for 15s, 60℃for 15s, 68℃ for 2min. The amplified PCR products were separated using 1.5 % agarose gel and purified using 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 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.

TUNEL assay

Metronidazole-treated, Paromomycin-treated, or NT trophozoites were fixed in 4% paraformaldehyde for 45 min at 4°C. After washing twice with PBS, 50 μl TUNEL reaction mixture (Roche®) was added and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Trophozoites were washed two times with PBS, loaded on slides, and observed with an Olympus fluorescence microscope (3).

Flow-cytometry assays and microscopic analysis

Changes in size and in the light-scattering properties of trophozoites were determined by flow cytometry, using a BD® FACSCalibur equipped with CellQuest software (BD®). Metronidazole- treated, Paromomycin-treated, or NT trophozoites (1 x10^6) were stained by Annexin-V-Alexa 568 and analyzed using a 568 nm argon laser. For microscopic analysis, Drug-treated or NT trophozoites were washed twice with PBS, stained by Annexin-V-Alexa 568, and placed on glass slides. Trophozoites were observed using an Olympus fluorescence microscope (3).

III. Results:

1) Isolation of non-pathogenic and pathogenic strains from clinical specimens

Eleven strains were isolated from clinical samples, 4 liver abscess, 2 severe diarrhea and 5 asymptomatic cases, and the work of collection and culturing of clinical specimens is still ongoing. Three strains had been sequenced and compared the phylogenetic relationship to other genotypes (Table 1 and Figure 1) by using the minimum spanning tree (MST). The genotype 1291 was indicated prevalence in HIV/ MSM populations and Japan in 2009, and belonged to the Cluster B, whereas genotypes1249 and 1245 were located in Cluster A branches and were most closest to two strains from two Taiwanese mental retardation institutions (Figure 1). The remaining eight strains isolated from the Philippines and Vietnam foreign worker strains, and HIV-infected patients have not yet carried out the genotyping. Due to the isolation and culturing of amebic parasites was very

time-consuming and high failure rates, and lack of funds, the expression microarray and genome sequencing was therefore not yet processed.

2) Isolation of drug resistant strains from clinical specimens

One strain was currently isolated from a post-treated HIV patient, which was suspected of drug-resistant strain, and cultured by the *E. coli* mono-xenic culture.

3) Analysis of the action mechanism of anti-*E. histolytica* drugs

Metronidazole with Paromomycin were currently used for amoebiasis treatment in Taiwan. Therefore, Metronidazole and Paromomycin were used to treat the standard strain *E. histolytica* HM1: IMSS seperately in order to analyze the anti-amebic mechanism. After 80 μM Paromomycin treatment for 4 hrs, the morphology and mobility of *E. histolytica* had no change, but some parasites occurred necrosis after 10 hrs even the drug was washed off by the new medium, and parasites were almost died completely after 24 hrs, indicating that the action mechanism was irreversible. After 40 μM Metronidazole treatment for 4 hrs, the morphology of amoebic parasites was significantly changed, it became round-up and lost attachment floating in the culture medium, such phenomenon was similar to apoptosis, but some of the floated parasites re-grew in new medium that indicated a reversible reaction (Figure 2). According Nasirudeen et al in 2004 (4), Blastocystis hominis could be induced the phenomenon of programmed cell death (Apoptosis) by the Metronidazole treatment. Therefore, we think that the Metronidazole treatment also might be induced E. histolytica Apoptosis. By using TUNEL assay, the apoptosis of amebic parasite treated by 40 μM Metronidazole or 80 μM Paromomycin for 4 hours was observed (Figure 3). The some parasites showed fluorescence after Metronidazole treatment indicated the DNA fragmentation of apoptosis, whereas there was no fluorescence seem in Paromomycin treatment.

In addition to using the TUNEL assay, the fluorescent Annexin-V was used as a probe to observe another characterization of apoptosis, the inner membrane phospholipids Serine (phosphatidylserine, PS) flip to the outer membrane surface. Increasing the PS in the outer membrane surface indicates the early characterization of apoptosis. Observed using fluorescence microscopy, the PS was significantly increasing in the outer membrane surface was observed after the Metronidazole treatment for 4 to 6 hours (Figure 4). Using flow cytometry analysis, about 47% of the cell surface of cells showing PS after the Metronidazole 8 hours of treatment (Figure 5). However, there were no significant changes after Paromomycin treatment for 8 hour (Figure 4, Figure 5).

4) Establishment of the genomic database of *E. histolytica* clinical strains

The total proteins of parasites stimulated different drug treatment had been collected to be analysed by the two-dimensional protein electrophoresis analysis (Figure 6), and the follow-

up will be carried out protein sequencing and repeatedly verify protein expression in order to establish a database for further analysis of the pathogenesis and drug action.

IV. Discussion:

Isolation of E. histolytica strains from clinical specimens could preserve the unique clinical strains for the establishment of a gene expression database of the virulence and drug tolerance strains, respectively. Therefore, 11 strains with different clinical characterization had been isolated and cultured from liver abscess, severe diarrhea and asymptomatic patients, and used to understand genetic differences. Eight strains were respectively isolated from Indonesia, the Philippines and Vietnam patients that could be used to investigate the geographic transmission and the pathogenesis of the different races. Analysis of specific gene expression of *E. histolytica* strains from liver abscess or severe diarrhea may assist in the prevention and treatment. Therefore, we hoped to massively produce amoeba cells for the studies of proteomics and transcriptome by using the 2D protein electrophoresis and protein sequencing and micriarray. Nevertheless, in the actual implementation, we found that the clinical strains can not be effective proliferation and amplified after isolation. Those experiments could not be achieved. The problem of effective amplification of ameba cells shall be overcome in the future.

About the drug treatment and tolerance, several patients have been found to remain positive after treatment in Taiwan. Therefore, it is necessary to further reveal whether the drug resistant strains have been produced. Currently the metronidazole is still the most important drugs for the amebiasis treatment in the world. If its resistance occurs, many countries will face with the dilemma of no drugs available. Clinically isolated strains of drug-resistance will help to understand the mechanisms of the drug resistance. We had found that the action mechanisms of metronidazole and paromomycin might differ (Figure 2 - 5). According Nasirudeen et al. Reported in the literature in 2004 (4), Metronidazole can induce *Blastocystis hominis* to produce apoptosis. We were also using the TUNEL assay and Annexin-V fluorescence probe for apoptosis experiments in *E. histolytica* HM1:IMSS and observed the results by fluorescence microscopy and flow cytometry. After the metronidazole treatment for 8 hour, the characters of programmed cell death could be found, including DNA fragmentation and phospholipidylserine increasing in the outer cell membrane surface. It can be speculated that an apoptosis signal was triggered by metronidazole treatment and promote amoeba into cell death. During the treatment, metronidazole may release free radical in amoeba cell and then induce the apoptotic signal (5, 6). But we also found that the programming death could be reversed if remove metronidazole before a checkpoint. The parasites grew well as normal parasites in standard culture. After the checkpoint, some irreversible damages might happen in the parasite. Parasite cells would eventually die (data not shown).

On the other hand, we found no apoptosis was observed by TUNEL and Annexin-V assay after paromomycin treatment for 8 hours in comparison to the control group (Figure 2 - Figure 5). The parasites were still shown necrosis phenomenon 20 hours later (Figure 7) and the phenomenon was irreversible even the paromomycin was removed. Therefore, we believe that the action mechanisms of metronidazole and paromomycin for E. histolytica HM1: IMSS may differ. When patient treat by metronidazole, the therapy shall complete and be confirmed by proper diagnosis to make sure there is no parasite shown in the clinical samples. If the amoeba parasites revered after metronidazole treatment, the parasites may gain the ability to resist the metronidazole and induce the drug resistance. Understanding the action mechanisms of those drugs may help us to develop new drugs and to know how to proper use those drugs.

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VI. Publication list for this work:

NIL

Tables

Table 1. Genotypes of clinical strains of *Entamoeba histolytica*

isolation	symptom		SD	SQ	DA	AL	RR	NK
1245	diarrhea	local	T1	4	6	10	5	T11
1249	asymptomatic	Indonesia	12	4	6	10	5	T15
1291	ALA	local	15	4	6	8	7	J4

Figure 1. Phylogenetic tree of the *E. histolytica* strains

MST of *E. histolytica* strains according the 6 tRNA-linked STRs loci

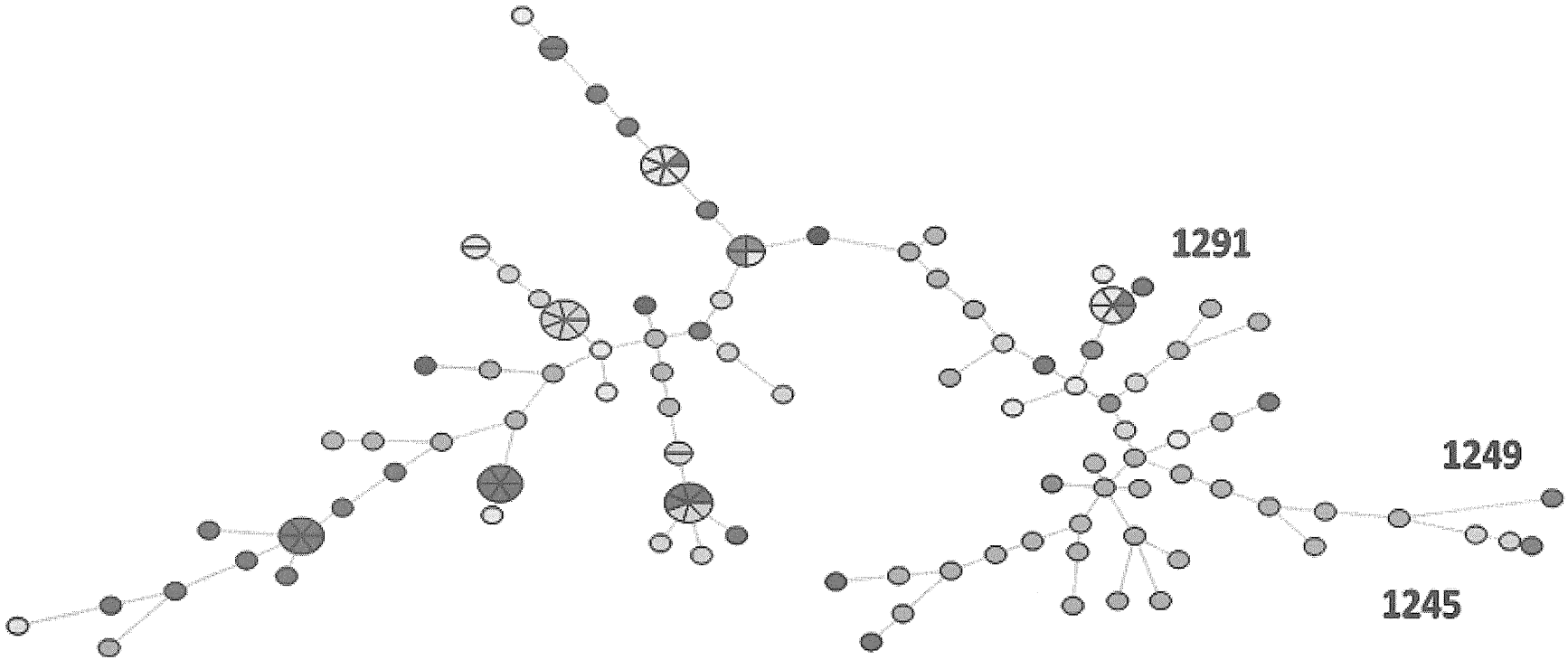
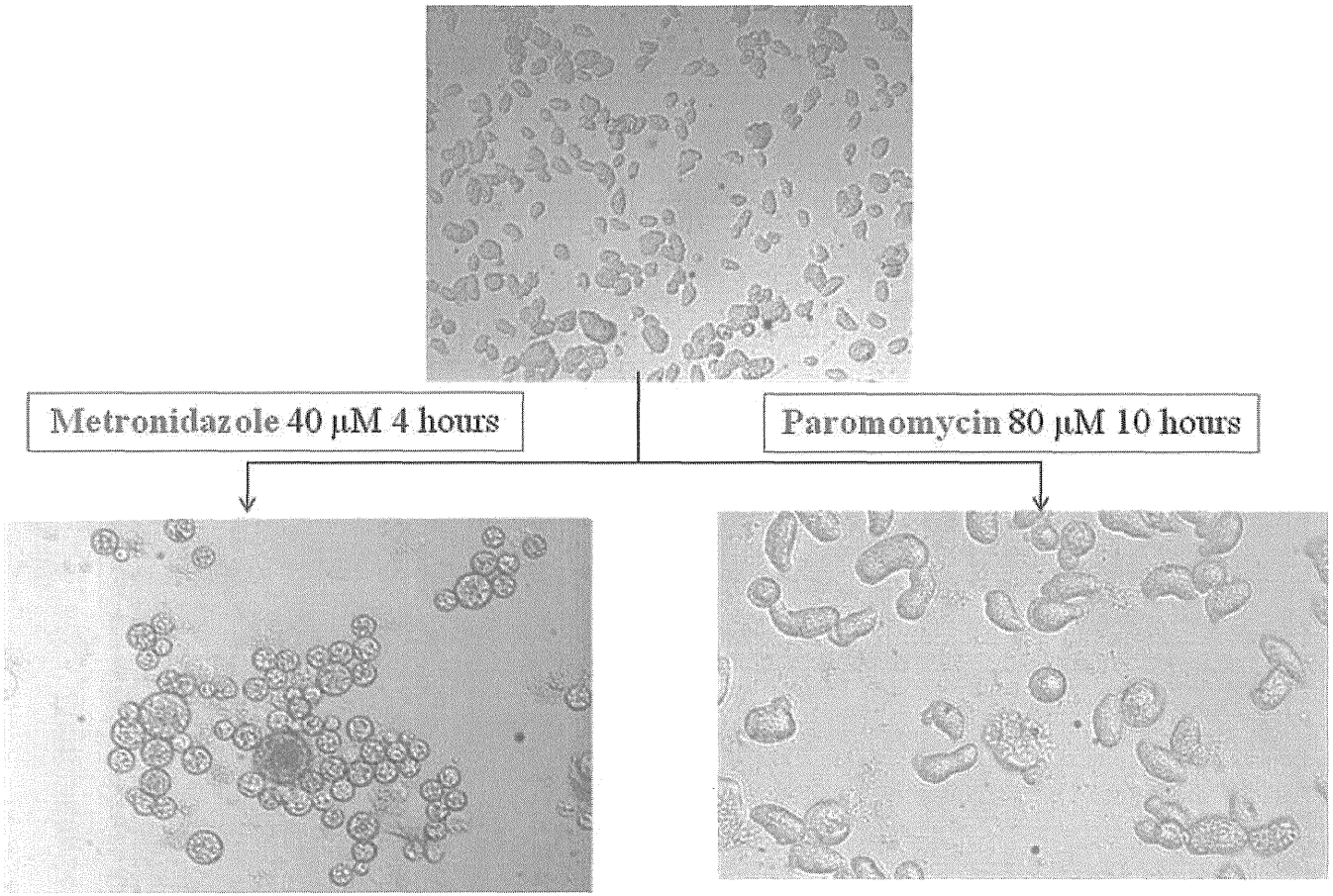
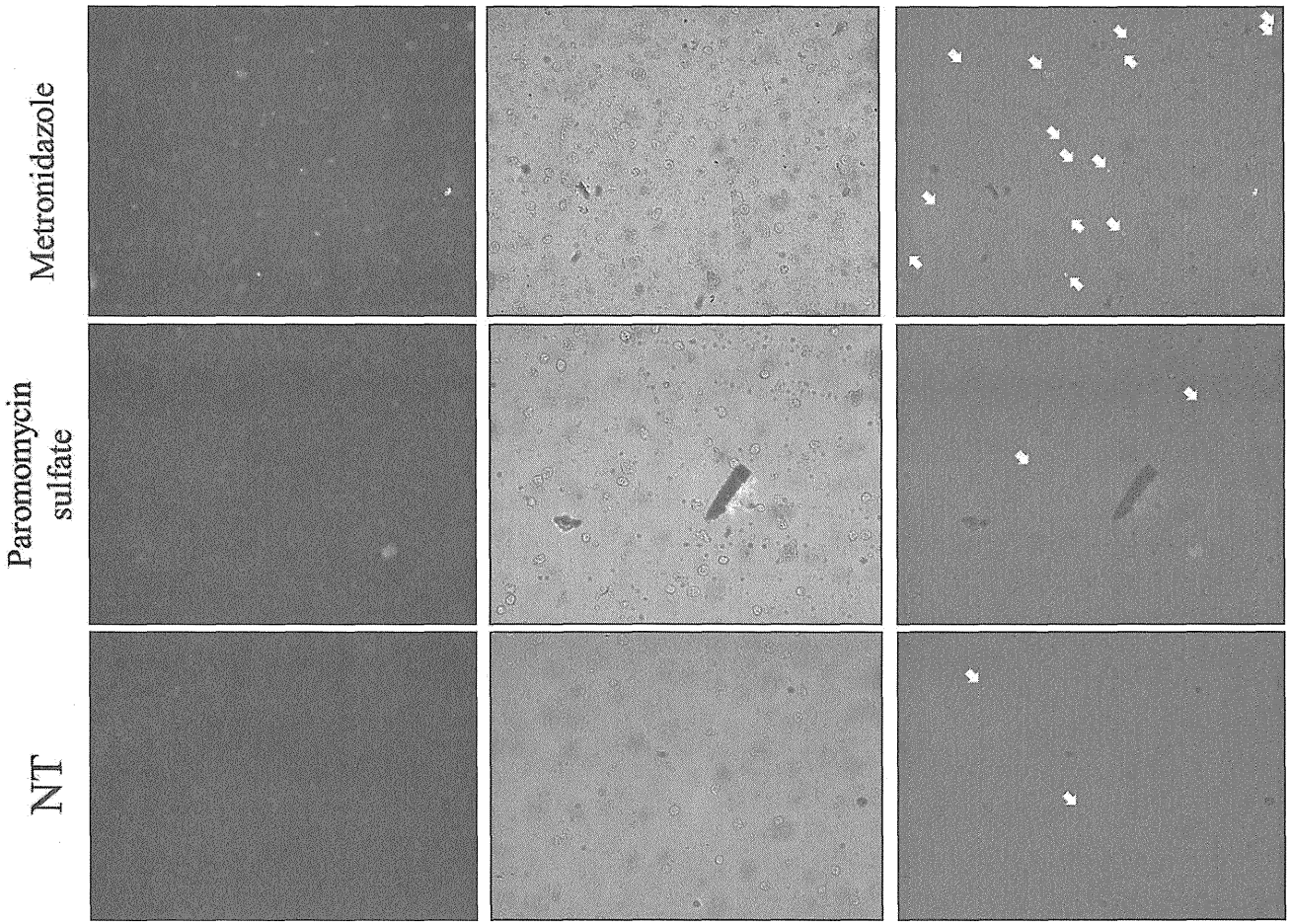


Figure 2. Observation of the drug treatment of the *E. histolytica* HM1:IMSS by microscopy



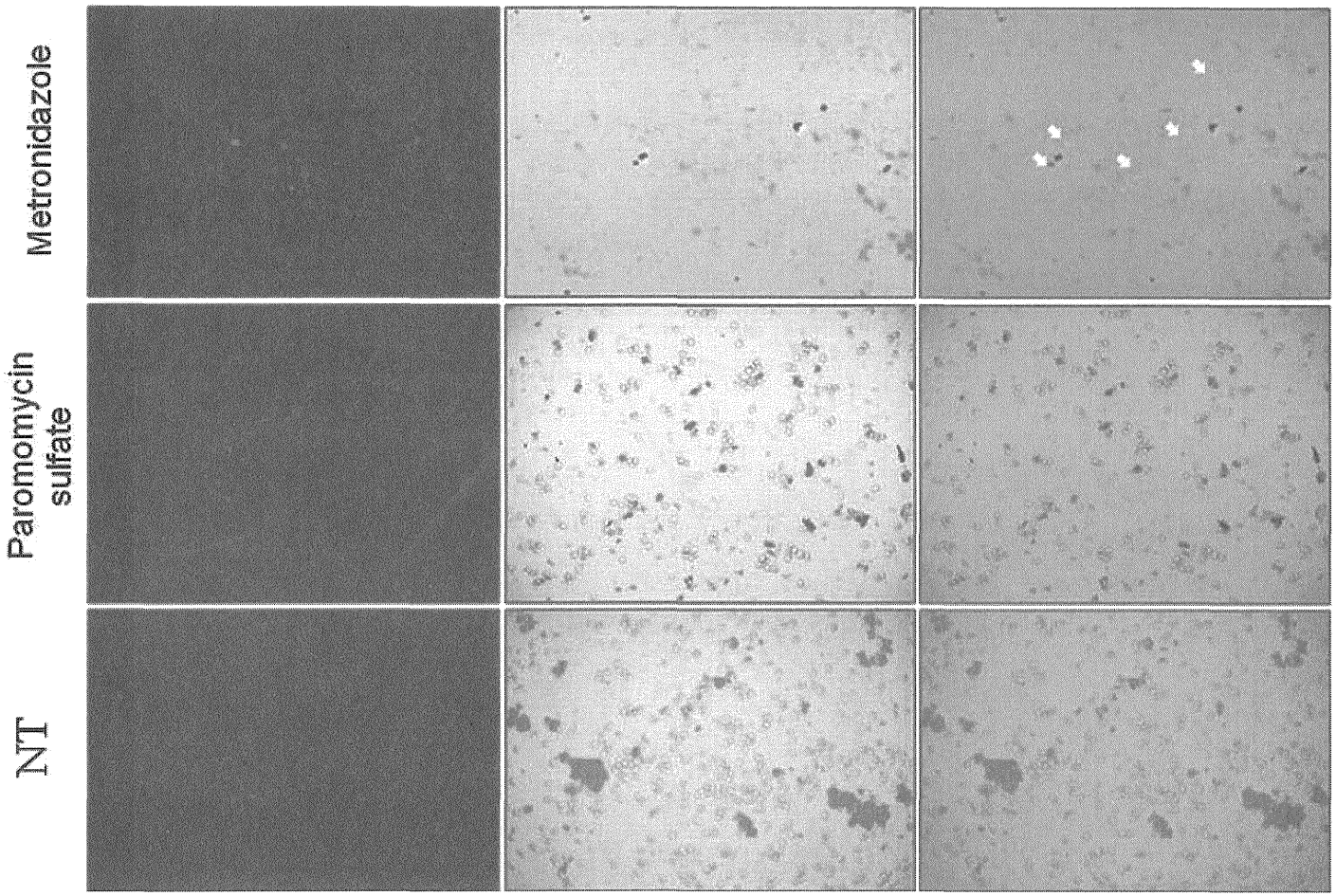
以 Metronidazole 與 Paromomycin 分別對標準蟲株 *E. histolytica* HM1:IMSS 引發蟲體死亡研究其機制。 Paromomycin 引發蟲體死亡的機制與表徵與 Metronidazole 不同。

Figure 3. Observation of the apoptosis of the *E. histolytica* HM1:IMSS by TUNEL assay



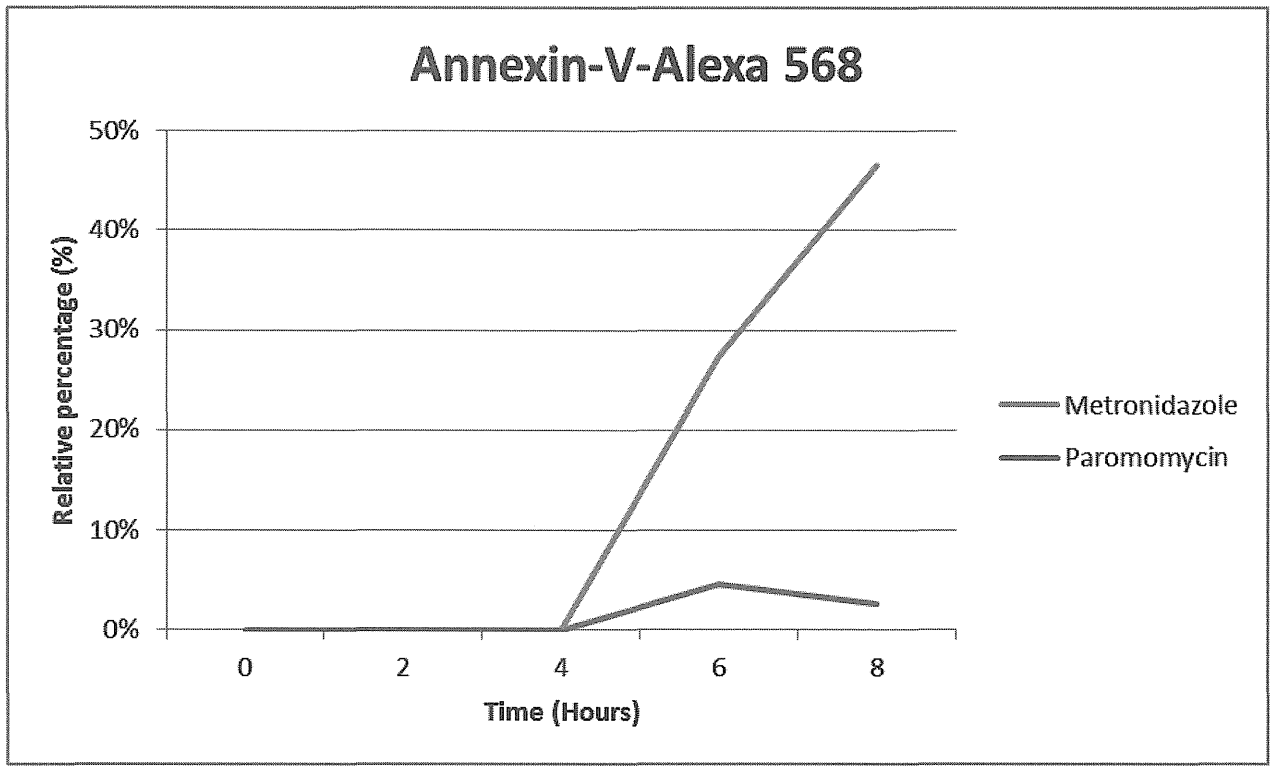
利用 TUNEL 法檢測，由 Metronidazole 與 Paromomycin 處理四小時的標準蟲株產生細胞程式死亡之現象。

Figure 4. Observation of the apoptosis of the *E. histolytica* HM1:IMSS by Annexin-V probe



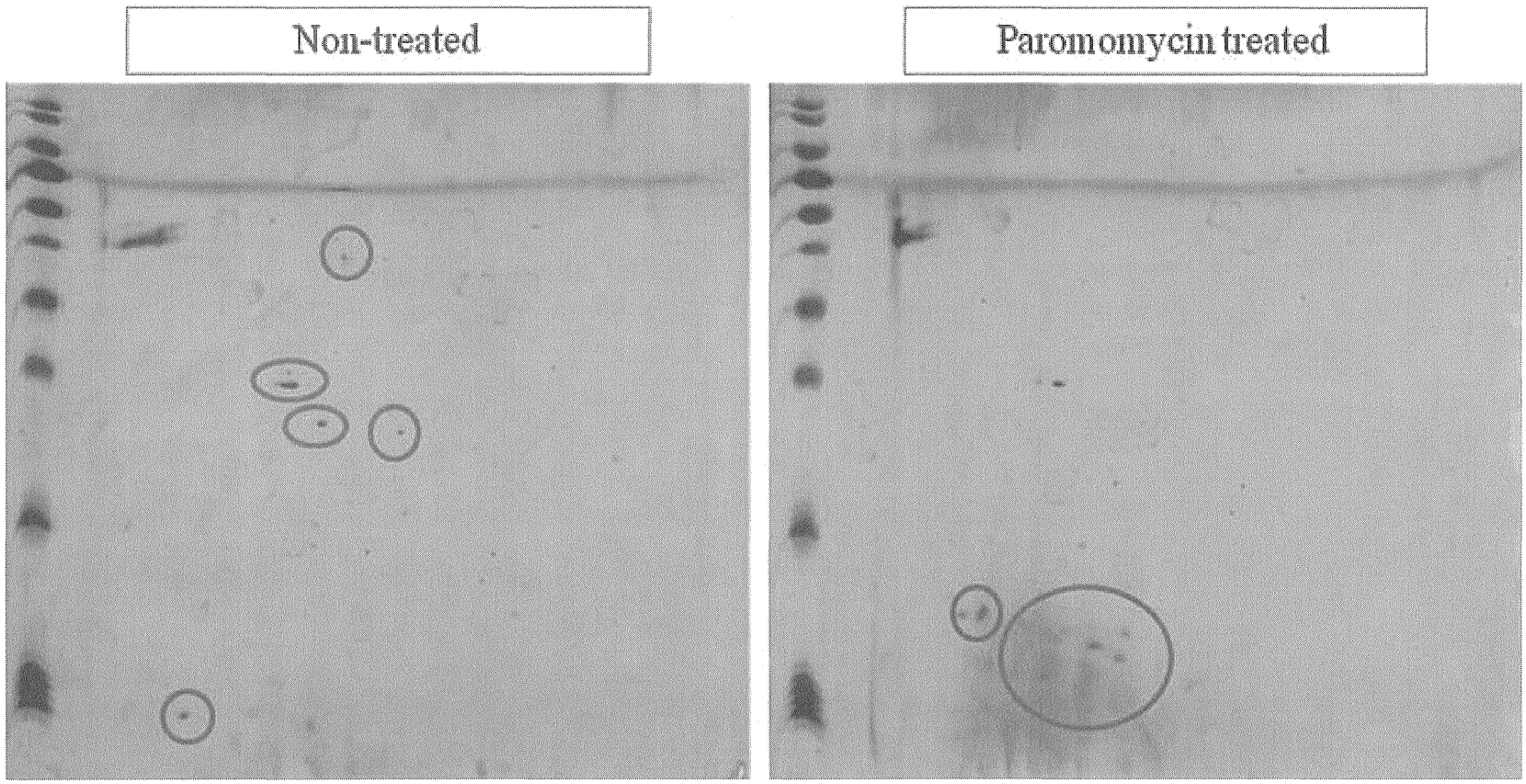
利用已標幟螢光的 Annexin-V 探針，去觀察細胞表面的磷脂質絲胺酸 (phosphatidylserine, PS)表現。

Figure 5. Quantitative analysis of the Annexin-V probed *E. histolytica* cells by Flow-cytometry



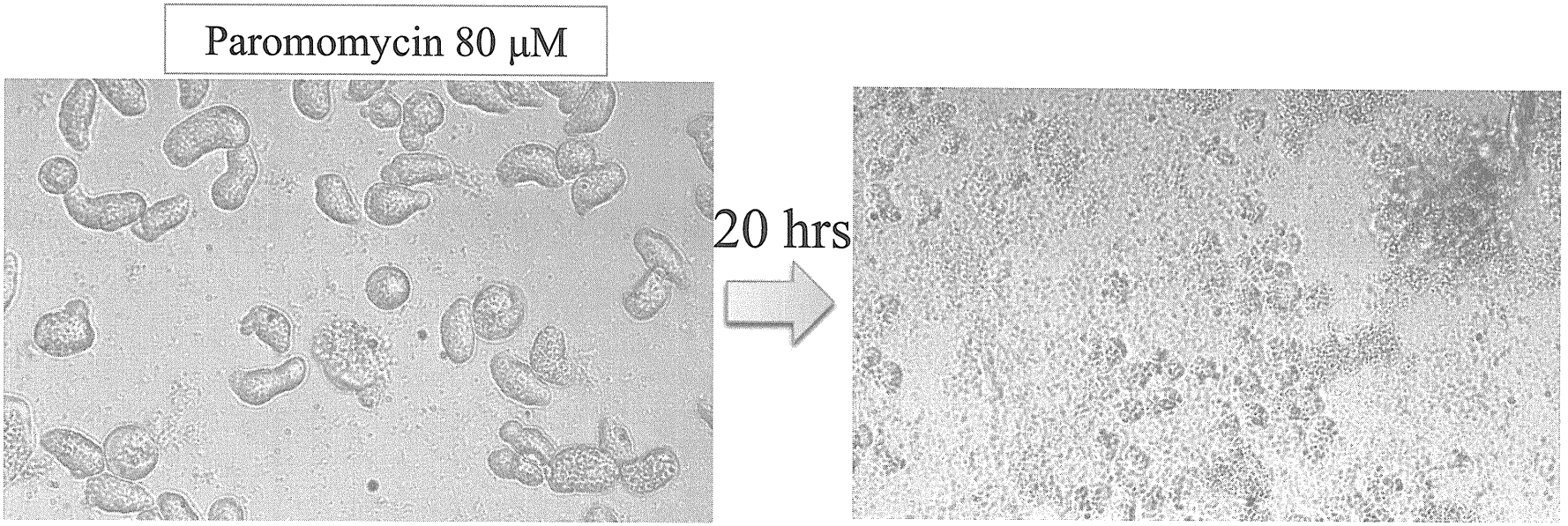
以流式細胞儀透過已標幟螢光的 Annexin-V 探針，量化分析細胞表面的磷脂質絲胺酸 (phosphatidylserine, PS)。
在標準蟲株藉由 Metronidazole 處裡的八小時內，大約 47%的細胞之細胞表面呈現出磷脂質絲胺酸；Paromomycin 處理八小時以內的標準蟲株，其細胞表面之磷脂質絲胺酸皆無明顯的變化。

Figure 6. Comparison of the protein variation after drug treatment by the two-dimensional protein



收集經由 Paromomycin 藥物對蟲體刺激所反應之蛋白質，進行二維蛋白質電泳分析

Figure 7. The morphological change of *E. histolytica* cells after Paromomycin treatment for 20 hours



當 Paromomycin 處理時間為二十小時後，則可以發現蟲株細胞呈現出類似細胞壞死 (Necrosis)的現象。

Drug-resistance mechanism, pathogenesis and genomics of tuberculosis: Gene mutations in isoniazid-resistant *Mycobacterium tuberculosis*

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Summary

One of the effective strategies to decrease incidence of tuberculosis (TB) is to find out patients as soon as possible. Therefore, the patients can be treated appropriately and avoid to transmit to other people. Regarding multi-drug resistant (MDR) TB patients, they need longer period of treatment and easily acquire extra drug resistance if they do not get correct regimen. Base on our preliminary study, of the 242 MDR *Mycobacterium tuberculosis* isolates, only 78.5% can be rapidly identified using the GenoType MTBDR*plus* commercial kit. Additional 13.5% of the MDR isolates can be detected by sequencing drug resistance associated genes, including the *rpoB* core-region, *katG*, *inhA*, *inhAr*, and *oxyR-ahpC* genes. The mechanisms of isoniazid (INH) resistant are more complicated than that of rifimpin (RMP) resistance. The INH-associated gene mutations still not yet completely identified. In order to improve the detection rate of MDR *M. tuberculosis* isolates using DNA sequencing, we focus on identification of novel INH resistant-associated genes including the *furA*, *ndh*, *kasA*, *mabA*, *efpA*, *Rv0340*, *iniB*, *iniA*, and *iniC* genes. In this study, we analyzed 57 INH-resistant isolates without mutations in the *katG*, *inhA*, *inhA* regulatory region, and *oxyR-ahpC* genes. The results showed that 14 mutations among 8 new sequenced INH resistant-associated genes (*mabA*, *kasA*, *ndh*, *furA*, *iniB*, *iniA*, *iniC*, and *efpA*) in 33.3% (19/57) of the INH-resistant isolates. Besides the 12-bp deletion of codon 222 in the *iniB* gene, we identified 13 novel mutations that were associated with INH resistance. While, 18 INH-susceptible isolates do not harbored any mutations in those 8 genes sequenced.

I. Purpose

The objective of this study is to identify new mutations of the INH resistant-associated genes to inctease the detection rate of INH-resistant isolates using the DNA sequencing method without waiting for results of time-consuming conventional drug susceptibility testing (DST). Therefore, prompt and proper treatment MDR TB patients can improve the treatment outcome.

II. Methods

Mycobacterium tuberculosis isolates. We received *M. tuberculosis* isolates from clinical mycobacteriology laboratories in Taiwan. One isolates was selected from individual TB case. In this study, 57 MDR *M. tuberculosis* isolates which were INH resistant confirmed by DST but harboring no mutation on the *katG*, *inhA*, *inhA* regulatory region, and *oxyR-ahpC* genes were selected to be sequenced. In addition, 18 INH-susceptible isolates were analyzed concordantly as a control.

Drug susceptibility testing. The agar proportion method on either Middlebrook 7H10 or 7H9 (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 RMP. Growth on the control medium was compared to growth on the drug-containing medium to determine susceptibility. 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 RMP.

Routine INH-resistant associated gene sequencing. Primer sets were used to analyze the variation at the *katG, inhA, inhA* regulatory region, *and oxyR-ahpC* genes and sizes of the PCR products were listed in Table1. The PCR reactions were performed as follows: 33 cycles at 95°C for 1 min; annealing at 55°C (*katG*) or 60°C (*inhA* and *inhA* regulatory region) for 1 min; and elongation at 72°C for 1 min. Thereafter, the PCR products were analyzed with an ABI Veriti automated sequencer (Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencher 4.7 Demo software.

Novel INH-resistant associated gene sequencing. Primer sets were used to analyze the variation at the *furA*, *ndh*, *kasA*, *mabA, efpA*, *Rv0340*, *iniB*, *iniA*, and *iniC* genes and sizes of the PCR products were listed in Table 2. The PCR reactions were performed as follows: 30 cycles at 95°C for 30 sec; annealing at 62°C for 30 sec; and elongation at 72°C for 1 min. Thereafter, the PCR products were analyzed with an ABI Veriti automated sequencer

(Applied Biosystems, USA), and the sequence data were assembled and edited using the Sequencher 4.7 Demo software.

III. Results

Gene sequencing analysis

The INH-resistant associated mutations identified in this study were list in Table 3 and Table 4. We identified 14 mutations among 8 additional INH resistant-associated genes (*mabA*, *kasA*, *ndh*, *furA*, *iniB*, *iniA*, *iniC*, and *efpA*) in 33.3% (19/57) of the INH-resistant isolates that did not harbored any mutations in the *katG*, *inhA*, *inhA* regulatory region, and *oxyR-ahpC* genes. Besides the 12-bp deletion of codon 222 in the *iniB* gene, we identified 13 novel mutations that were associated with INH resistance.

IV. Discussion

Based on the sequence analyses of the new INH resistant-associated genes, we found 14 mutation sites in the INH-associated genes in 33.3% (19/57) of the INH-resistant isolates with no mutations in the *katG*, *inhA*, *inhA* regulatory region, and *oxyR-ahpC* genes. These results can be applied in a high throughput system to detect these mutation sites simultaneously. In this study, the results showed that using additional mutations that associated with the INH resistant-associated genes will be helpful in identifying MDR *M. tuberculosis* isolates without waiting for time-consuming conventional DST results. In addition, a rapid molecular diagnosis kit can be as developed with an improved sensitivity.

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VI. Publication list for this work

NIL

VIII. Tables

Table 1. Primer sequences used to analyze the variation at the *katG, inhA, inhA* regulatory region, *and oxyR-ahpC* genes

Gene	Primer name	Primer sequence	Size (bp)
katG	katG-F	5′-GTC ACA CTT TCG GTA AGA C-3′	658
	katG-R	5′-TTG TCG CTA CCA CGG AAC G-3′	
inhA	inhA 1713-F	5′-CCG AGG ATG CGA GCT ATA TC-3′	543
	inhA 1713-R	5′-GGC TCG GGT CGA AGT CCA TG-3′	
	inhA 2194-F	5′-AGG CGC TGC TGC CGA TCA TG-3′	456
	inhA 2194-R	5′-CCG AAC GAC AGC AGC AGG AC-3′	
inhA regulatory region	inhA locus-F	5′-AAT TGC GCG GTC AGT TCC AC-3′	453
	inhA locus-R	5′-GTC GGT GAC GTC ACA TTC GA-3′	
oxyR-ahpC	ahpC-F	5′-GCT TGA TGT CGG AGA GCA TCG-3′	701
	ahpC-R	5′-GGTCGC GTA GGC AGT GCC CC-3′	

Table 2. Primer sequences used to analyze the variation at the *furA*, *ndh*, *kasA*, *mabA*, *efpA*, *Rv0340*, *iniB*, *iniA*, and *iniC* genes

Gene	Primer name	Primer sequence	Size (bp)
furA	furA-F	5'-CTCATCGGAACATACGAAGGCT-3'	700
	furA-R	5'-ATTTCATATGACCCACGACGGGAC-3'	
ndh	ndh-1F	5'-GCACGCTGTGGTGTGGCTGATGAC-3'	906
	ndh-1R	5'-CGGAGTCCTTGACGGTGATGCCGT-3'	
	ndh-2F	5'-CCGGCACATCGACTCGACCAA-3'	888
	ndh-2R	5'-GCATTCACCGACGCCATCGACG-3'	
kasA	kasA-1F	5'-GGTGACGTTGTCGCCTACATCC-3'	884
	kasA-1R	5'-CGTCTCGATGAGCATCAGCGCA-3'	
	kasA-2F	5'-CGTCAGATCGTGATGGGCGAC-3'	854
	kasA-2R	5'-GCGATGCCGGTGACGACTACGTA-3'	
mabA	mabA-1F	5'-GCAATTGCGCGGTCAGTTCCA-3'	674
	mabA-1R	5'-CGACCGAATTTGTTGCGCTGC-3'	
	mabA-2F	5'-GCTGTTTGGCGTCGAATGTGAC-3'	685
	mabA-2R	5'-TACCCGTGCGATGTGAAACGCGAT-3'	
efpA	efpA-1F	5'-AACAGACGTTGCGGGCCACCCT-3'	1019
	efpA-1R	5'-CGCTCCACGATGACAAACGCGAC-3'	
	efpA-2F	5'-GCCTTCTCGATCGGTCCTGAA-3'	952
	efpA-2R	5'-GGTGCGCAAGAACAACTCGGACAT-3'	
Rv0340	Rv0340-F	5'-TAATGCGGCCATCCCCTAACG-3'	797
	Rv0340-R	5'-ATCGACGCTATGGATTCCGCCT-3'	
iniB	iniB-1F	5'-GCCGATCCCGATAGGTGTTTGG-3'	981
	iniB-1R	5'-GCATAGCAGCGCCGTTCAAGG-3'	
	iniB-2F	5'-CGCTAGCCAGATCGGTGTCTC-3'	837
	iniB-2R	5'-GCTCGTTTACGCCTCAGATCACG-3'	
iniA	iniA-1F	5'-TCGGTGTTTGACGTCGGTCACGAG-3'	891
	iniA-1R	5'-CAGATGTGCTGCATTGGCATTGAC-3'	
	iniA-2F	5'-CGATGCCGTCTTGGTGGTCAG-3'	800
	iniA-2R	5'-CGAAGTCGGTGCCCATGACGTG-3'	
	iniA-3F	5'-TGTGACCCGACTGCGCATTGG-3'	835
	iniA-3R	5'-CGGTCCAGCTGGCAAAAAACGTCG-3'	
iniC	iniC-1F	5'-CGGAAACCGAGCGGGACAATCG-3'	890
	iniC-1R1	5'-TCAGCGCAAGAAGTCCGGATACC-3'	
	iniC-2F	5'-CTCAAACAGATCGGTGGGCTGGT-3'	1009
	iniC-2R	5'-GCTCGAAAACATGTTCCACCCGGT-3'	

Table 3. Mutations identified in novel INH-resistant associated genes, *mabA, kasA*, *ndh*, and *furA* genes

katG, inhA, inhAr, oxyR-ahpC	DST result			*mabA*	*kasA*	*ndh*	*furA*			
				codon 74	codon 253	codon 68	codon 40	codon 40	codon 61	codon 122
				Ala→Thr	His→Tyr	Ile→Thr	Thr→Pro	Thr→Ala	Asp→His	Asp→Lys
wild type	R*	57	mutation	**1**	**1**	**5**	**1**	**1**	**1**	**1**
			wild type	56	56	52	56	56	56	56
	S*	18	mutation	0	0	0	0	0	0	0
			wild type	18	18	18	18	18	18	18

*: R, resistant; S, susceptible