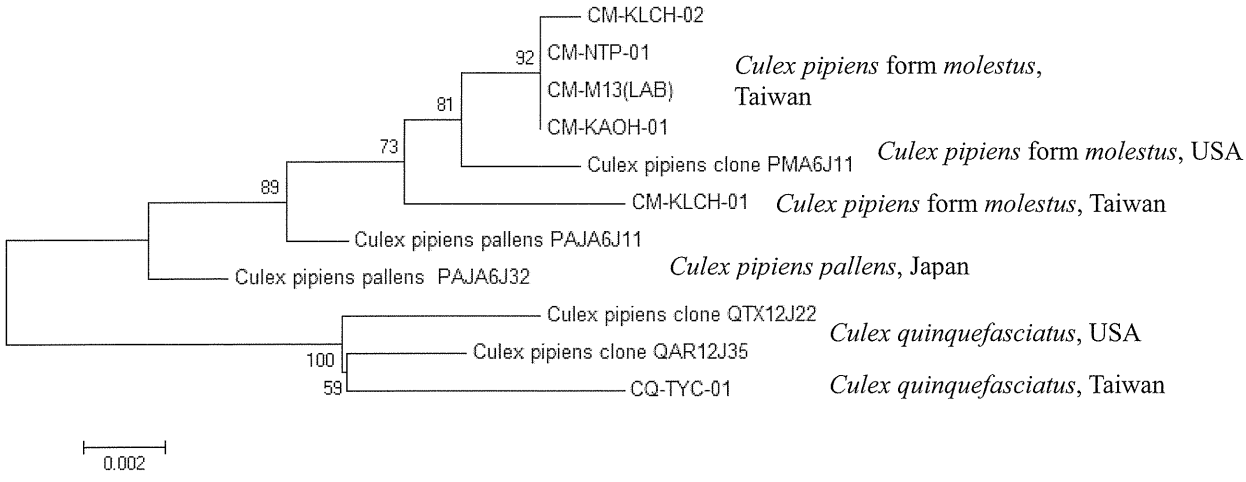

Fig. 5. The phylogenetic analysis of *Cx.pipiens complex* on 18S ribosomal DNA。

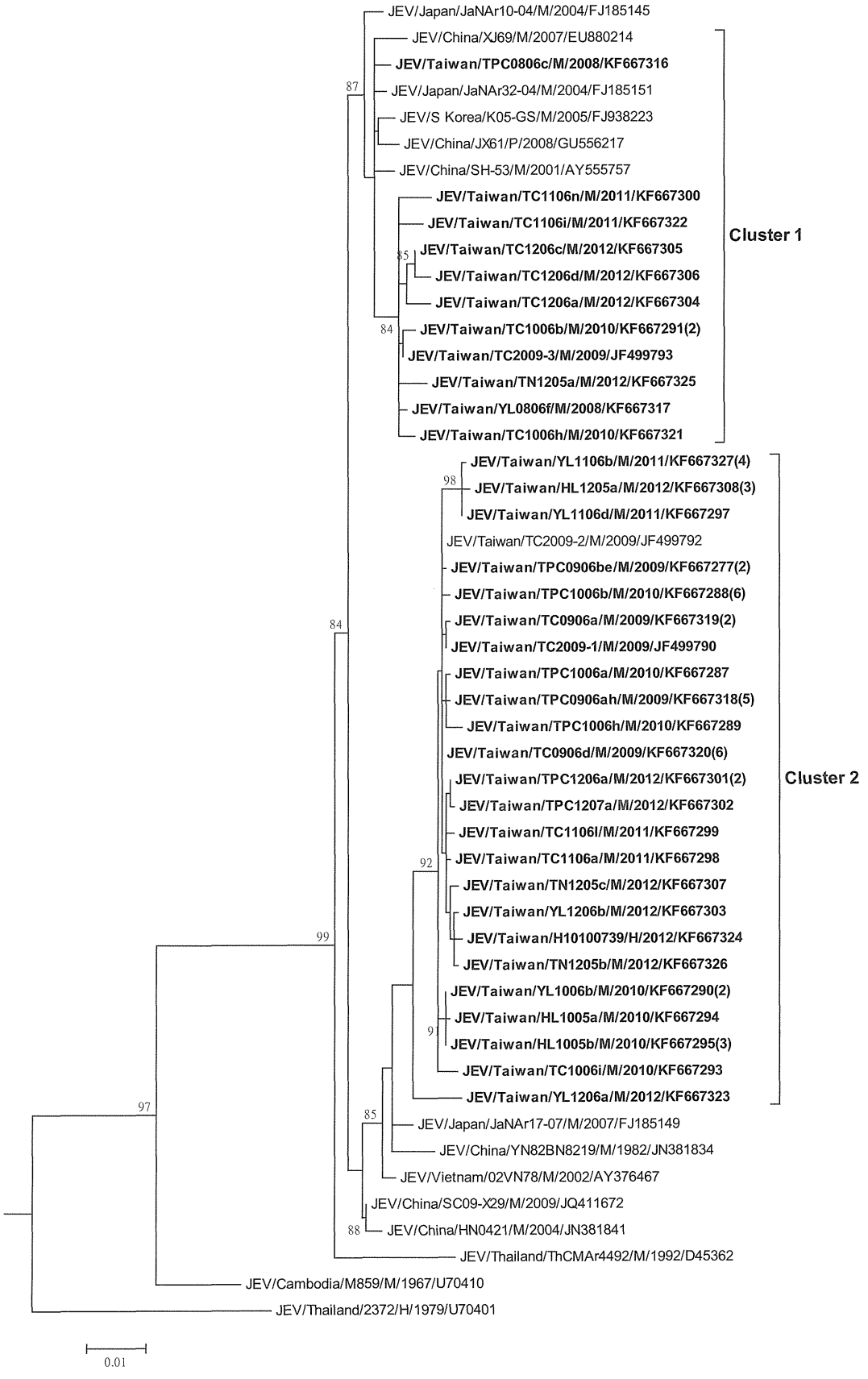

Fig. 6. Phylogenetic tree showing the genetic relationship among genotype I of Japanese encephalitis virus (JEV) isolates. The tree was constructed on the basis of the nucleic acid sequences of complete envelope (E) genes of JEV

strains. Sequences obtained in this study are indicated in **boldface**. Viruses were identified by using the nomenclature of virus/country/strain/source/year of isolation/GenBank accession number. Numbers in parentheses indicate the number of isolates that showed 100% nucleotide homology. Virus isolates with the same sequences were collected at the same time from the same location in this study. CH=Changhua County; HL=Hualien County; KH=Kaohsiung City; TC=Taichung City; TN=Tainan City; TP=New Taipei City; TPC=Taipei City; TY=Taoyuan County; YL=Yilan County; M=mosquito pool; P=pig serum; H=human sample. Analysis was performed by using MEGA 5 software and the maximum likelihood method based on the general time-reversible model.

Publication list for this work:

NIL

The study of genetic diversity and drug action mechanisms of *Entamoeba histolytica* in Taiwan

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Summary:

Amoebiasis still is a very important parasitic disease, which results in severe and invasive disease all over the world and causes about 100 thousand deaths each year. Study of the variations of *Entamoeba histolytica* strains can help to investigate the virulence factors and drug resistance from the genetic information. Two of *E. histolytica* clinical strains TCDC-1198 and TCDC-1446 had been isolated from two asymptomatic foreign labors belonged to different 6 tRNA-STR genotypes and had been used to investigate the drug tolerance and pathogenic factors. Only strain TCDC-1198 has *AIG1* gene, an important pathogenic factor of *E. histolytica*. The paromomycin tolerance of *E. histolytica* clinical strains and standard strain HM1-IMSS had been tested. The clinical strains were more tolerant than standard strain. The paromomycin might induce necrosis in *E. histolytica*, whereas metronidazole induced apoptosis. Study of the pathogenicity and drug tolerance of *E. histolytica* may help the development of anti-amebic drugs for future amebiasis treatment and control.

Purpose:

The aims of this project were to establish an applicable *Entamoeba histolytica* clinical isolation technique and investigate the drug resistance mechanism of paromomycin that is currently used for the treatment of *E. histolytica* in Taiwan, and analysis of the pathogenic and genetic differences of the highly virulent strains by phylogenic methods for the further disease prevention and control.

Method:

Entamoeba histolytica clinical isolation

Fecal sample collection

Fresh Stool samples of amoebiasis patient were collected to Taiwanese, foreign spouses, foreign labors and HIV patient from Taiwan local mental hospital and hospital.

Clinical sample preparation and storage

The clinical specimen processing modifies according to Nollau et al. (1996) protocol (1). Fresh stool samples take about 0.5g in 1% 6M guanidine thiocyanate vortex to mix that

be heated in 95°C at 30 min. Let the samples cool down in the room temperature, and then centrifuge at 13,000 rpm for 3 min. These samples would be extracted the DNA through Roche MagNA Pure LC system or stored in -20°C.

Monoxenic culture from clinical sample

These clinical isolates were cultured in monoxenic condition using yeast extract–iron–maltose–dihydroxyacetone-serum (YIMDHA-S) medium supplemented with *Crithidia fasciculata*. Brief, filter the suspension from fecal sample by BD filter (40 μm funnel) (put on the 50 ml tube) and then using sucrose gradient centrifugation. Then take out the central layer from sucrose gradient centrifugation, it includes entamoeba cysts. To activate entamoeba cysts, we add acid (1% HCl) in separated sediment for 30 min. The sediment is inoculated into fresh YIMDHA-S medium (9 ml) containing 15% adult bovine serum, polymyxin B sulfate (130 unit/ ml), penicillin G (100 units/ml), amphotericin B (25 ng/ml), streptomycin (10 U/ml) and *C. fasciculata*. The culture tube is inoculated at horizontal position for 1 day at 37°C. After overnight attachment, we changed the culture medium from YIMDHA-S to LYI-S-2. Observe the growth of amoeba and monitor contamination. If culture medium be derby, on ice five minutes, then wash again and fill new medium. If there is a great quantity of cells, put the tube on ice five minute and transfer 1 ml to 4 ml into the tube with fresh complete LYI-S-2 medium.

Polymerase chain reaction (PCR) and DNA sequencing

The polymerase chain reaction were used 5 μl template DNA in 25μl mixture containing 0.3 μM primer mix, 1X Pfx Amplification buffer, 1.0 μM MgSO4, 0.3 mM dNTP mixture and 1.25 U Pfx DNA polymerase (Platinum® Pfx DNA polymerase). The PCR conditions were followed by 35 cycles of 94°C for 15 s, 60°Cfor 15 s, 68°C for 2min. The STR fragments were amplified using 6 *E. histolytica*-specific tRNA-linked STR primers (DA-H, AL-H, NK2-H, RR-H, SQ-H, and STGAD-H) under the conditions previously described (2). The amplified PCR products were separated using 1.5 % agarose gel and purified using the QIAquick Gel Extraction Kit. And then, using cloning (TOPO TA Cloning® Kit) found the correct PCR products. Sequence analysis was performed by Genomics BioSci & Tech company (Taiwan). Nucleotide sequences were analyzed using the BioNumerics v6.5 software (Applied Meths, Belgium) to identify.

Parasite and growth conditions

E. histolytica (strain HM1:IMSS) was cultured axenically in the LYI-S-2 medium. Metronidazole-treated, Paromomycin-treated trophozoites were added and incubated with Metronidazole or Paromomycin (40 μM or 80 μM) for different periods of time, as

indicated.

Trypan blue cell counting

The cell suspension was prepared by taken the culture tube on ice for 10 min and then mixed by up and down the culture tube gently. One ml of suspension was aliquot into a 1.5 ml eppendorf and vortex. Ten μl of cell suspension was mixed with 10 μl trypan blue and putted into the cell counter. The total cell number included pale blue and complete cells, which might be dying.

Result:

1. **Genetic typing and characteristic analysis of *Entamoeba histolytica* clinical strain**

To have better study of the virulence factors and drug tolerance of *Entamoeba histolytica* strains, an applicable isolation protocol of clinical strains should be setted up. Two clinical strains of *E. histolytica*, called TCDC-1198 and TCDC-1446, had been isolated and monoxenic cultured with with *Crithidia fasciculata* successfully (fig. 1A). The *AIG1* gene had been indicated to be an important pathogenic factor for *E. histolytica*. According to the report of Gilchrist and Biller et al (3,4), we found that only strain TCDC-1198 has the *AIG1* gene (fig. 1B). The two clinical strains were isolated from two asymptomatic foreign labors belonged to two different 6 tRNA-STR genotypes (fig. 2). In the same culture condition, the strain TCDC-1446 grew slower than TCDC-1198. But strain TCDC-1446 had better adhesion on the surface of culture tube than TCDC-1198. After 20 μM paromomycin treatment for 24 hours, the cell morphology of strain TCDC-1198 and TCDC-1446 would continuously kept the normal trophozoite form, whereas the standard strain HM1 would become necrosis. The result indicated that TCDC-1198 and TCDC-1446 might be more tolerant to paromomycin than HM1 (fig. 3).

2. **Analysis of paromomycin susceptibility of *E. histolytica***

To analyze the paromomycin susceptibility of *E. histolytica*, the seven different doses of paromomycin treatment were selected (fig. 4). After 20 μM paromomycin treatment for 16 hours, we found that paromomycin could inhibit the growth of strain HM1. The 20 μM paromomycin alone could inhibit 40% growth of HM1 strain. With increase of the dose, we could see the dose-dependent phenomenon.

3. **Time series analysis of paromomycin cytotoxicity of clinical and standard strain**

To do the time series analysis of drug cytotoxicity, we chose the 80 μM paromomycin as a curve model. When increased the time of treatment, the dead HM1 cells were increased as well (fig. 5). After the paromomycin treatment for 24 hours, HM1 cell number has only 27.8% left in compared to the control group. In the same time, the

morphology of amoebic parasites was significantly changed, it became round-up and lysis in the culture medium (fig. 6). On the other hand, both clinical strains TCDC-1198 and TCDC-1446 showed better survival rate than HM1, and indicated their higher tolerance to paromomycin than HM1 (fig. 7).

Discussion:

Establishment of isolation and culture technique of *E. histolytica* from clinical patient samples could provide a good tool to conduct researches on strain virulence and drug resistance, and the different gene expressions of various strains could be established transcriptomic and proteomic databases. Two clinical strains TCDC-1198 and TCDC-1446 had been isolated and cultured from two asymptomatic foreign labors, and the analysis of *AIG1* gene showed that the TCDC-1198 wais *AIG1* gene PCR (+) and the TCDC-1446 was PCR (-) (fig. 1B). The *AIG1* genes had been indicated to be an important virulence factor (3,4). Therefore, strains TCDC-1198 and TCDC-1446 can be used in vivo animal experiment that test their *AIG1* gene effect in the mouse model. The low dose of paromomycin and long-term culture condition had been used to understand the drug tolerance between HM1 strain and clinical isolates (fig. 3). These results showed that two clinical strains in compared with standard strain had higher drug tolerance (fig. 3, fig. 7). In long-term culture with paromomycin or metronidazole, clinical strains would increase drug tolerance, which had the development potential of drug resistance. Nevertheless the strain HM1 was still very sensitive that could not survive in long-term drug treated condition. Becourse the strain HM1 had been cultured in vitro for more than 30 years without any anti-amebic drug treatment, the HM1of course are still sensitive. The clinical strains are continuously contact anti-amebic drugs and may have been increased their drug tolelance. However, some drug-resistant related genes of the HM1 could be discarded during this period that might also resulted in the clinical strains are more tolerant. The possible reasons have to be clarified by more studies. We would continiue the analysis of transcriptome and proteome to identify possible drug-resistant mechanisms. In Taiwan, metronidazole is currently the first-line treatment of amoebiasis, which had been listed as the U.S. National Institutes of Health in carcinogens list (5). This announcement reminded us that the drug selection and usage of amoebiasis treatment must be cautious in the near future.

Acknowledgment:

We would like to thank Dr. Kumiko Tsukui to providing us the *Crithidia fasciculata* for monoxenic culture and Dr. Tomoyoshi Nozaki for his kindly sugguestions to this study.

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Publication list for this work:

NIL

Fig. 1 Morphology of *Entamoeba histolytica* clinical strains and analysis of *AIG1* gene

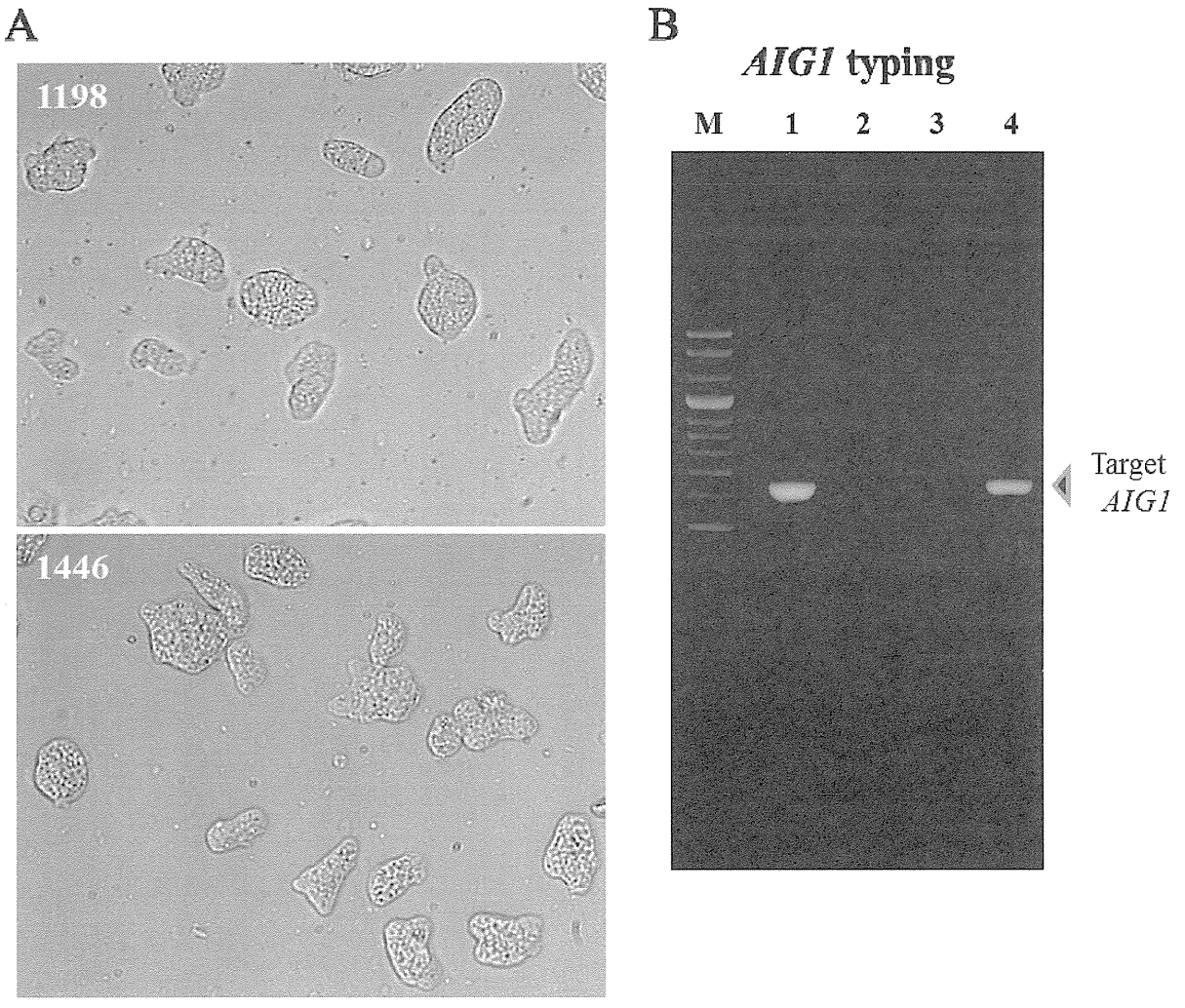


Fig. 1A:

Morphology of *Entamoeba histolytica* clinical strains, TCDC-1198 and TCDC-1446, both co-cultured with *Crithidia fasciculata*.

Fig. 1B:

Line 1: HM1 standard strain (positive control); line 2: Negative control; line 3: TCDC-1446 clinical strain; line 4: TCDC-1198 clinical strain.

Fig. 2 Six tRNA-STR genotyping analysis of *Entamoeba histolytica* clinical strain

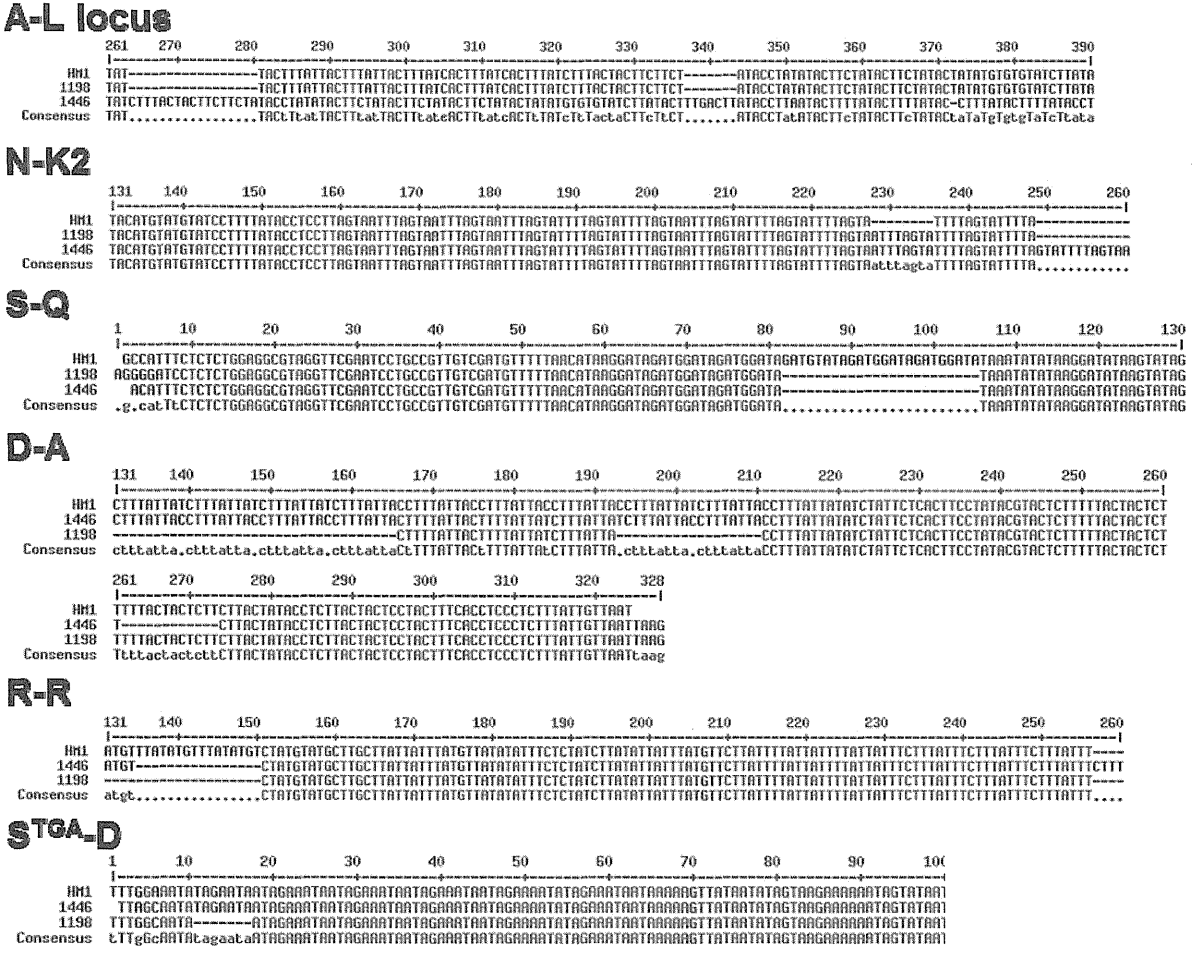


Fig. 2: These were part of tRNA-STR loci sequence data.

Fig. 3 Observation of the paromomycin treatment of the *E. histolytica* clinical and standard strains by microscopy

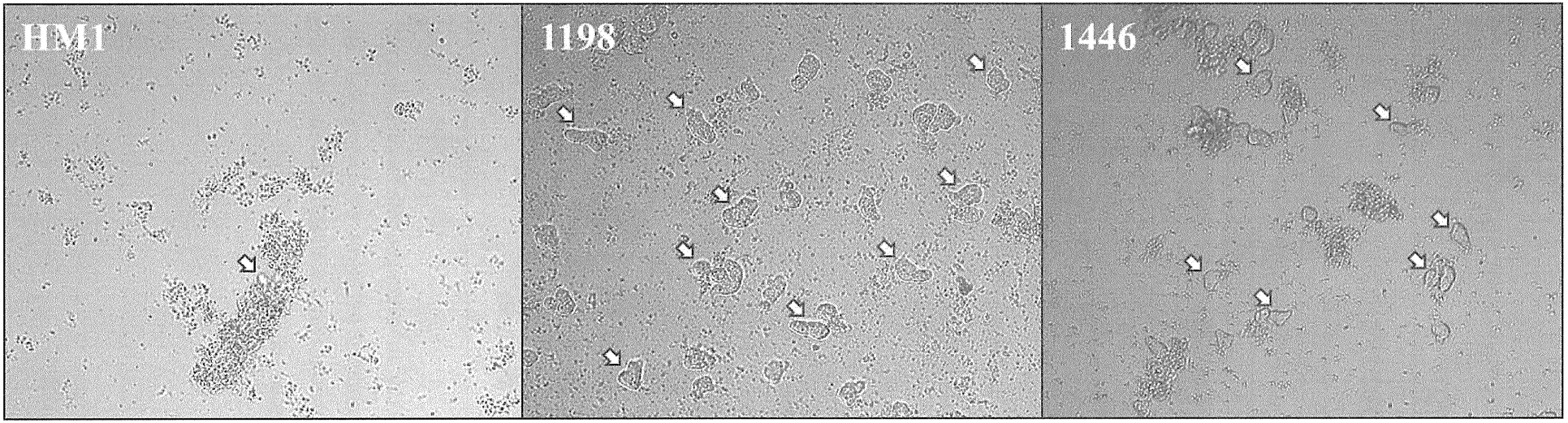
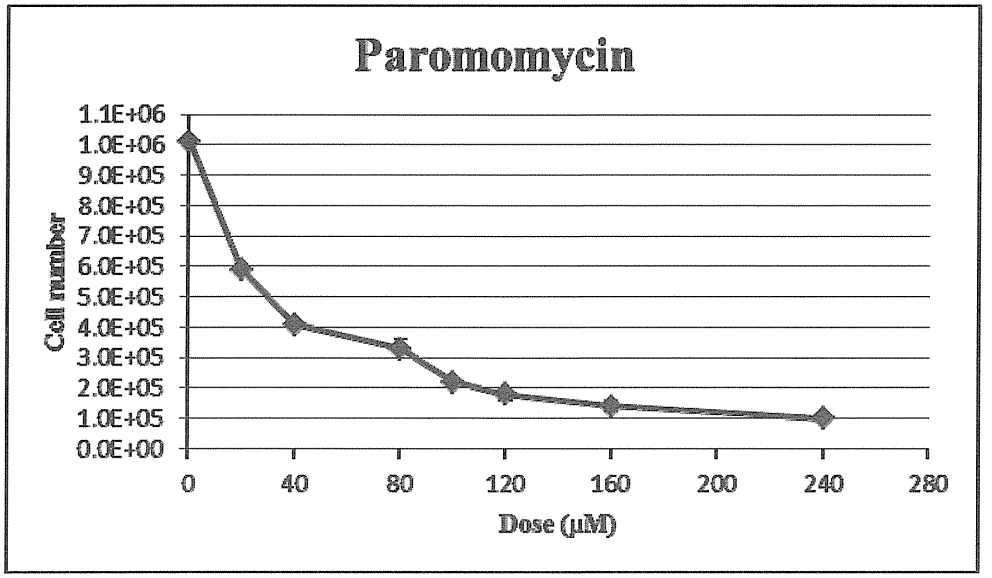


Fig. 3:

This experiment was cell seeding about $5x10^5$ for overnight, all cell co-culture with *Crithidia fasciculata*. After 20 μM paromomycin treatment for 24 hours, we could observe that the clinical strains still kept trophozoite form, which has clearly pseudopodia.

Fig. 4 Analysis of paromomycin susceptibility of *Entamoeba histolytica*



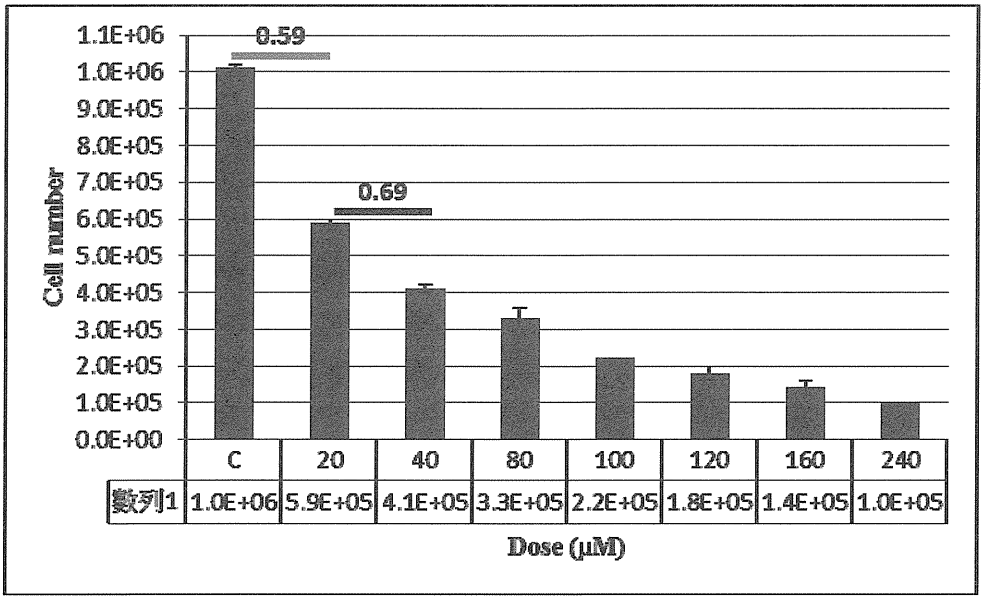


Fig. 4:

This experiment was cell seeding about $5x10^5$ for overnight with Nocodazole (100 nM), which was synchronization drug. Before paromomycin treatment, we changed from the LYI-S-2 medium with Nocodazole to fresh. After incubated with indicated concentration paromomycin for 16 hours, we calculated the total cell number.

Fig. 5 Time series analysis of paromomycin cytotoxicity of HM1 strain

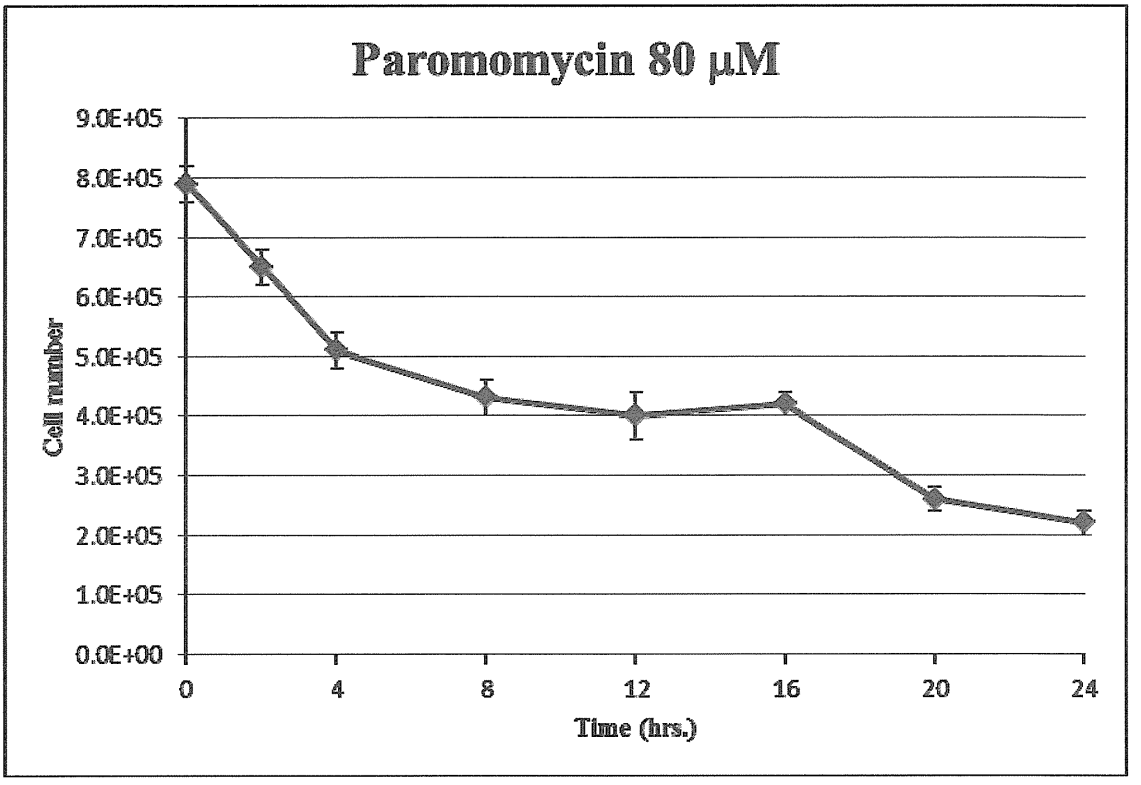


Fig. 5:

This experiment was cell seeding about $5x10^5$ for overnight with Nocodazole (100 nM), which was synchronization drug. Before paromomycin treatment, we changed from the LYI-S-2 medium with Nocodazole to fresh. After incubated with 80 μM paromomycin for indicated time point, we calculated the total cell number.

Fig. 6 Observation of the paromomycin treatment of the HM1 by microscopy

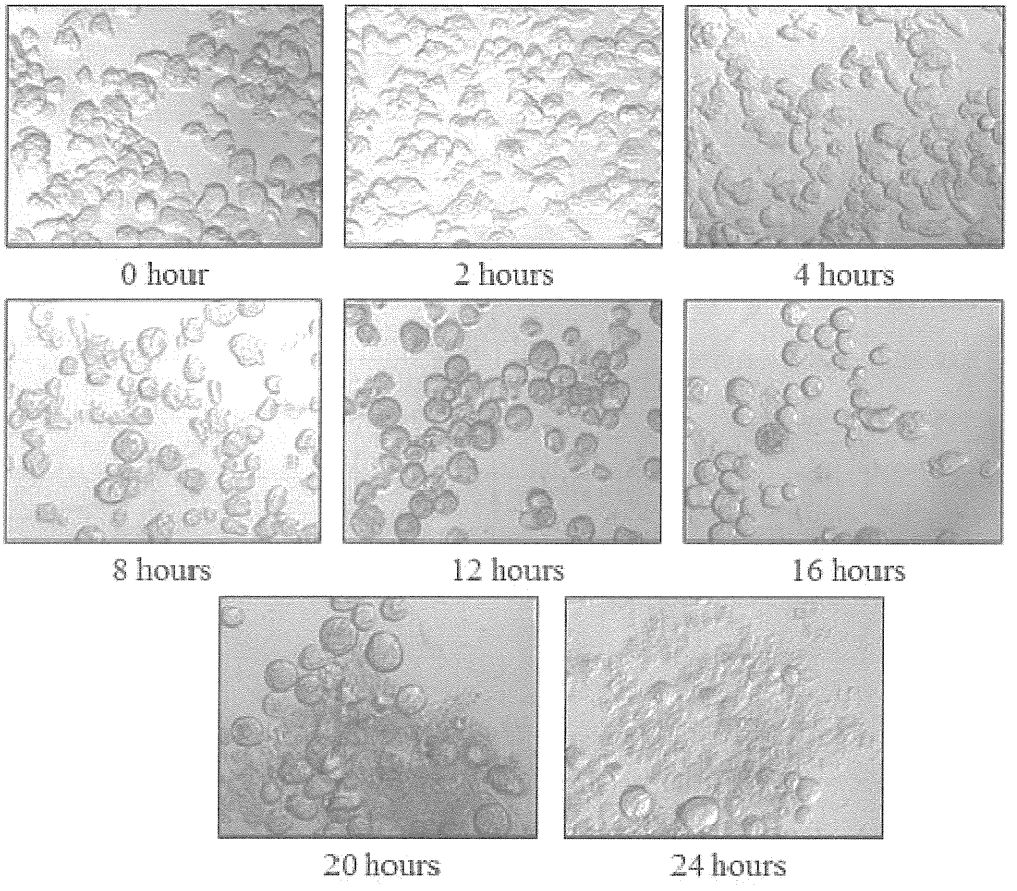


Fig. 6:

This experiment was cell seeding about $5x10^5$ for overnight with Nocodazole (100 nM), which was synchronization drug. Before paromomycin treatment, we changed from the LYI-S-2 medium with Nocodazole to fresh. After incubated with 80 μM paromomycin for indicated time point, we took the photo respectively.

Fig. 7 Time series analysis of paromomycin cytotoxicity of HM1 and clinical strains

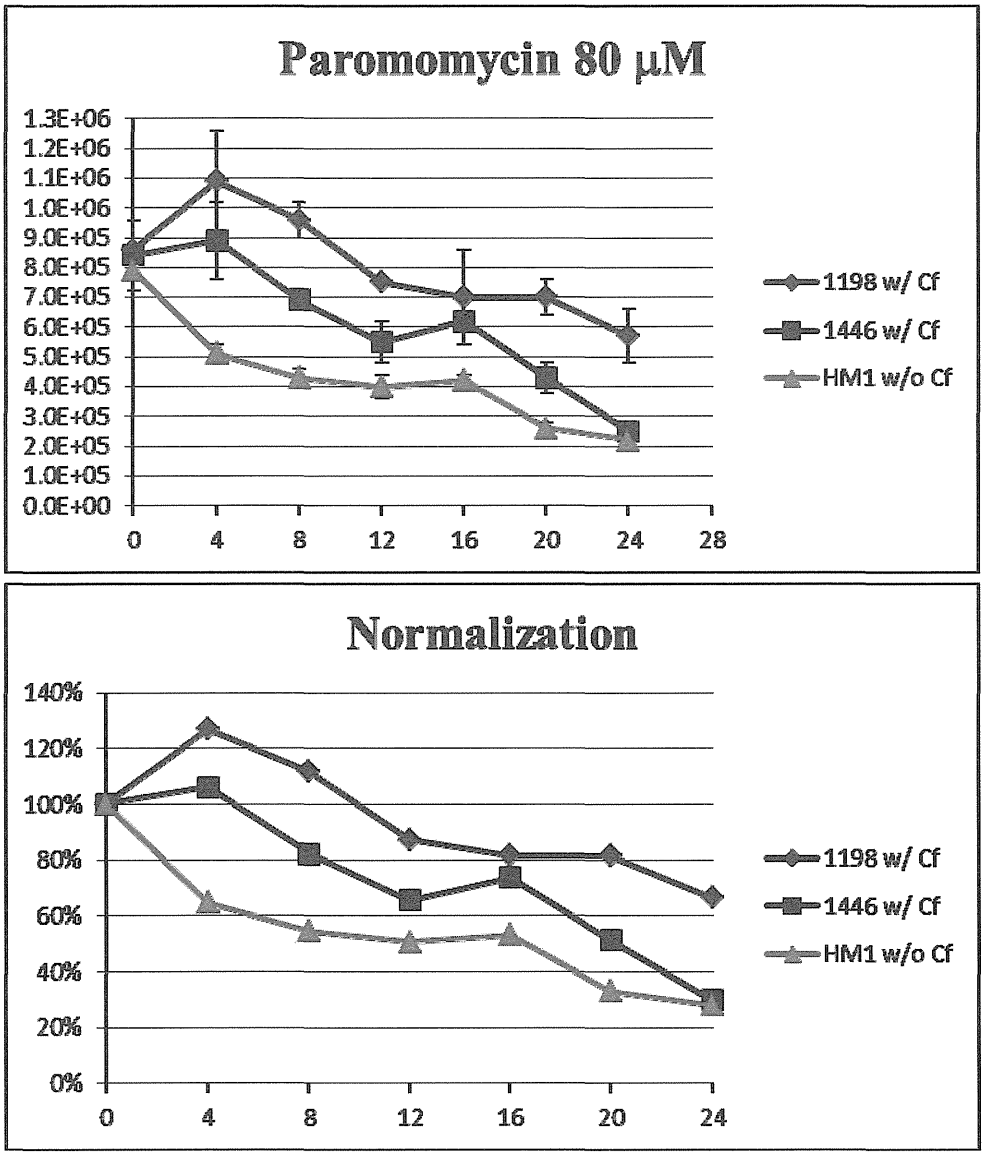


Fig. 7:

This experiment was cell seeding about $5x10^5$ for overnight with Nocodazole (100 nM), which was synchronization drug. Before paromomycin treatment, we changed from the LYI-S-2 medium with Nocodazole to fresh. After incubated with 80 μM paromomycin for indicated time point, we calculated the total cell number. The clinical strains co-culture with *Crithidia fasciculata*, but HM1 strain was not. Normalization was each strain compared with control group of himself (below).

Drug-resistance mechanism, pathogenesis and genomics of tuberculosis: Molecular characteristics and associations of rifampicin resistance to other antituberculosis drugs in *Mycobacterium tuberculosis*

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Summary

Rifampin (RIF) is bactericidal, and acts on both intracellular and extracellular organisms. RIF is a potent drug for tuberculosis (TB) treatment and is a marker for multidrug-resistant (MDR) TB. However, RIF has limitation in treating MDR-TB and cases with side-effects and co-infected with HIV. To understand genetic alterations of the *rpo*B gene conferring RIF resistance, genetic differences to rifabutin (RBT), and the association with other anti-tuberculosis drugs, we conducted a population-based analysis of 800 MDR *Mycobacterium tuberculosis* isolates. Of the 800 isolates, predominant *rpoB* mutations conferred RIF resistance were S531L (61.8%), H526Y (6.7%) and H526D (4.4%). We found that the cross-resistant rate between RIF and RBT was 87.0%. Among isolates with single mutation in the *rpoB* gene, mutations at codon 146, 513 and 531 were only observed in RBT-resistant isolates, whereas, mutations at codons 143, 511, 516, 522 and 529 were only in RBT-susceptible ones. Interestingly, isolates with amino acid substitutions at codon 526 (H to C, L, T, N) and codon 529 (R to L) were susceptible to RBT. We further analyzed 568 isolates with results of the first and second-line drug susceptibility testing (DST), and found codon 531 mutation of the *rpoB* gene was significantly associated with ofloxacin and ethionamide resistance, and negatively associated with kanamycin resistance ($p<0.05$). Whereas, codons 513 and 533 mutations were not found in isolates resistant to

amikacin or capreomycin. Consequently, specific mutations of the *rpoB* gene can be used to determine RBT susceptibility and to predict drug resistance to other anti-TB drugs.

Purpose

The objective of this study is to understand genetic alterations of the *rpo*B gene conferring RIF resistance, genetic differences to RBT, and the association with other anti-TB drugs, we conducted a population-based analysis of 800 MDR *Mycobacterium tuberculosis* isolates.

Materials and Methods

Study populations. *M. tuberculosis* isolates identified as MDR based on bacteriological (culture in Löwenstein-Jensen or MGIT® medium), biochemical and molecular identification were collected from each of the 803 cases in certified clinical Mycobacteriology laboratories throughout Taiwan from January 2006 to December 2010. We excluded one confirmed mixed culture and two isolates without RBT DST results. The spare 800 isolates were used to evaluate the cross-resistant rate between RIF and RBT. In addition, we also evaluated the relationships between specific *rpo*B mutations and the percentage of resistance to the first and second-line anti-TB drugs from 568 isolates with complete DST data.

Drug susceptibility testing. The agar proportion method on either Middlebrook 7H10 or 7H11 (Creative Microbiologicals or Sancordon, Taiwan), and BACTEC™ MGIT™ 960 SIRE Kits (Becton Dickinson Diagnostic Systems, Sparks, MD) with a

liquid culture system were used. The critical first-line drug concentrations for the agar proportion method on 7H10 were 0.2 μg/ml and 1.0 μg/ml for INH, 1.0 μg/ml for RIF, 5.0 μg/ml and 10μg/ml for ethambutol (EMB), 2.0 μg/ml and 10μg/ml for streptomycin (STR). Isolates resistant to at least INH and RIF were considered MDR and were subjected to the second-line drug DST. The critical concentrations of second-line drugs for the agar proportion method on 7H11 were 2 μg/ml for ofloxacin (OFX), 6 μg/ml for AMK, 6 μg/ml for KAN, 10 μg/ml for CM, 8μg/ml for p-aminosalicylic acid (PAS), 10μg/ml for ethionamide (ETH), and 0.5μg/ml for RFB. Growth on the control medium was compared to growth on the drug-containing medium to determine susceptibility. The DST results were categorized as resistant or susceptible. The tests were validated by comparison to the susceptibility of *M. tuberculosis* H37Rv included in the same DST. MDR was defined as *M. tuberculosis* isolates resistant to at least INH and RIF.

DNA sequencing of the *rpo*B gene. Two primer sets were used to analyze the variation at the *rpo*B gene. A 541-bp fragment which contains the 81-bp hotspot region was amplified and sequenced with the oligonucleotide primers *rpoB*-F (5′-TCGGCGAGCCCATCACGTCG-3′) and *rpoB*-R (5′-GCGTACACCGACAGCGAGCC-3′) [1]. A 365-bp fragment targeting the V146F (V176F according to the *M. tuberculosis* numbering system) mutation was amplified and sequenced with the oligonucleotide primers TB-176-F (5′-CTTCTCCGGGTCGATGTCGTTG-3′) and TB-176-R (5′-CGCGCTTGTCGACGTCAAACTC-3′) [2]. The PCR reactions were performed as follows: 35 cycles at 96°C for 1 min; annealing at 64°C for 1 min; and elongation at 72°C for 1 min. Thereafter, the PCR products were analyzed with an ABI 3730 automated sequencer (Applied Biosystems, USA), and the sequence data were

assembled and edited using the Sequencing Analysis 5.2.0 software (Applied Biosystems, USA). In this study, codons were numbered according to the *E. coli* numbering system.

Statistical analysis. The percentage of resistant to various anti-TB drugs between wild-type and mutated groups at specific location of the *rpo*B gene was compared using binomial test. *P*-value < 0.05 was considered as statistically significant.

Results

***rpoB* gene sequence conferring RIF resistance.** Predominant *rpoB* mutations conferring RIF resistance of the 800 MDR *M. tuberculosis* isolates were at codons S531L (61.8%), H526Y (6.7%) and H526D (4.4%). The cross-resistant rate between RBT and RIF was 87.0% (696/800). Of the 800 isolates, 740 (92.5%) had single mutation, 40 (5%) had double mutations, 1 had triple mutations, 6 had deletion, and 13 (1.6%) showed wild-type. We revealed 13 novel *rpoB* gene variations conferring RIF resistance including 11 isolates with single mutation (R143C, V144A, Q148R, S164P, D444V, E458K, T480I, A501T, R529Q, R529L, M558K) and two deletions (509-511 or 510-512, and 9 bp discontinuous deletion at 513-516).

***rpoB* gene sequence conferring RIF but not RBT resistance.** Correlations between specific *rpoB* mutations confer RBT resistance among 740 MDR *M. tuberculosis* isolates with single mutation in the *rpoB* gene, and DST results using minimum inhibitory concentration (MIC) tests were summarized in Table 1. Of the 740 isolates, 68.5% harbored mutations in codon 531, 16.8% in codon 526, 5.9% in codon 516, 3.6% in codon 533 and 3.0% in codon 513. Nevertheless, 12.3% (91/740) of the

studied isolates with single mutation in the *rpoB* gene were RBT-susceptible. Interestingly, 11 single mutations (R143C, L511P, D516Y, D516V, D516F, S522L, H526C, H526L, H526T, H526N, R529L) were identified only in RBT-susceptible isolates (Table 1). In addition, for MDR isolates with more than one mutated codon, changes in their resistance to RBT could be found in isolates with multiple mutations at codon 511 (71.4%, 5/7) and 516 (84.6%, 11/13).

Drug susceptibility of MDR *Mycobacterium tuberculosis* isolates. Since PZA, PAS and ETH resistance were not tested in the beginning of our laboratory program, only 568 MDR *M. tuberculosis* isolates had complete results of the first and second-line DST were included in the analysis. The drug susceptibility patterns of the 568 clinical MDR isolates were summarized in Table 2. Of the 568 isolates, 52.8% were resistant to EMB, 43.3% to STR, 34.0% to PZA, 23.4% to OFX, 11.4% to PAS, 29.0% to ETH, 5.5% to AMK, 9.9% to KAN, and 4.9% to CM (Table 2). Overall 27.8% (158/568) were resistant to all four first-line drugs, 23.4% (133/568) were pre-XDR and 5.3% (30/568) were XDR. Moreover, DST profiles were highly diversified with 120 patterns identified. Of the 568 MDR isolates, 15 (2.6%) isolates were susceptible to all other tested drugs and 88 (15.5%) isolates had concurrent resistance to RBT. Major patterns were MDR isolates with concordant mono-resistant to RFB (88, 15.5%), concordant double-resistant to RBT and EMB (55, 9.7%), followed by concordant double-resistant to RBT and STR (37, 6.5%) (Table 2).

Correlations between *rpoB* mutations and drug resistance. Interestingly, we found associations between specific *rpoB* mutations and drug-resistant rates of various drugs. Isolates with a mutation at codon 516 had higher resistant rate to PZA ($p<0.05$), PAS, and KAN. Isolates with a mutation at codon 526 had higher resistant rate to STR