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.

Reference list:

1. Nollau P, Moser C, Weinland G, Wagener C. Detection of K-ras mutations in stools of patients with colorectal cancer by mutant-enriched PCR. Int J Cancer. 1996 May 3;66(3):332-6.
2. Ali IKM, Zaki M, Clark CG. Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping *Entamoeba histolytica*. J Clin Microbiol 2005;43:5842–7.
3. Gilchrist CA, Houpt E, Trapaidze N, Fei Z, Crasta O, Asgharpour A, Evans C, Martino-Catt S, Baba DJ, Stroup S, Hamano S, Ehrenkaufer G, Okada M, Singh U, Nozaki T, Mann BJ, Petri WA Jr: Impact of intestinal colonization and invasion on the *Entamoeba histolytica* transcriptome. Mol Biochem Parasitol. 2006 Jun;147(2):163-76.
4. Biller L, Davis PH, Tillack M, Matthiesen J, Lotter H, Stanley SL Jr, Tannich E, Bruchhaus I: Differences in the transcriptome signatures of two genetically related *Entamoeba histolytica* cell lines derived from the same isolate with different pathogenic properties. BMC Genomics. 2010 Jan 26;11:63.
5. Report on Carcinogens, Twelth Edition (2011); http://ntp.niehs.nih.gov/ntp/roc/twelfth/ profiles/Metronidazole

Publication list for this work:

NIL

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

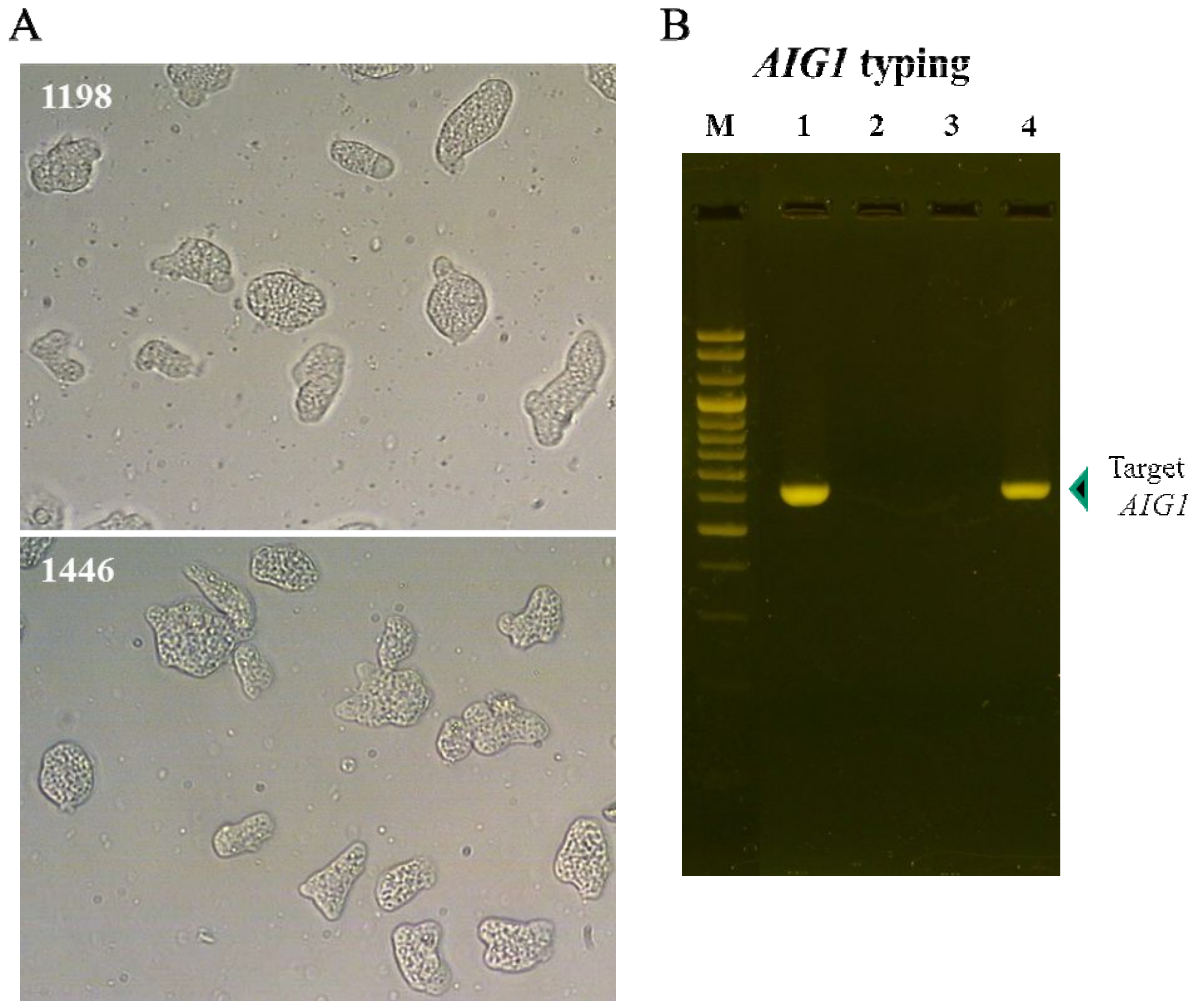

Fig. 1A:

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

A-L locus

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          261      270       280       290       300       310       320       330       340       350       360       370       380       390
HM1       TAT-----------------TACTTTATTACTTTATTACTTTATCACTTTATCACTTTATCTTTACTACTTCTTCT-------ATACCTATATACTTCTATACTTCTATACTATATGTGTGTATCTTATA
1198      TAT-----------------TACTTTATTACTTTATTACTTTATCACTTTATCACTTTATCTTTACTACTTCTTCT-------ATACCTATATACTTCTATACTTCTATACTATATGTGTGTATCTTATA
1446      TATCTTTACTACTTCTTCTATACCTATATACTTCTATACTTCTATACTTCTATACTATATGTGTGTATCTTATACTTTGACTTATACCTTAATACTTTTATACTTTTATAC-CTTTATACTTTTATACCT
Consensus TAT.................TACtTtatTACTTtatTACTTtatcACTTtatcACTtTATcTtTactaCTTcTtCT.......ATACCTatATACTTcTATACTTcTATACtaTaTgTgtgTaTcTtata
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N-K2

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          131      140       150       160       170       180       190       200       210       220       230       240       250       260
HM1       TACATGTATGTATCCTTTTATACCTCCTTAGTAATTTAGTAATTTAGTAATTTAGTATTTTAGTATTTTAGTAATTTAGTATTTTAGTATTTTAGTA--------TTTTAGTATTTTA------------
1198      TACATGTATGTATCCTTTTATACCTCCTTAGTAATTTAGTAATTTAGTAATTTAGTATTTTAGTATTTTAGTAATTTAGTATTTTAGTATTTTAGTAATTTAGTATTTTAGTATTTTA------------
1446      TACATGTATGTATCCTTTTATACCTCCTTAGTAATTTAGTAATTTAGTAATTTAGTATTTTAGTATTTTAGTAATTTAGTATTTTAGTATTTTAGTAATTTAGTATTTTAGTATTTTAGTATTTTAGTAA
Consensus TACATGTATGTATCCTTTTATACCTCCTTAGTAATTTAGTAATTTAGTAATTTAGTATTTTAGTATTTTAGTAATTTAGTATTTTAGTATTTTAGTAatttagtaTTTTAGTATTTTA............
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S-Q

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          1        10        20        30        40        50        60        70        80        90        100       110       120       130
HM1        GCCATTTCTCTCTGGAGGCGTAGGTTCGAATCCTGCCGTTGTCGATGTTTTTAACATAAGGATAGATGGATAGATGGATAGATGTATAGATGGATAGATGGATATAAATATATAAGGATATAAGTATAG
1198      AGGGGATCCTCTCTGGAGGCGTAGGTTCGAATCCTGCCGTTGTCGATGTTTTTAACATAAGGATAGATGGATAGATGGATA------------------------TAAATATATAAGGATATAAGTATAG
1446        ACATTTCTCTCTGGAGGCGTAGGTTCGAATCCTGCCGTTGTCGATGTTTTTAACATAAGGATAGATGGATAGATGGATA------------------------TAAATATATAAGGATATAAGTATAG
Consensus .g.catTtCTCTCTGGAGGCGTAGGTTCGAATCCTGCCGTTGTCGATGTTTTTAACATAAGGATAGATGGATAGATGGATA........................TAAATATATAAGGATATAAGTATAG
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D-A

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          131      140       150       160       170       180       190       200       210       220       230       240       250       260
HM1       CTTTATTATCTTTATTATCTTTATTATCTTTATTACCTTTATTACCTTTATTACCTTTATTACCTTTATTATCTTTATTACCTTTATTATATCTATTCTCACTTCCTATACGTACTCTTTTTACTACTCT
1446      CTTTATTACCTTTATTACCTTTATTACCTTTATTACTTTTATTACTTTTATTATCTTTATTATCTTTATTACCTTTATTACCTTTATTATATCTATTCTCACTTCCTATACGTACTCTTTTTACTACTCT
1198      -----------------------------------CTTTTATTACTTTTATTATCTTTATTA------------------CCTTTATTATATCTATTCTCACTTCCTATACGTACTCTTTTTACTACTCT
Consensus ctttatta.ctttatta.ctttatta.ctttattaCtTTTATTACtTTTATTAtCTTTATTA.ctttatta.ctttattaCCTTTATTATATCTATTCTCACTTCCTATACGTACTCTTTTTACTACTCT

          261      270       280       290       300       310       320    328
HM1       TTTTACTACTCTTCTTACTATACCTCTTACTACTCCTACTTTCACCTCCCTCTTTATTGTTAAT
1446      T------------CTTACTATACCTCTTACTACTCCTACTTTCACCTCCCTCTTTATTGTTAATTAAG
1198      TTTTACTACTCTTCTTACTATACCTCTTACTACTCCTACTTTCACCTCCCTCTTTATTGTTAATTAAG
Consensus Ttttactactctt CTTACTATACCTCTTACTACTCCTACTTTCACCTCCCTCTTTATTGTTAATtaag
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R-R

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          131      140       150       160       170       180       190       200       210       220       230       240       250       260
HM1       ATGTTTATATGTTTATATGTCTATGTATGCTTGCTTATTATTTATGTTATATATTTCTCTATCTTATATTATTTATGTTCTTATTTTATTATTTTATTATTTCTTTATTTCTTTATTTCTTTATTT----
1446      ATGT----------------CTATGTATGCTTGCTTATTATTTATGTTATATATTTCTCTATCTTATATTATTTATGTTCTTATTTTATTATTTTATTATTTCTTTATTTCTTTATTTCTTTATTTCTTT
1198      --------------------CTATGTATGCTTGCTTATTATTTATGTTATATATTTCTCTATCTTATATTATTTATGTTCTTATTTTATTATTTTATTATTTCTTTATTTCTTTATTTCTTTATTT----
Consensus atgt................CTATGTATGCTTGCTTATTATTTATGTTATATATTTCTCTATCTTATATTATTTATGTTCTTATTTTATTATTTTATTATTTCTTTATTTCTTTATTTCTTTATTT....
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S^TGA-D

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          1        10        20        30        40        50        60        70        80        90        100
HM1       TTTGGAAATATAGAATAATAGAAATAATAGAAATAATAGAAATAATAGAAAATATAGAAATAATAAAAAGTTATAATATAGTAAGAAAAAAATAGTATAAT
1446       TTAGCAATATAGAATAATAGAAATAATAGAAATAATAGAAATAATAGAAAATATAGAAATAATAAAAAGTTATAATATAGTAAGAAAAAAATAGTATAAT
1198      TTTGGCAATA-------ATAGAAATAATAGAAATAATAGAAATAATAGAAAATATAGAAATAATAAAAAGTTATAATATAGTAAGAAAAAAATAGTATAAT
Consensus tTTgGcAATAtagaataATAGAAATAATAGAAATAATAGAAATAATAGAAAATATAGAAATAATAAAAAGTTATAATATAGTAAGAAAAAAATAGTATAAT
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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

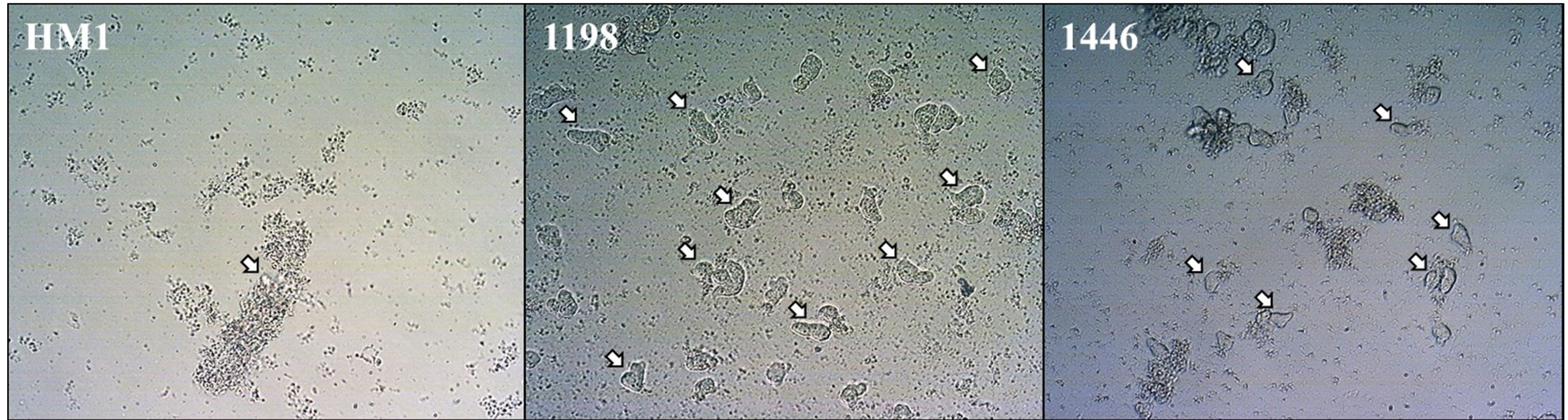

Fig. 3:

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*

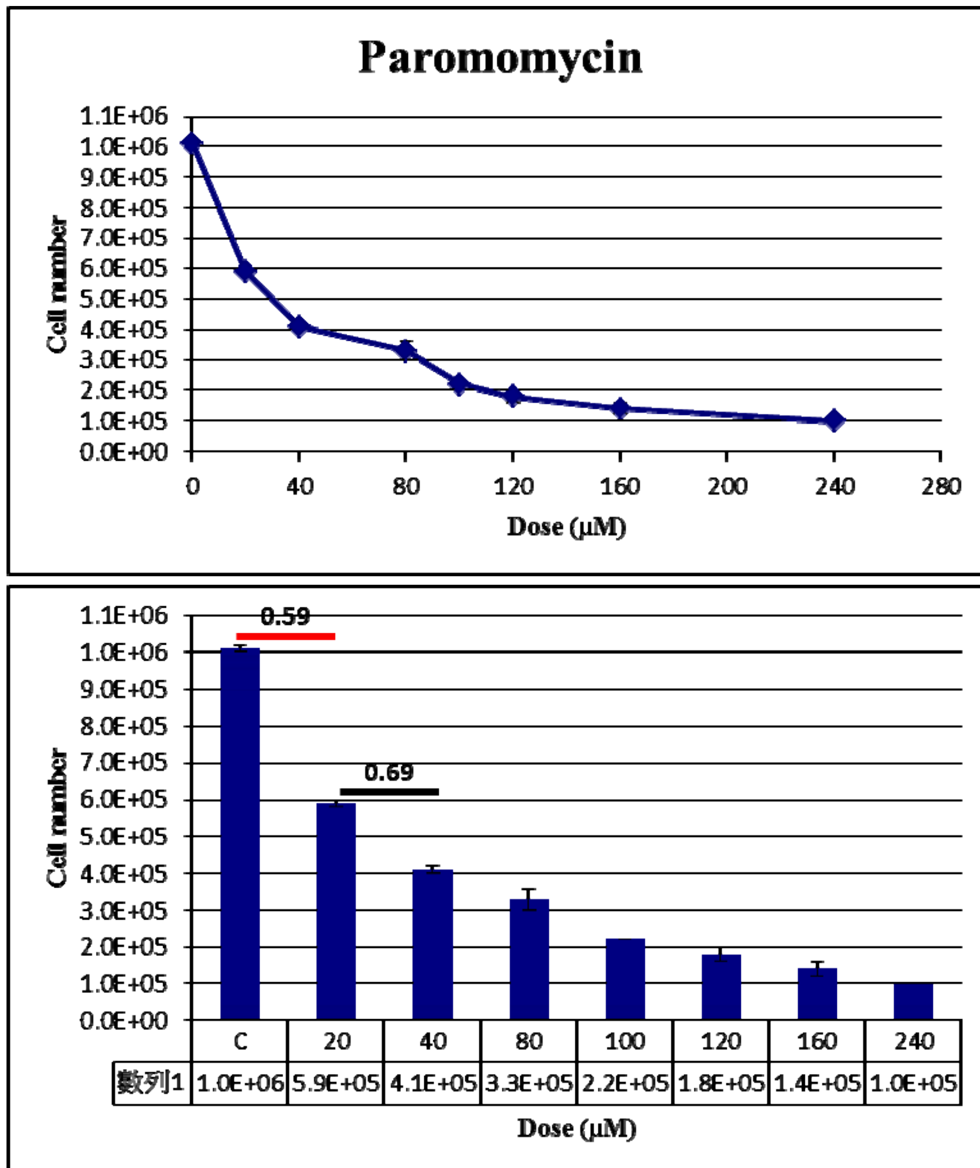

Fig. 4:

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

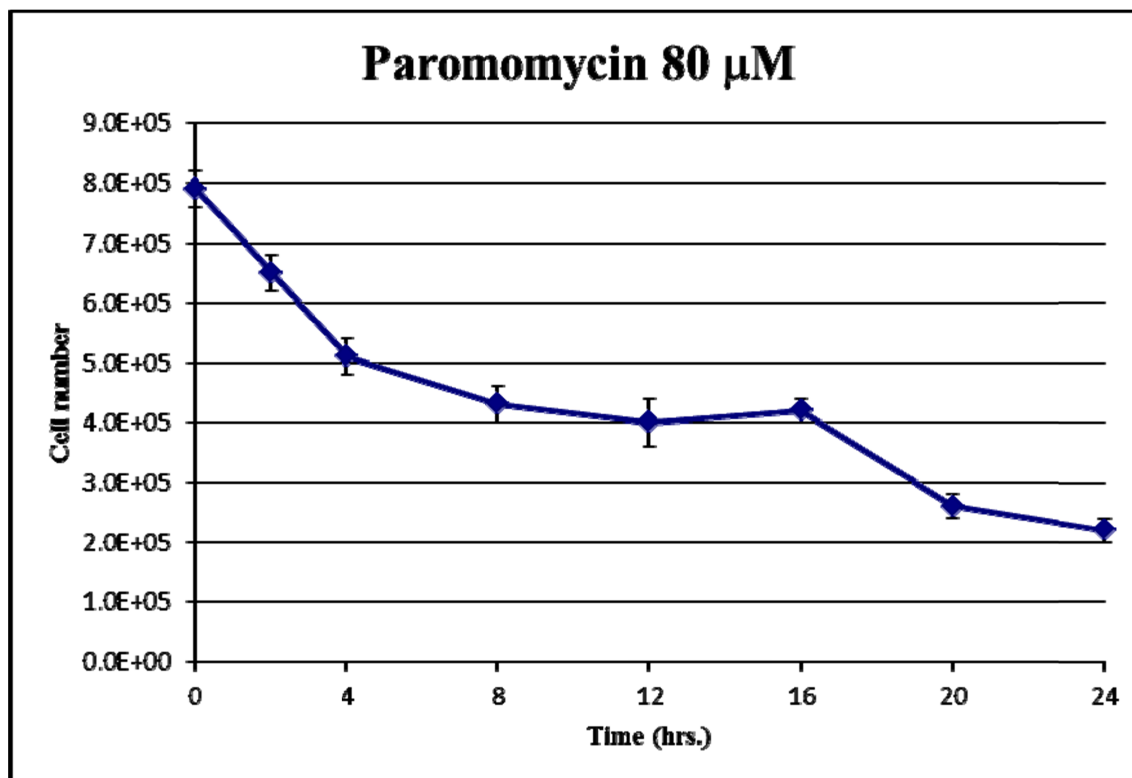

Fig. 5:

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

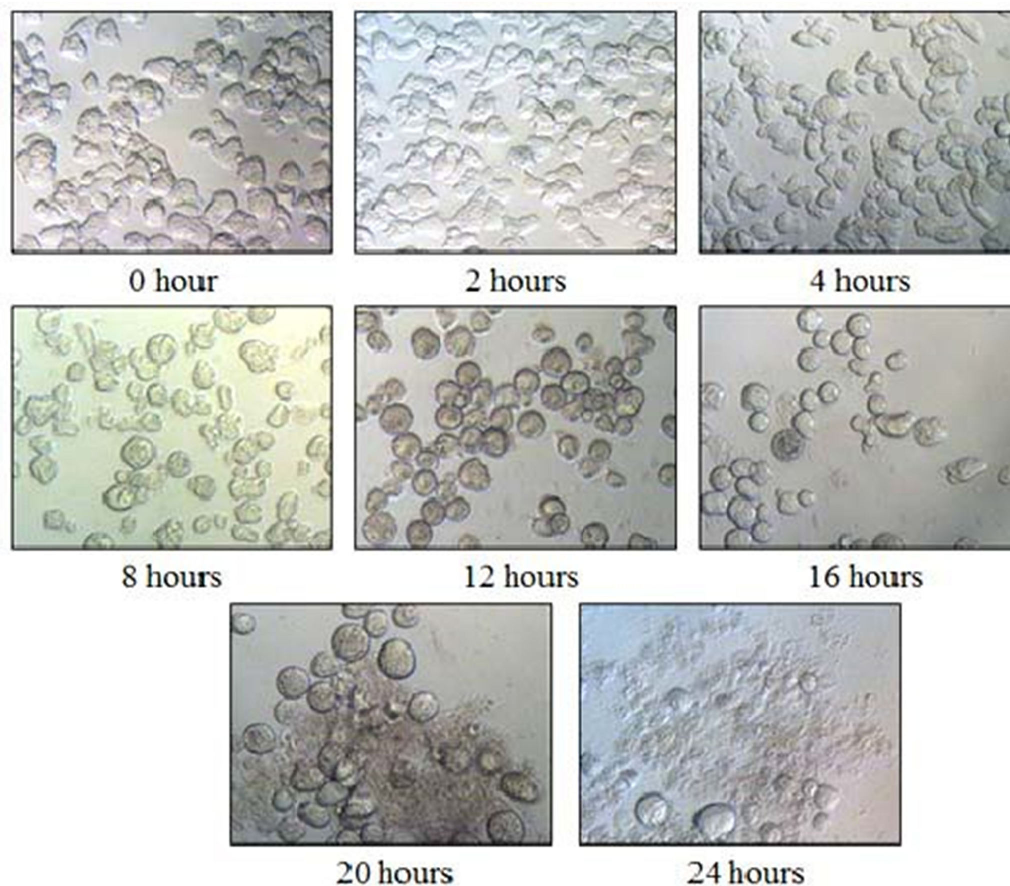

Fig. 6:

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

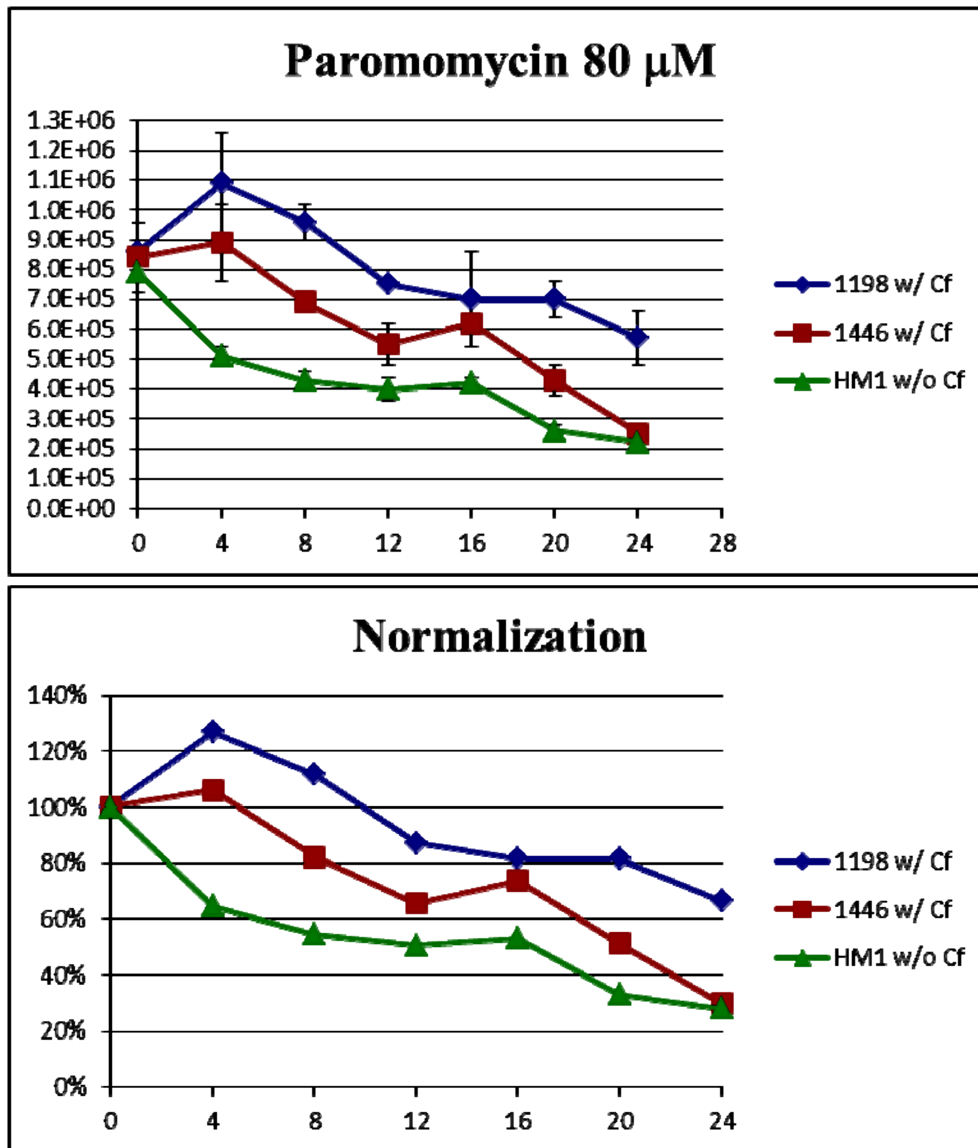

Fig. 7:

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).