Genetic analysis of highly virulent strains of Entamoeba histolytica in the high risk groups between Taiwan and Japan: 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 thousands death each year. Eleven of *Entamoeba histolytica* clinical strains had been isolated from different foreign groups with different clinical symptoms and will be used to find out the virulence factors which cause the strain differences in pathogenicity. In addition, we found the parasiticidal mechanism of the two anti-amebic drugs, metronidazole and paramomycin, was different. The 40μM tissue drug Metronidazole induced ameba produced apoptosis with chromatin DNA fragmentation and inner cell membrane reversal, whereas the 80μM luminal drug paramomycin caused ameba necrosis. Previous mechanism was reversible but later was irreversible. Once cell initiates the cell lysis, the cell is dying. This study intended for the study of pathogenicity and drug tolerance of *E. histolytica*, and tries to understand the parasiticidal mechanism of the two anti-amebic drugs for future amebiasis treatment and control.

I. Purpose:

The aims of this project were to investigate action and drug resistance mechanisms of metronidazole and paromomycin that are currently used for the treatment of *Entamoeba 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.

II. Methods:

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.

Clinial sample preparation and storage

The clinical specimen processing modify according to Nollau protocol. Fresh stool samples take about 0.5 g in 1% 6M guanidine thiocyanate vortex to mix. In room temperature for 10 min, then be DNA extraction or storage in -20℃. The sample centrifuge 14000 rpm 5 min to save supernatant and separate DNA by phenol/chlorofom/isoamyl alcohol method.

Culture Methods: Xenic culture

The clinical stool sample which takes about half of a peanut was xenically cultured at 35.5°C in basal amoebic (BR) medium with 250 µl rice starch solution (5 mg/ml) and 120 µg erythromycin for 24 hours. In the second day, remove the BR medium and keep the stool sample and rice starch powder layer in the bottle. Add the phthalate solution, bacto-peptone solution, erythromycin in to the bottle and fill with the BRS medium (BR medium with 10 % serum) to the bottle neck. The next day, take about 10 µl faeces and the starch for checking the *E. histolytica* trophozoites. If cannot see them, incubate the tube for another 24 hours. If trophozoites exist, transfer 100µl of faecal-starch layer into per new bottles and fill the bottles with phthalate solution, bacto-peptone solution, erythromycin and BRS medium (the complete BRS medium) to bottle neck. After cultured days, if there are a great mounts of trophozoites, transfer to the monoxenic culture medium.

Culture Methods: Monoxenic culture from trophozoites

These clinical isolates were cultured in monoxenic condition using yeast extract–iron– maltose–dihydroxyacetone-serum (YIMDHA-S) medium supplemented with *E. coli* (1, 2). Brief, filter the suspension from xenic culture bottles by BD filter (40 µm funnel) (put on the 50 ml tube) and transfer to glass culture tube at 35.5^{o}C 30 minute to 1 hour for attachment. Then pipette out the BRS medium and wash the sediment by centrifugation (1200 rpm, three minute) for three times with fresh BI-S-33 medium or LYI-2 medium. The sediment is inoculated in to fresh YIMDHA-S medium (5.5 ml) containing 15% adult bovine serum, potassium penicillin G (1000 units/ml), gentamycin (130 units/ml), streptomycin sulfate (1 mg/ml). The culture tube is inoculated at stand upright position for 30 minute at 35.5^{o}C. Centrifuge the tube (1200 rpm, 3 minute) and remove the supernatant gently. Add 5.5 ml the complete YIMDHA-S medium in the tube. The culture tube is incubated at stand upright at 35.5^{o}C about 3 days. Observe the growth of amoeba and monitor contamination. If culture medium to be derby, on ice five minute then wash again and fill new medium. If there is a great mount of cells, put the tube on ice five minute and transfer 1 ml to 4 ml in to the tube with fresh complete YIMDHA-S medium.

Gene expression database

We will set up the gene expression database about *E. histolytica* from HM1:IMSS, treated HM1:IMSS, clinical strain, high symptomatic and asymptomatic through the microarray comparison. We therefore collaborated with Dr. Graham Clark in University of London, London School of Hygiene & Tropical Medicine and Dr. Kumiko Tsukui in NIID. This database may help to compare gene expression about the international different strain in Future.

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 conditiona were followed by 35 cycles of 94°C

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^{o}C. After washing twice with PBS, 50 μl TUNEL reaction mixture (Roche®) was added and incubated for 60 min at 37 ^{o}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.

V. Reference list (if have):

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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

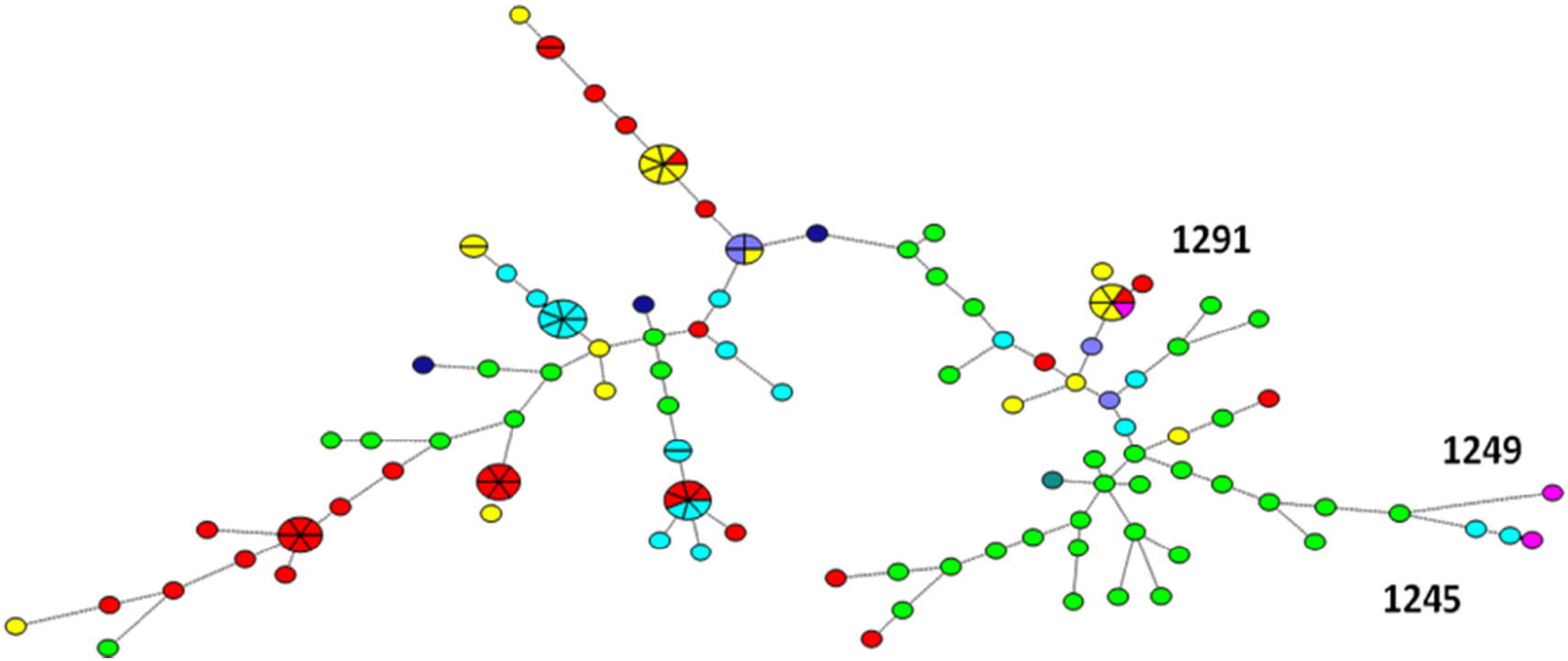
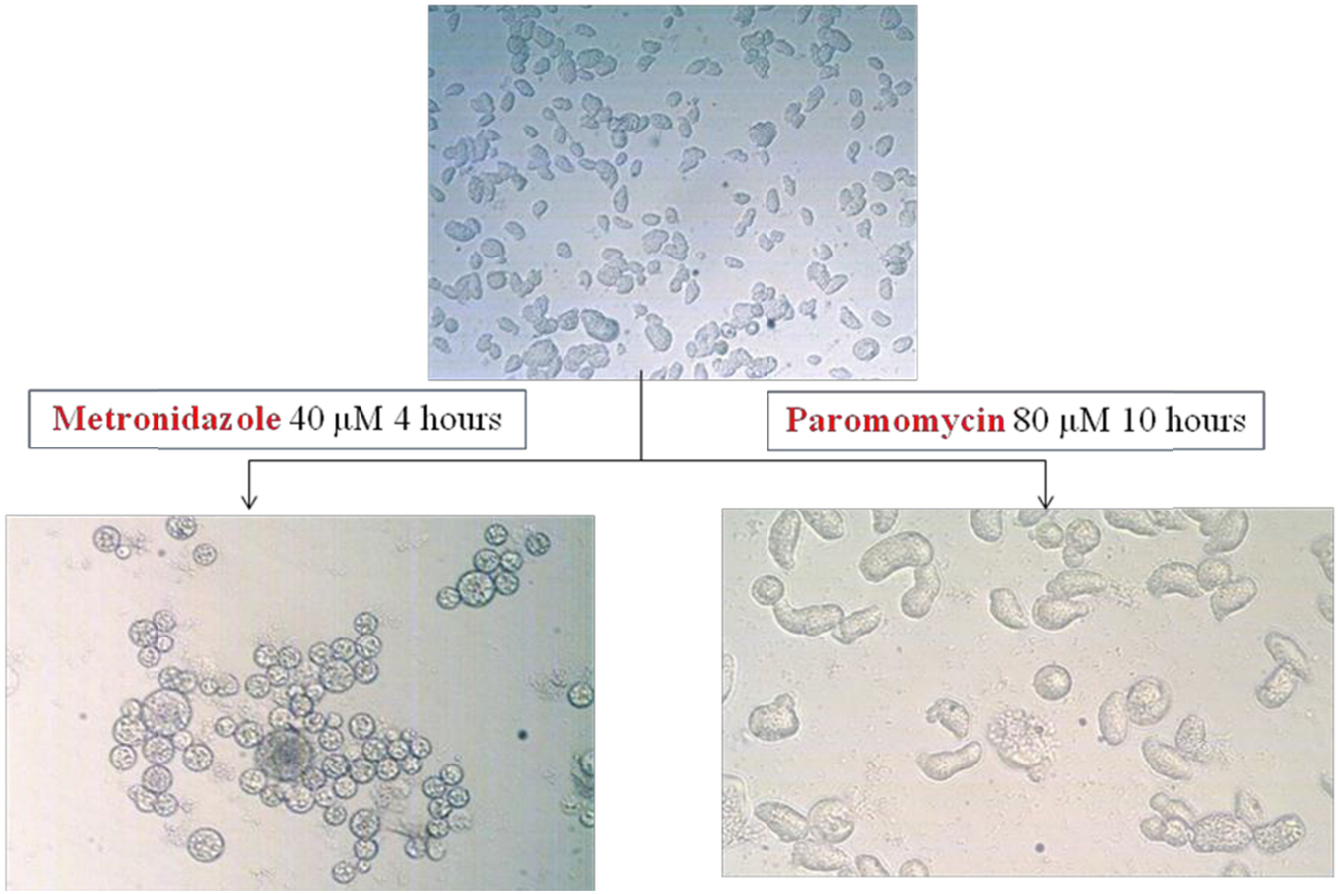
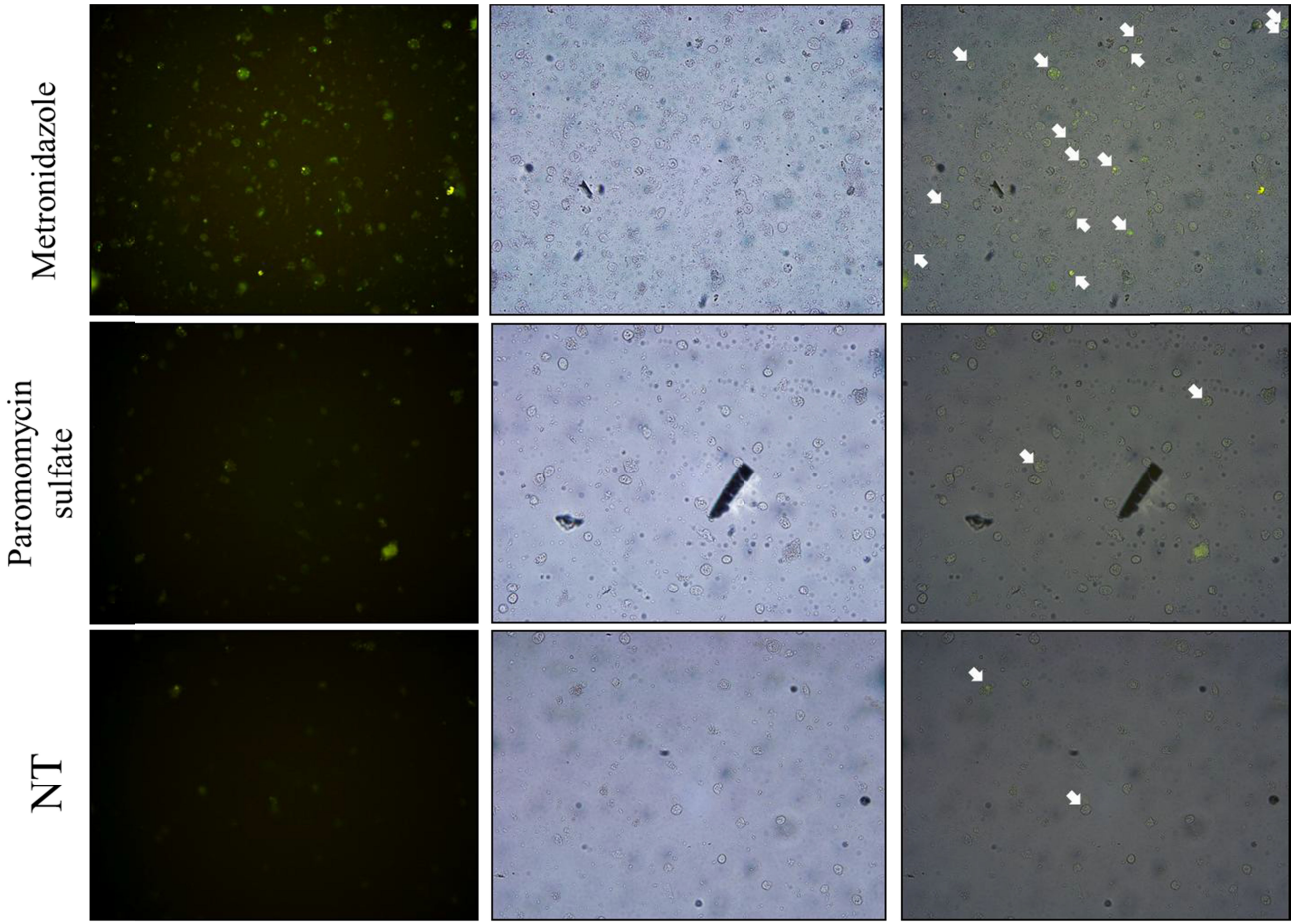


Figure 2. Observation of the drug treatment of the *E. histolytica* HM1:IMSS by microscopy



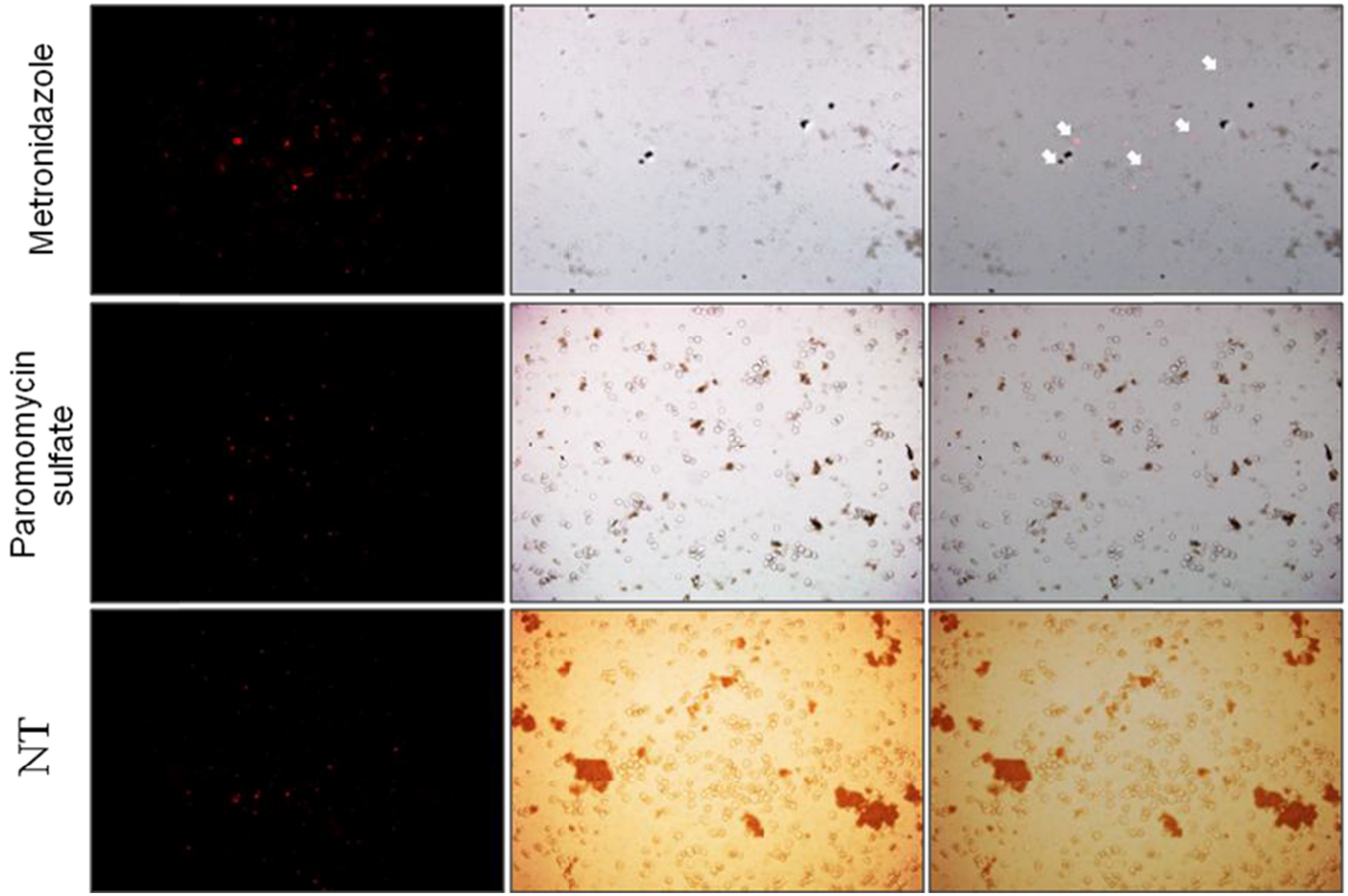
* Metronidazole與 Paromomycin 分別對標準蟲株 *E. histolytica* HM1:IMSS 引發蟲體死亡研究其機制。 Paromomycin 引發蟲體死亡的機制與表徵與 Metronidazole 不同。

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



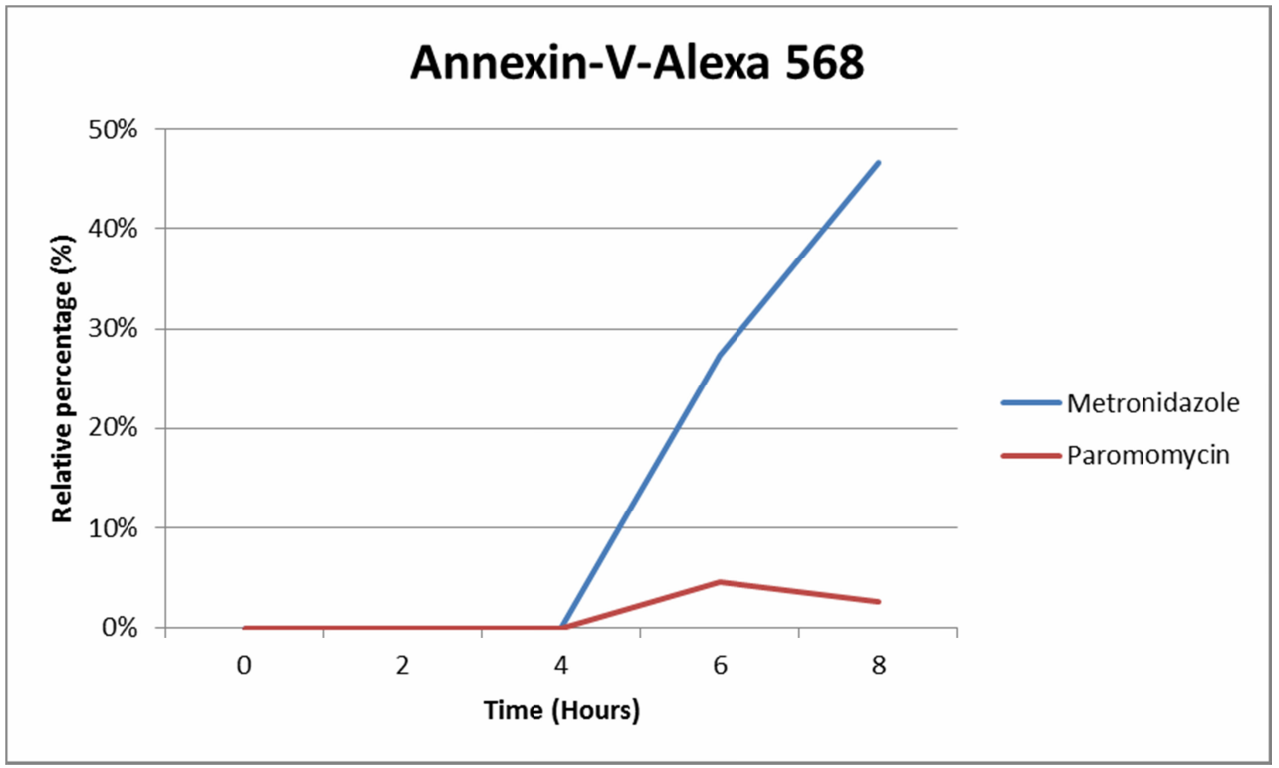
利用 TUNEL 法檢測，由 Metronidazole 與 Paromomycin 處理四小時的標準蟲株產生細胞程式死亡之現象。

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。

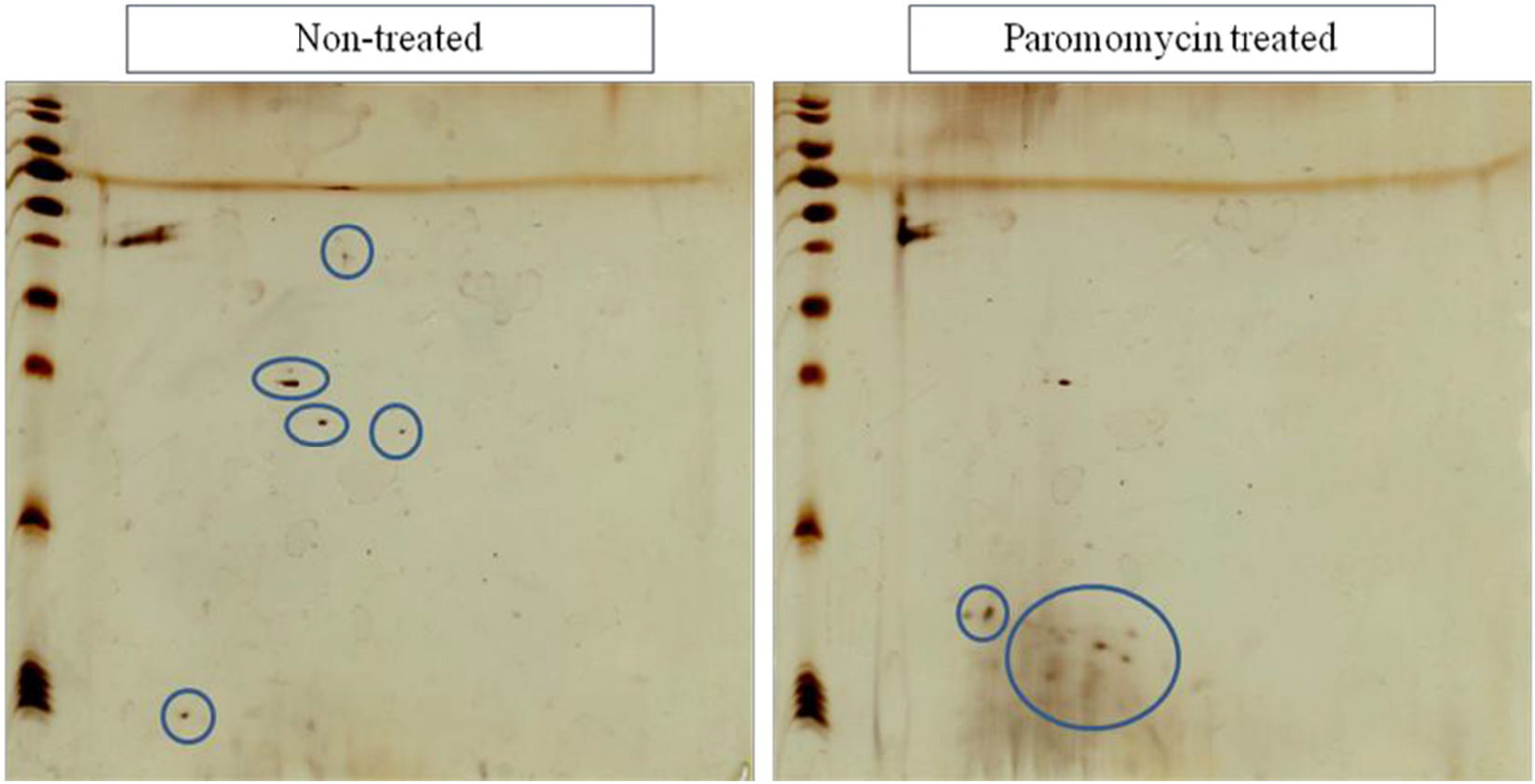
Figure 5. Quantitative analysis of the Annexin-V probed *E. histolytica* cells by Flow-cytometry



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。

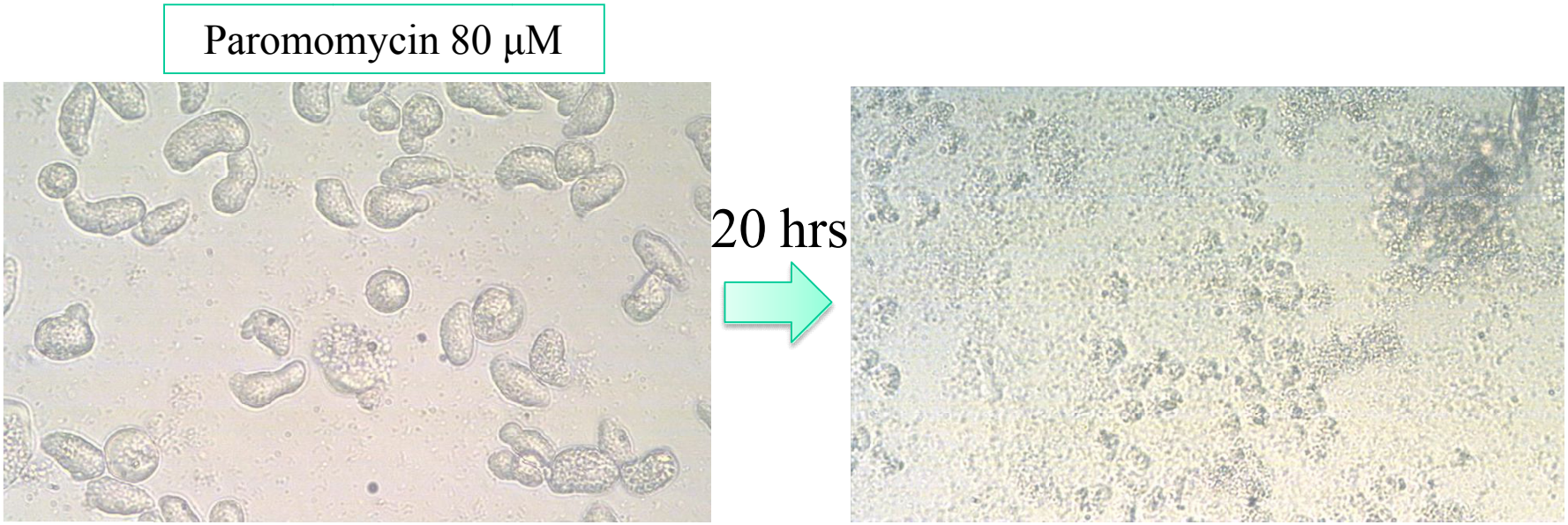
Figure 6. Comparison of the protein variation after drug treatment by the two-dimensional protein

electrophoresis analysis



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。