL^{-1} (RIF), Ethambutol 5,00 μg mL^{-1} (ETB), Kanamycin 2,5 μg mL^{-1} (KAN), Ofloxacin 2,0 μg mL^{-1} (OFX), Capreomycin 2,5 μg mL^{-1} (CAP), Moxifloxacin 0,25 μg mL^{-1} (MOXI)) with the presence and absence of 100 μL verapamil 40 μL mL^{-1} (Sigma-Aldrich, St. Louis, MO, USA). The critical proportion of resistant bacillus necessary to define a resistant strain is 1% for all tested drugs[35]. The MGIT tubes were registered in the EpiCenter (version 6.20A) software (BD Bioscience, Erembodegem, Belgium) and placed in BACTEC MGIT 960 instrument to continuously monitor the growth for 14 consecutive days[36].

The reference strain of *M. tuberculosis* H37RV (ATCC 27294) was included in each batch of tests. All bacterial suspensions used in DST MGIT 960 were checked for purity from contamination by ZN staining and culture blood agar plate[37-39]. Growth control (GC) contained verapamil (40 μg mL^{-1}) only for each DST series in every isolate.

Fractional inhibitory concentration formula index (FIC): The fractional inhibitory concentration (FIC) formula index was used to assess the interaction of anti-TB drugs and inhibitor. The FIC determined as a ratio of the Growth Unit (GU) assessed in an inhibitor and drug combination to the GU of the same drug tested alone. This study used GU because the verapamil experiment was done in the MGIT system. The interaction values were classified as synergistic when FIC index $\leq$0,5-0,9, indifference/additive when FIC index +1-1,9 and antagonistic when FIC index $\geq$2 FIC[38,39].

Research ethics: This study has attained an ethical approval from the Institutional Research Board of Medical Faculty of Hasanuddin University, Makassar, Indonesia. Registration number 42/H4.8.4.5.31/PP36-KOMETIK/2018, dated January 18th, 2018. The informed consent for this study was obtained written from all participants or their parents/guardians accompanied by the authorized nurses who were in charge of managing TB patients.

Statistical analysis: The SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used to perform ANOVA one-way F-test evaluating the significant difference of *M. tuberculosis* growth in the presence of anti-TB drugs alone or in combination with verapamil. The T paired test was also performed to see the effect of efflux pumps inhibitor verapamil that were studied, if it is a quantitative data (intervals and ratios) and normally distributed. If it was not normally distributed, a non-parametric Wilcoxon test was administered with a significance level of 5% ($p<0.05$).

RESULTS

Drug-resistant frequency: Total of 65 isolates of *M. tuberculosis* were tested for drug susceptibility test (DST) using first and second-line as described in Table 1. Screening of *M. tuberculosis* isolates for drug resistance identified in Table 2 consist of 14 (21.54%) isolates with monoresistant phenotype, 4 isolates (6.15%) with polyresistant phenotype,

Table 1: Drug-resistant profile of each *M. tuberculosis* isolates

Strain ID	STR	INH	RIF	ETB	KAN	OFX	CAP	MOXI
TB1	S	R	R	S	S	S	S	S
TB2	S	R	R	S	S	S	S	S
TB3	R	R	R	S	S	S	S	R
TB4	S	R	R	R	S	R	S	R
TB5	S	S	R	S	S	S	S	S
TB6	S	R	R	S	S	S	S	S
TB7	R	R	R	S	S	S	S	S
TB8	R	R	R	R	S	R	S	R
TB9	R	R	R	R	S	R	S	R
TB10	S	R	R	S	S	S	S	S
TB11	S	R	R	R	S	R	S	R
TB12	R	R	R	R	S	R	S	R
TB13	S	R	R	R	S	S	S	S
TB14	R	R	R	R	S	S	S	S
TB15	S	R	R	S	S	S	S	S
TB16	S	R	R	S	S	R	S	R
TB17	S	R	R	S	S	S	S	S
TB18	R	R	R	S	S	S	S	S
TB19	S	S	R	S	S	S	S	S
TB20	R	R	R	R	S	S	S	S
TB21	S	S	R	S	S	S	S	S
TB22	R	R	R	R	S	S	S	S
TB23	S	R	R	R	S	R	S	S
TB24	R	R	R	R	S	R	S	R
TB25	S	R	R	R	S	R	S	R
TB26	S	R	R	S	S	S	S	S
TB27	R	R	R	R	S	R	S	R
TB28	S	S	R	S	S	S	R	S
TB29	R	R	R	S	R	S	R	S
TB30	R	R	R	R	R	R	R	R
TB31	R	R	R	R	S	R	S	S

Table 1: Continue

Strain ID	STR	INH	RIF	ETB	KAN	OFX	CAP	MOXI
TB32	R	R	R	R	R	S	R	S
TB33	S	S	R	S	S	S	S	S
TB34	R	R	R	R	S	R	R	R
TB35	S	R	R	R	S	S	S	S
TB36	S	R	S	S	S	S	S	S
TB37	S	S	R	S	S	S	S	S
TB38	R	R	R	R	R	R	R	R
TB39	R	R	R	S	R	R	S	S
TB40	R	R	R	R	S	R	R	R
TB41	S	S	R	S	R	S	R	S
TB42	S	S	R	S	S	S	S	S
TB43	S	R	R	R	S	R	S	R
TB44	S	R	R	S	S	S	R	S
TB45	S	R	R	S	R	R	R	S
TB46	S	S	R	S	S	S	S	S
TB47	R	R	R	S	S	S	S	S
TB48	R	R	R	R	R	S	R	S
TB49	S	S	R	S	S	S	S	S
TB50	R	R	R	S	R	R	R	R
TB51	S	R	S	R	S	S	S	S
TB52	R	R	R	R	S	S	S	S
TB53	R	R	R	S	S	S	S	S
TB54	S	R	R	R	S	R	S	R
TB55	S	R	S	S	S	S	S	S
TB56	R	R	S	S	S	R	S	R
TB57	S	R	R	S	S	S	S	S
TB58	R	R	R	R	S	R	S	R
TB59	R	R	R	R	S	S	S	S
TB60	R	R	R	R	S	R	S	R
TB61	S	R	R	S	S	S	S	S
TB62	S	S	S	S	S	R	S	S
TB63	S	S	S	S	S	R	S	S
TB64	S	S	S	S	S	R	S	S
TB65	S	S	S	S	S	S	R	S

Source: Primary data. STR: Streptomycin, INH: Isoniazid, RIF: Rifampicin, ETB: Ethambutol, KAN: Kanamycin, OFX: Ofloxacin, CAP: Capreomycin, MOXI: Moxifloxacin, R: Resistant, S: Sensitive

Table 2: Frequency of resistance pattern in *Mycobacterium tuberculosis* isolates

Resistance pattern	n	Percentage
Monoresistant:	14	21.54
INH-resistant	2	3.07
RIF-resistant	8	12.30
OFX-resistant	3	4.61
CAP-resistant	1	1.54
Polyresistant	4	6.15
Multi drug resistant (MDR)	20	30.76
Pre extensively drug resistant (Pre-XDR)	20	30.67
Extensively drug resistant (XDR)	7	10.77
Total	65	100.00

Source: Primary data

20 isolates (30.76%) with MDR-TB phenotype, 20 isolates (30.67%) with Pre XDR-TB phenotype, 7 isolates (10.77%) with XDR-TB phenotype.

Verapamil (40 μg mL^{-1}) effect on *M. tuberculosis* growth: The data in Table 3 showed the average growth rates of 65 *M. tuberculosis* isolates in the presence of drugs and combination of drugs and verapamil (40 μg mL^{-1}). ANOVA one way F-test evaluating the significant difference of *M. tuberculosis* growth with $p = 0.000$ ($p<0.05$) between two groups. There were 8 drugs that were tested showed a decreased of mean values in STR (1.00 μg mL^{-1}), INH (0.10 μg mL^{-1}), RIF (1.00 μg mL^{-1}), CAP (2.5 μg mL^{-1}), OFX (2.0 μg mL^{-1}) and MOXI (0.25 μg mL^{-1}) after the addition of efflux pump inhibitor. Conversely, there was an increased of mean values in ETB (5.00 μg mL^{-1}) and KAN (2.5 μg mL^{-1}) with the combination of verapamil. The Table 4 showed the significant effect of verapamil (40 μg mL^{-1}) was only seen in STR and combination of STR+verapamil with $p = 0.000$ ($p<0.05$). The overall effect of verapamil towards all groups of tested drugs showed p value of 0.001 ($p<0.05$).

Synergistic properties of verapamil and anti-TB drugs: The results in Table 5 showed fractional inhibitory concentrations (FIC) of *M. tuberculosis* isolates that were calculated to assess

Table 3: Growth rate of *M. tuberculosis* isolates in different anti-TB drugs with the absence or presence of verapamil (40 μg mL^{-1})

Anti-TB drugs	n	Mean±SD drug only	Minimum	Maximum	Mean±SD drug+verapamil (40 μg mL^{-1})	Minimum	Maximum	p-value (ANOVA one-way F-test)
STR (1,00 μg mL^{-1})	65	172.32±199.60	0	400	83.85±162.28	0	400	0.000
INH (0,10 μg mL^{-1})		313.85±165.72	0	400	307.69±169.84	0	400	
RIF (1,00 μg mL^{-1})		350.77±132.43	0	400	348.40±132.89	0	400	
ETB (5,00 μg mL^{-1})		163.26±191.17	0	400	187.26±192.73	0	400	
KAN (2,5 μg mL^{-1})		53.88±135.40	0	400	55.49±139.19	0	400	
OFX (2,0 μg mL^{-1})		156.78±195.03	0	400	140.85±188.39	0	400	
CAP (2,5 μg mL^{-1})		78.83±158.64	0	400	59.94±135.98	0	400	
MOXI (0,25 μg mL^{-1})		124.14±185.44	0	400	104.58±173.22	0	400	

Source: Primary data, STR: Streptomycin, INH: Isoniazid, RIF: Rifampicin, ETB: Ethambutol, KAN: Kanamycin, OFX: Ofloxacin, CAP: Capreomycin, MOXI: Moxifloxacin

Table 4: Effect of verapamil (40 μg mL^{-1}) on drug susceptibility test (DST) result of all *M. tuberculosis* isolates

Paired Z test	n	Mean difference	p-value (Wilcoxon signed rank test)
Drugs-Drugs+verapamil	65	3.419	0.001
STR-STR+verapamil		3.819	0.000
INH-INH+verapamil		1.000	0.317
RIF-RIF+verapamil		0.000	1.000
ETB-ETB+verapamil		1.550	0.121
KAN-KAN+verapamil		0.535	0.593
OFX-OFX+verapamil		1.572	0.116
CAP-CAP+verapamil		1.332	0.183
MOXI-MOXI+verapamil		1.863	0.063

Source: Primary data, STR: Streptomycin, INH: Isoniazid, RIF: Rifampicin, ETB: Ethambutol, KAN: Kanamycin, OFX: Ofloxacin, CAP: Capreomycin, MOXI: Moxifloxacin

Table 5: FIC indices (Fractional inhibitory concentration) for verapamil (40 μg mL^{-1}) in combination with different anti-TB drugs

Anti-TB drugs	Verapamil (FIC)	Inhibitor/drug interaction
STR	0.5	Synergistic
INH	0.9	Synergistic
RIF	0.9	Synergistic
ETB	1.1	Indifferent/additive
KAN	1.0	Indifferent/additive
OFX	0.9	Synergistic
CAP	0.7	Synergistic
MOXI	0.8	Synergistic

Source: Primary data, STR: Streptomycin, INH: Isoniazid, RIF: Rifampicin, ETB: Ethambutol, KAN: Kanamycin, OFX: Ofloxacin, CAP: Capreomycin, MOXI: Moxifloxacin

the interaction between verapamil (40 μg mL^{-1}) with different anti-TB drugs. Synergistic interaction was observed in the presence of verapamil (40 μg mL^{-1}) in combination with STR, INH, RIF, OFX, CAP and MOXI but indifference interaction was found in combination with ETB and KAN.

DISCUSSION

This study revealed that the addition of efflux inhibitor verapamil decreased bacterial growth in the existence of STR, INH, RIF, OFX, CAP or MOXI indicating an extended susceptibility towards these drugs (synergy observed). Conversely, verapamil enhanced resistance to ETB and KAN as measured by the observed escalated growth in different drug interaction. Yet, there were 4 isolates with ETB-resistant phenotype showed a cut down of growth unit in the presence of verapamil and turned out to be sensitive toward ETB.

Ethambutol was the last drug that has been added to the first-line drugs regimen, to compensate the function of INH, RIF and Pyrazinamide during the first week of therapy and prevent INH- and RIF-resistant phenotypes. Pharmacokinetics of other anti-TB drug was clearly known, except for ETB[40].

The outcome was in concurrence with previous data that implied verapamil could partially restore drug efficacy of RIF[3,9,15], INH[15] and fluoroquinolones[24] on *M. tuberculosis* resistant strains. Balganesh *et al.*[20] suggested efflux pumps play an important role against anti-TB drugs activity. The study used *M. tuberculosis* WT (wild type) and KO-mutant (knocked-out) strains where the WT strain was unable to withstand antibiotic stress even with the addition of efflux inhibitor, while KO-mutant strain showed sensitive phenotype after the addition of efflux inhibitor.

The addition of verapamil increased susceptibility towards CAP, it might suggested that aminoglycosides and cyclic peptides are extruded multiple efflux pumps. The results showed the engagement of MFS, ABC, RND superfamilies efflux pumps in designating the level of intrinsic resistance to various anti-TB drugs[7,8,14,20,41]. The results might support the novel inhibitors improvement to target these efflux pump targets and thereby strengthen MDR- or XDR-TB regimens. In order to intensify the action of anti-TB that targeted to efflux mechanisms, the drug should be coregulated with an efflux inhibitor, therefore assimilating the compound to be effective, even in resistant isolates[42,24,43].

Verapamil is a Ca^{2+} channel blocker, phenylalkylamine group prototype, which inhibits the multidrug ATP-dependent transporters and MDR pumps, inhibiting the transport process of proton motive force, acting as an inhibitor of efflux pump activity in prokaryotic cell[24]. Machado *et al.*[16] conducted a research in restoring the susceptibility of *M. tuberculosis* INH-resistant strains that were induced to be sensitive to INH with the addition of verapamil. Gupta *et al.*[2] stated the addition of verapamil to standard TB chemotherapy causes antibiotic acceleration in killing bacteria cell and decreases the average relapse (4 months of treatment) in tested mice infected with *M. tuberculosis*. The long duration of antibiotic treatment of *M. tuberculosis* might be due to microbe entering the dormant state in the host and it rendered the *M. tuberculosis* to be phenotypically resistant to anti-TB drugs[44].

Chen *et al.*[30] mentioned that the verapamil is not entirely inhibited the efflux mechanism in *M. tuberculosis* that could causes accumulation of drugs inside bacterial cell but cationic amphiphilic verapamil also generates disruption of membrane function and induces stress responses in cell membrane, thus giving a direct effect on the balance of energetic process which occurred in *M. tuberculosis* cell membrane.

The limitation of this study was lacking of efflux pump gene expression analysis and genetic background observation on the clinical isolates that were used. However, future study should investigate efflux pump gene expression and mutation in targeted gene on each isolates.

CONCLUSION

Efflux pumps played a crucial role in determining the level of intrinsic resistance. However this is the first investigation to study the effect of different efflux pumps on the level of intrinsic resistance to a broad spectrum of anti-TB drugs in drug resistant *M. tuberculosis* with different phenotype background. There were 6 drugs that showed a decrease of mean growth unit in STR, INH, RIF, CAP, OFX and MOXI after the addition of efflux pump inhibitor and the overall effect of verapamil towards all groups of drugs tested was significant at 0.001 ($p<0.05$)

SIGNIFICANCE STATEMENT

This study reveals that efflux inhibitor verapamil at 40 μg mL^{-1} concentration could manage to reduced bacterial growth in the existence of STR, INH, RIF, OFX, CAP or MOXI in *M. tuberculosis* isolates with different pattern of resistance and that can be beneficial to support the search of novel inhibitors and drugs development in targeting efflux pump mechanism and thereby could strengthen TB treatment.

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