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ITS and pB2.5 gene expression of Naegleria fowleri in drug resistance

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ABSTRACT

***Naegleria fowleri* was causative agent of primary amoebic meningoencephalitis (PAM). Accroding to the failure of treatment, several researches reported the activity of chemotherapeutic drugs against *N. fowleri* but we did not know the drug resistance of the amoebae. The purpose of this study was to examine the effects of drugs (amphotericin B, artesunate, azithromycin, voriconazole, chlorpromazine, fluconazole and gentamicin sulphate) on *ITS* and *pB*2.3 genes of *Naegleria fowleri* trophozoites. Our study demonstrated gene expression of treated *N. fowleri* by RT-PCR. The results reviewed that *ITS* gene of *N. fowleri* showed up regulate to amphotericin B, azithromycin and gentamicin sulphate, while *pB*2.3 gene showed up regulate to artesunate. These results compared with *beta actin* (house keeping gene) expression at time intervals 15 - 120 min. The change of gene expression of treated *N.fowleri* was possibly to cause of drug resistance. The mechanism of drug resistance genes *ITS* and *pB*2.3 of *N. fowleri* should be clarified in further study.**

Keywords: Naegleria Fowleri; ITS; pB2.3; Drug Resistance

1. INTRODUCTION

Naegleria fowleri causes severe meningoencephalitis mainly in children and young adults. Due to the treatments have not been succeeded, most of patients die from *N. fowleri* infection [1]. The effect of drug against *N. fowleri* has been carried out both *in vitro* and *in vivo* studies which provided for clinical trends for treatment [2]. A prelude of drug resistant has been frequently documented in recent years [3]. Several researches on antifungal resistant have been focused on elucidating the molecular basis and transcriptional regulation of azole in *Candida* spp. [4]. The resistance to azole uptake of *C. albicans* can be achieved with the introduction of key point mutation in and/or up regulation of gene expression which encodes on efflux pump; *EGR11*, *MDR* including ATP-binding cassette transporter molecules *CDR*2 [5]. In addition, antifungal resistant was involved sterol uptake which was controled by *UPC*2 gene [6]. Owing to failure treatment of PAM, one of the major problems was drug resistant from gene alteration. Up to date, a study has been addressed the intrinsic resistant on *nfa1* and *Mp2Cl5* genes which regulated pathogenesis of the amoebae. The results demonstrated that either *nfa1* resistant to fluconazole or *Mp*2*Cl*5 resistant to amphotericin B, azithromycin and artesunate of *N. fowleri* were found [7]. Owing to the dominant *ITS*, located in the 5.8S rRNA gene and species-specific chromosomal DNA *pB*2.3 genes were used for identify pathogenic *N. fowleri* [8] and diversity of *N. fowleri* at molecular level [9], we investigated the activity of drugs on *ITS* and *pB*2.3 genes of *N. fowleri*. This report revealed the ability of *Naegleria* genes against drugs of choice.

2. MATERIALS AND METHODS

2.1. *Naegleria Fowleri* Cultivation

Free living *N. fowleri* trophozoites (Khon-Kaen strain) were cultured in Nelson's medium supplemented with 5% heat-inactivated fetal calf serum (FCS) without antibiotics at 37˚C. Trophozoites were tested with the concentration of amphortericin B, voriconazole, fluconazole, chlorpromazine, artesunate, azithromycin and gentamicin at IC_{50} [10] during 15 - 120 min, triplicate. Untreated trophozoite was used for negative control. At indicated times, trophozoites were twice washed with normal saline and frozen at –80˚C until required.

2.2. RNA Extraction

Total RNA was extracted from untreated or treated

amoebae trophozoites using Tri Reagent (Sigma-Aldrich, USA). For positive control, *nf actin* gene (housekeeping gene) of *N.fowleri* was confirmed by primer 5′- ACT CTG GTG ATG GTG TCT CTC ACA C-3′ and 5′- CTC TGA CAA TTT CTC TCT CAG TGG-3′. The amplicons of amoebae were prepared from primers of *ITS* (*ITS1*; 5′-GAACCTGCGTAGGGATCATTT-3′ and *ITS*2; 5′-TTTCTTTTCCTCCC CTTATTA-3′) and *pB*2.3 (p3f; 5′-GTGAAAACCTTTTTTCCATTTACA-3′) and p3r; 5′-AAATAAAAA TTACCATTTGAAA-3′) by one – step Super-script PCR (Invitrogen, Gransland). MW of each amplicon was detected by 1.5% Agarose Gel Electrophoresis at 100 V for 30 min.

3. RESULTS AND DISCUSSION

In our studies we demonstrated the responsibility of *N. fowleri* to the effects of drugs (amphotericin B, artesunate, azithromycin, voriconazole, chlorpromazine, fluconazole and gentamicin sulphate) on *ITS* and of *pB*2.3 gene by RT-PCR. The number of *N. fowleri* treated or untreated *N. fowleri* was not significant different between two groups at time intervals (the results was not shown) ($t < 0.005$). Total RNA was extracted from this two groups using Tri Reagent (Sigma-Aldrich, USA) at indicated time. As a positive control, we used *nf actin* to amplify under the same RT-PCR condition and regulation of expression of *nf actin* gene was detected at 170 bp at 15 - 120 min. The up regulate of *nf actin* was compared with treated or untreated amoebae at every point of time. We found that untreated *N.fowleri* showed up regulate of *ITS* gene at 450 while *ITS* gene expression from treated trophozoites with voriconazole, fluconazole or chlorpromazine was not observed during 120 min. In contrast, trophozoites treated with amphotericin B was found at least 30 min whereas trophozoites treated with azithromycin or gentamicin was shown at least 45 min (**Figure 1**). Similarly, we tested the drugs activity against *pB*2.3 gene expression of amoebae trophozoites. The untreated trophozoites showed bright band fragment at 310 bp as shown in **Figure 2**. Interesting, we did not observe *pB*2.3 gene expression from treated trophozoites with a panel of drugs at 120 min, except artesunate. It is possibly suggested that *ITS* gene of *N. fowleri* trophozoite was resisted to amphotericin B, azithromycin or gentamicin including the *pB*2.3 gene was resisted to artesunate.

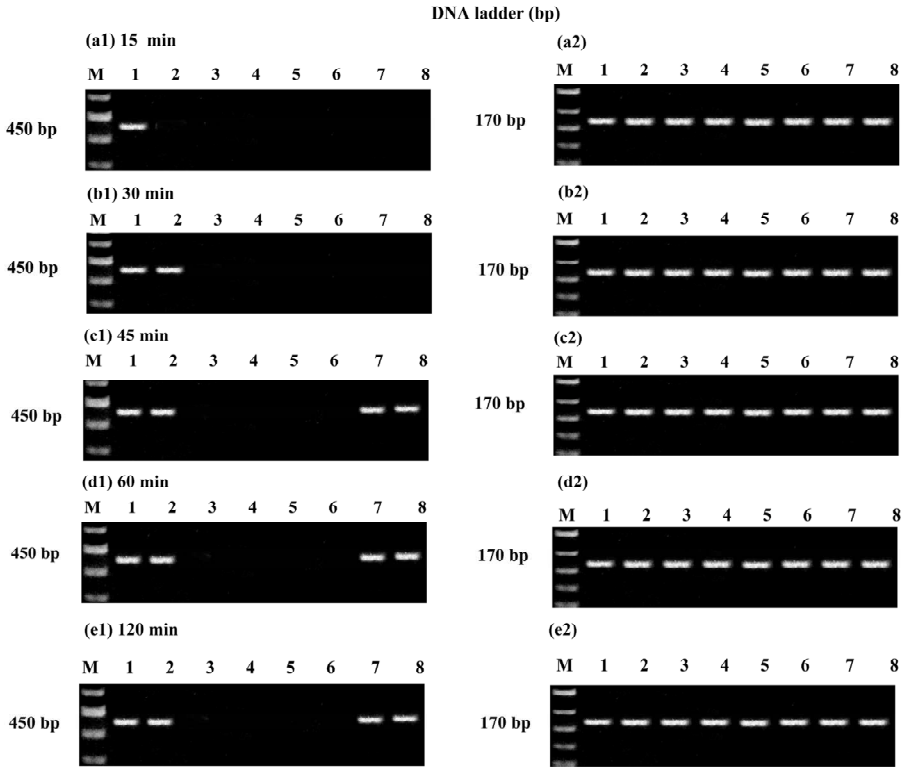

Figure 1. Effect of drugs on *ITS* gene of *N. fowleri* by RT-PCR at 15, 30, 45, 60 and 120 min (a1-e1) compared with the positive expression control, *nf actin* gene at the same time (a2-e2). Untreated N. fowleri showed bright band fragment at 450 bp (lane1). Treated *N. fowleri* with amphotericin B (lane 2), voriconazole (lane 3), fluconazole (lane 4), chlorpromazine (lane 5), artesunate (lane 6), azithromycin (lane 7), and gentamicin sulphate (lane 8), respectively.

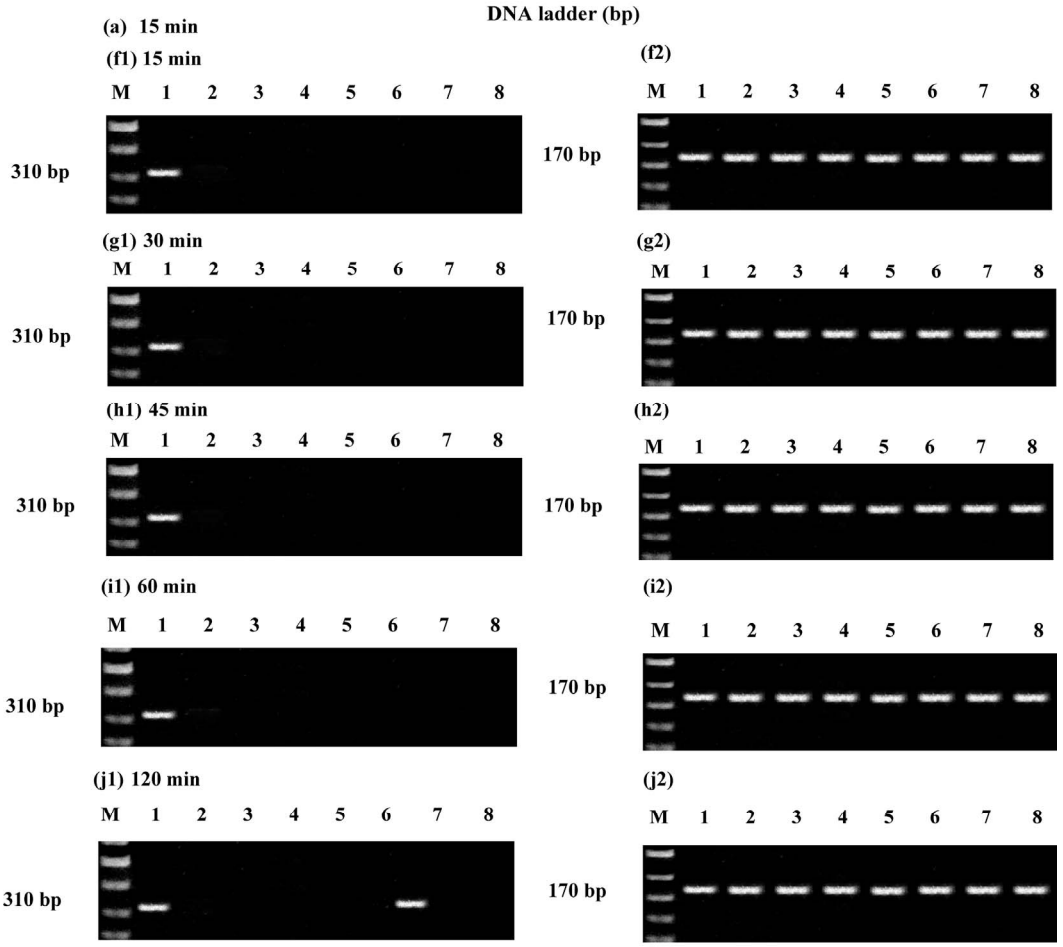

Figure 2. Effect of drugs on *pB*2.3 gene of *N. fowleri* by RT-PCR at 15, 30, 45, 60 and 120 min (f1-j1) compared with the positive expression control, *nf actin* gene(170) at the same time (f2-j2). Untreated *N. fowleri* showed bright band fragment at 310 bp (lane1). Treated *N. fowleri* with amphotericin B (lane 2), voriconazole (lane 3), fluconazole (lane 4), chlorpromazine (lane 5), artesunate (lane 6), azithromycin (lane 7), and gentamicin sulphate (lane 8), respectively.

Amphotericin B has been generally recognized for fungal and protozoa treatment, many publications reported amphotericin B-resistant genes in *Candida lusitaniae*, *Saccharomyces cerevisiae* [11] and *Cryptococcus neoformans* [12]. However, it has been reported in pathogenic protozoa; *Entamoeba histolytica*, *Giardia lamblia*, *Trichomonas vaginalis* and also occurred anaerobic protozoa; *Blastocystis hominis*, *Cryptosporidium parvum*, *Isospora* spp., *Cyclosporssporidia* spp. [13]. In addition, amphotericin B resistant gene was also found in vector born protozoa, *Leishmania tarentolae* [14].

The mechanism of amphotericin B, polyene resistant of *C. albicans* and *S. cerevisiae* caused by mutation of *EGR* genes which control the production of ergosterol and sensitivity to polyenes. As the result of *EGR6* mutant strain of *C. lusitaniae* transcription, the reduced ergosterol content was appeared [15]. A study of *EGR6* mutant caused unable to form amphotericin B-generated pores in the cell membrane on *Candida* spp [16]. Our study revealed amphotericin B resistant *ITS* gene of *N. fowleri* was firstly appeared. Moreover, drug resistant; azithromycin, gentamicin sulphate to *ITS* and artesunate to *pB*2.3 of *N. fowleri* were also demonstrated. A few publications reported the azithromycin resistant in *Pseudomonas aeruginosa* [17] and genetic mutation at 23S rRNA region of *Ureaplasma urealyticum* and *Neisseria gonorrhoeae* [18]. Gene resistant, *ermG* , to azithromycin was established in *Bacteroides*. Mechanism of azithromycin resistant established at MexCD-OprJ pump of *Pseudomonas aeruginosa* biofims [19].

Gentamicin has been general used in cultivation; *Enterococcus faecali* [20], *Pseudomonas aeruginosa* [21]. The occurrence of gentamicin-resistant genes of gram negative bacteria; *Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter* were isoloated from different environment mainly originating from sewage, faces, coastal water

polluted with wastewater and the distribution of these resistant genes could broadly transfer to hosts [22]. Gentamicin-resistant to *N. fowleri* has been found *in vitro* since 2009 [10].

This study showed that gentamicin resistant concerned both gene expression and aminoglycoside activity [20]. Artesunate was used to treatment for *falciparum* malaria due to artesunate accumulated lipids bodies and induced oxidative membrane damage [23]. Furthermore, it blocked protein synthesis of yeast cells [24] and inhibited replication of cytomegalovirus [25]. According to general use of artesunate, the artemisinin resistance to malaria was found in clinical trial [26]. The resistance gene *mdr1*, *cg10*, *tctp*, and *atp6* to artemisinin of *Plasmodium chabaudi chabaudi* were developed and transmitted to its derivatives [27]. The *pB*2.3 resistant gene of *N. fowleri* was appeared, thereby it located in mitochondria and chromosome of the amoebae. In conclusion, one of the drug treatment failure focused on *ITS* and *pB*2.3 genes of *N. fowleri*.

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