Bali Medical Journal (Bali Med J) 2016, Volume 5, Number 1: 8-12
P-ISSN.2089-1180, E-ISSN.2302-2914

SUSCEPTIBILITY OF RIFAMPICIN-ISONIAZID RESISTANT MYCOBACTERIUM TUBERCULOSIS ISOLATES AGAINST LEVOFLOXACIN

[1]Alvin Hartanto Kurniawan, [2,3,4]Ni Made Mertaniasih, [4,5]Soedarsono

[1]Faculty of Medicine, Airlangga University, Surabaya-Indonesia;
[2]Department of Clinical Microbiology, Faculty of Medicine, Airlangga University, Surabaya-Indonesia;
[3]Institute of Tropical Disease, Airlangga University, Surabaya-Indonesia;
[4]Dr. Soetomo General Hospital, Surabaya-Indonesia;
[5]Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Airlangga University, Surabaya-Indonesia

Background: Tuberculosis (TB) is a high burden disease in Indonesia with multidrug-resistant (MDR) TB incidence started to increase. Treatment success of MDR-TB globally was low in number than it was targeted which was especially caused by fluoroquinolone resistance. One of the fluoroquinolone is levofloxacin, an antibiotic that has been widely used irrationally as antimicrobial treatment. Therefore, this study investigated the sensitivity and MBC of MDR *Mycobacterium tuberculosis* isolates against Levofloxacin. **Method:** The susceptibility test for MDR-*Mycobacterium tuberculosis* on levofloxacin by standard method with levofloxacin were on concentrations 0,5 µg/ml, 1 µg/ml, and 2 µg/ml. Sample of 8 strains MDR-*Mycobacterium tuberculosis* were cultured with each concentrations on Middlebrook 7H9 for 1 week incubation. Next, each of the incubated concentration was subcultured on solid media Middlebrook 7H10 for 3 weeks incubation. Colonized agar plates after 3 weeks incubation were confirmed with acid-fast stain. **Results:** On MB 7H10 with levofloxacin concentration 2 µg/ml showed bactericidal effect 100% by no MDR *Mycobacterium tuberculosis* colony grew (0/8) while the MB 7H10 with levofloxacin concentration 1 µg/ml and 0,5 µg/ml showed the bactericidal effect 37,5% and 25% respectively. The colonized agar plate implied that the MDR *Mycobacterium tuberculosis* with levofloxacin concentration 1 µg/ml (5/8) and 0,5 µg/ml (6/8) grew well. **Conclusion:** Levofloxacin concentration 2 µg/ml was susceptible on MDR *Mycobacterium tuberculosis*. The concentration 2 µg/ml of levofloxacin could be considered as MBC.

Keywords: Tuberculosis; *Mycobacterium tuberculosis*; Levofloxacin. Susceptibility Test

INTRODUCTION

Tuberculosis (TB) is one of high burden diseases in Indonesia with incidence in 2014, according to WHO global report in 2015, is about 1 000 000 cases, the second most in the world after India, with mortality rate about 100 000 cases.[1] The number of TB incidence in 2014 is hugely increased, compared to 2013, which was only about 460 000 cases and mortality rate approximately 68 000 cases.[2]

The other problem is antibiotic resistance. Antibiotic resistance has been a major problem in antibiotic era that is caused by the bacteria adaptation and mutation to modify target of antibiotic action on either enzymes or genes.[3]

Address for correspondence:
Alvin Hartanto Kurniawan
Faculty of Medicine, Airlangga University,
Surabaya-Indonesia
Email: alvinhartanto@yahoo.co.id

Antibiotic resistance is also occurred on *Mycobacterium tuberculosis*. Resistant to first line agents especially rifampicin and isoniazid, *Mycobacterium tuberculosis* is going to be multidrug-resistant (MDR) *Mycobacterium tuberculosis.* Resistant to second line agents primarily fluoroquinolone or injected drug aminoglycoside, MDR *Mycobacterium tuberculosis* is going to be extremely drug resistant (XDR) *Mycobacterium tuberculosis.*[4]

Indonesia is one of 27 high MDR-TB burden countries with new MDR-TB incidences in 2014 are sub nationally reported about 1.9% of every new TB incidences while 12% of TB cases in Indonesia were reported on retreatment of MDR-TB cases. Another major problem is the treatment outcome of MDR-TB. WHO globally targeted that MDR-TB treatment success was at least 75%.[1] While in Indonesia, the outcome was targeted about 80% for case detection rate and 75% for treatment success.[1,5] However, it was reported that the

treatment for MDR-TB in Indonesia has been far below the target with merely 65% gave response effects and 56% managed to be success treatment.[6] One of causes of MDR-TB treatment failure is drug resistance especially fluoroquinolone.[7]

Levofloxacin is one the newest generations of fluoroquinolone that has been widely accepted not only as MDR-TB treatment, but also as broad spectrum antibiotic for microbial treatment.[7,8] Levofloxacin is commonly used because it is a broad spectrum antibiotic with well tolerated side effect and good distribution that could reached many tissues and body fluids.[9] Despite its uses and benefits, unwise and irrational use of antibiotics will lead to bacterial resistance including *Mycobacterium tuberculosis*.[3] Patients, who received levofloxacin during course of TB treatment, were more likely to get levofloxacin resistance during MDR TB treatment.[10] This study aims to investigate sensitivity of MDR *Mycobacterium tuberculosis* isolates against levofloxacin and establish the minimal bactericidal concentration of levofloxacin on MDR *Mycobacterium tuberculosis*.

MATERIAL AND METHODS

The susceptibility test was carried out with experimental laboratory on standard method of in vitro indirect conventional tuberculosis test.[11] The susceptibility test was held on Institute of Tropical Disease of Airlangga University, Surabaya-Indonesia.

Bacterial Strain

Rifampicin and isoniazid resistant strains of *Mycobacterium tuberculosis* randomly selected from isolate submitted to microbiology laboratory of Dr. Soetomo General Hospital on Surabaya. The isolate originated from sputum of patients with pulmonary MDR-TB. The *Mycobacterium tuberculosis* isolate with concentration 10^7 cfu/ml were diluted with Middlebrook (MB) 7H9 until to get 10^5cfu/ml suspension. The process was started by blending 10^7 cfu/ml concentration of *Mycobacterium tuberculosis* with vertex for 1 minute. Next, the *Mycobacterium tuberculosis* concentration was taken 500 µl by micropipette and diluted with 4.5 ml MB 7H9 to get 10^6 cfu/ml suspension. These steps repeated once again to get 10^5 cfu/ml suspension.[12]

Levofloxacin Solution

Levofloxacin solution with concentration 500mg/100ml were diluted with sodium chloride 0.9% to get levofloxacin solution final concentration 0.5 µg/ml, 1 µg/ml, and 2 µg/ml.

Dilution Test

Sample of the 10^5 cfu/ml suspension of *Mycobacterium tuberculosis* was replicated 8 times for each of levofloxacin solution final concentration. The dilution test was carried out in broth medium MB 7H9 with 0.5 ml of the *Mycobacterium tuberculosis* suspension and 0.5 ml of levofloxacin solution from each concentration. One control media was made in broth medium MB 7H9 with 0.5 ml of the *Mycobacterium tuberculosis* suspension and 0.5 ml of sodium chloride 0.9% without any levofloxacin solution. The 25 broth media MB 7H9 were incubated for one week in incubation cabinet at 37°C and CO_2 5%.

Subculture test

Each of the 25 incubated cultured broth media with *Mycobacterium tuberculosis* and levofloxacin were subculture on solid medium MB 7H10. The subculture process were done by took 100 µl of each incubated cultured broth media with micropipette and transferred to the surface solid medium MB 7H10. The subculture solid media MB 7H10 were incubated for 3 weeks in incubation cabinet at 37°C and CO_2 5%.

Analysis

Analysis of the susceptibility test was done visually macroscopic and microscopic. The macroscopic visual analysis on MB 7H9 media was done by comparing the turbidity between control medium and each of concentration medium. The macroscopic visual analysis on MB 7H10 media by finding colony that characterize as *Mycobacterium tuberculosis* colony which is rough, granular, and creamy white.[13] The solid media that were grown with colony of *Mycobacterium tuberculosis* were analyzed microscopically with acid fast staining (Ziehl Neelsen).[4] The concentration, which was qualified to be sensitive and have bactericidal effect on *Mycobacterium tuberculosis*, had to be no colony grew in all of 8 replication samples (99.9%,).[14]

RESULTS

After 7 day of incubation in broth media MB 7H9, samples and control were observed macroscopically by comparing the turbidity. The result is there were no differences in turbidity between samples and control (Figure 1A). Next, this study continued with subcultured each samples and a contol in solid media MB 7H10 for 3 weeks. The control showed many colonies with characteristics as *Mycobacterium tuberculosis* colony which was rough, granular, and creamy white (Figure 1B) while the subcultured *Mycobacterium tuberculosis* with levofloxacin solution 0.5 µg/ml showed 6 out of 8 samples (6/8) grew colonies with characteristics as *Mycobacterium tuberculosis* colony (Figure 1C, 1D, and 1E). The subcultured with 1 µg/ml showed 5 out of 8 samples (5/8) grew colonies that characterized as *Mycobacterium tuberculosis*

colony (Figure 2A, 2B, 2C, and 2D) while the subcultured with 2 µg/ml pointed out no colony (0/8) that panned out grew on solid media MB 7H10 (Figure 2E, 2F, 2G, and 2H). The colony that grew on each solid media MB 7H10 were stained with Ziehl Neelsen method to confirm the colonies were *Mycobacterium tuberculosis* colonies.

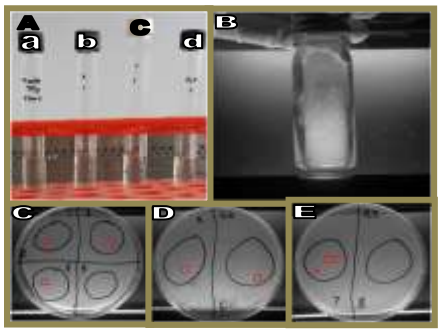

Figure 1
A The result of cultured *Mycobacterium tuberculosis* on MB 7H9 without any levofloxacin a) and with levofloxacin solution 2 µg/ml b), 1 µg/ml (c), and 0.5 µg/ml (d) after 1 week incubation.
B The result of subcultured *Mycobacterium tuberculosis* after 3 weeks incubation without levofloxacin on MB 7H10 that showed many colonies grew and
C, D, E with levofloxacin 0.5 µg/ml which also showed 6 out of 8 samples grew colonies.

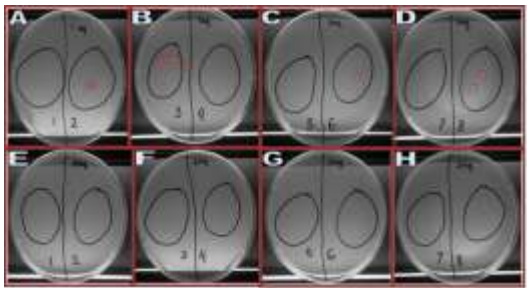

Figure 2
The result of subcultured *Mycobacterium tuberculosis* after 3 weeks incubation
A, B, C, D with levofloxacin 1 µg/ml that showed 5 out of 8 samples grew colonies and
E, F, G, H with levofloxacin 2 µg/ml that pointed out no colony grew.

DISCUSSION

With the increasing in number of MDR-TB incidence in Indonesia and one major cause of it because of fluoroquinolone resistant, this study was to investigate sensitivity of the MDR *Mycobacterium tuberculosis* against levofloxacin with susceptibility test.[1,7]

Levofloxacin was chosen in this study because it was better than the other fluoroqionolone. The other fluoroquinolone such as ciprofloxacin were reported very high in number while offloxacin, which is the L-isomer of levofloxacin, was less bactericidal than levofloxacin. Gatifloxacin was avoided because of the side effect. Moxifloxacin, one of the newest generation of fluroquinolone, was less bactericidal and more potent to be resistant than levofloxacin.[15]

The susceptibility test was begun with dilution test on MB 7H9 with 1 week incubation. After 1 week, observation was done visually by comparing the turbidity between control and samples. The result was there was no difference of turbidity on both control and samples which was explained because *Mycobacterium tuberculosis*, unlike other common bacteria, needed 20 hours to replicate itself with heavily clumped. Therefore, common observation on turbidity in *Mycobacterium tuberculosis* dilution test was hardly done unless using some expensive reagants such as BACTEC or MB-7H12.[16]

Because of the undifferented turbidity, the next process was subculture. The media in the study was preferred MB 7H10 to Lowenstein-Jensen (LJ) because LJ is an egg-based solid media that much more potent to be contaminated and less sensitive than MB 7H10 which is using antibiotic.[17] Disadvantage of using MB 7H10 are incubation time that is shorter than LJ and the price that is more expensive than LJ.[18]

After 3 weeks incubation, the subculture on MDR *Mycobacterium tuberculosis* with levofloxacin solution 0.5 µg/ml and 1 µg/ml on MB 7H10 were colonized consecutively 75.00% and 62.50%. The result indicated that these concentrations were categorized as resistant because of inadequate bactericidal dose of levofloxacin solution that allowed several strain to adapt, mutate, and modify the target of antibiotic action. Contrarily on 2 µg/ml that showed no colony implied that the concentration was adequate dose so that no bacteria able to either adapt or mutate.[4] The concentration 2 µg/ml was categorized as sensitive in this study and the only concentration that had bactericidal effect because no colony grew on all the replications.[19]

Comparation this study with other study, Angeby *et al* in 2010 represented levofloxacin, one of four fluoroquinolone that was used in the study, had critical minimal inhibitory concentration (MIC) on *Mycobacterium tuberculosis* was at 2 µg/ml.[20] Sanders *et al* in 2006 represented levofloxacin critical concentration with BACTEC 460 and BACTEC MGIT 960 was at 2 µg/ml while with agar plate method was at 1 µg/ml.[21] Niward *et al* in 2016 showed in their study that levofloxacin 1 µg/ml was resistant to Fluoroquinolone resistant-*Mycobacterium tuberculosis* which resulted in increased MIC of levofloxacin (2-8 µg/ml).[22] Ahmed *et al* in 2013 showed in their study in Pakistan that levofloxacin with 1 µg/ml on pre-XDR TB was almost completely resistant

(91.20%).[23] Kim *et al* in 2013 with their study on some of *Mycobacterium tuberculosis* strains showed that on levofloxacin with concentration 2 µg/ml was sensitive to all strains.[24]

CONCLUSION

This study demonstrated that levofloxacin solution with concentration 2 µg/ml was susceptible against MDR *Mycobacterium tuberculosis* isolates and also the MBC of this study. Management of rational and wise antibiotic use especially levofloxacin is required to maintain the sensitivity of levofloxacin on *Mycobacterium tuberculosis*. Further studies are also needed to update the susceptibility of levofloxacin on *Mycobacterium tuberculosis*.

ACKNOWLEDGEMENT

We would like to thank Dean of Faculty of Medicine, Universitas Airlangga, Director of Institute of Tropical Disease, Universitas Airlangga, and Director of Dr. Soetomo General Hospital.

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