Study of genetic diversity and drug action mechanisms of *Entamoeba histolytica* clinical isolates

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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. The new of *E. histolytica* clinical strain TCDC-2126 had been isolated from one asymptomatic foreign labors that had been treated by standard therapy. The tRNA-STR genotype of TCDC-2126 was different to another clinical strain TCDC-1198 and used to investigate the drug tolerance. The tolerance of paromomycin and metronidazole had been tested to *E. histolytica* clinical strains and standard strain HM1-IMSS. 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:

This project is to investigate action and drug resistance mechanisms of paromomycin that are currently used for the treatment of *Entamoeba histolytica* in Taiwan, and analysis of the genetic differences of the highly virulent strains.

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 S^{TGA}D-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 (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.

Mass spectrometry-based proteomics analysis

Mass spectrometry will be performed in National Yang-Ming University, Taiwan. The expressed protein

profiles will be established by compared to E. histolytica genome database. Brief, in-gel Digestion was performed by manually excising selected gel pieces and placed into 1.5 mL Eppendorf tubes. Then the gel pieces were destainedand dehydrated. Thereafter, trypsin (Promega, Madison, WI, USA) was added to the dried gel pellets, and the reaction was incubated at 4°C for 40 min. Finally, the gel pieces were sonicated and these final products were ready for protein identification by using the following mass-spectrometric analysis. The LTQ Orbitrap (Discovery) hybrid mass spectrometer was used to perform all Mass spectrometry analyses with a nanoelectrospray ion source (Thermo Electron, San Jose, CA) coupled to a nano flow HPLC (Agilent Technologies 1200 series). The conventional MS survey scan was acquired at high resolution over the acquisition range m/z 200-2000 and a series of precursor ions were selected for the MS/MS scan. The former examined the accurate mass and charge state of the selected precursor ions and the latter acquired the spectrum (MS/MS spectrum) of the fragment ions generated by collision-induced dissociation. Mass spectra were processed using the MassLynx 4.0 software (Micromass) and the protein identities were analyzed using the MS/MS peak lists generated from MassLynx. Protein identification was carried out by interpreting MS/MS data based on NCBInr gene database

Indirect Immunofluorescence Assay

The amoebae were washed by PBS and fixed with acetone/methanol (1:1) for 30 min. After washing with PBS solution, coverslip were blocking with 5% BSA for 30 min and then reacted with primary antibody diluted at 1:500 (anti-ATP sulfurylase antiserum) for 1 h. The samples were then reacted with Alexa Fluor 488-conjugated anti-rabbit secondary antibody (1:1,000) for 1 h.

Result:

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

    In previous report, we successfully set up an applicable protocol and isolated two of *E. histolytica* clinical strains, TCDC-1198 and TCDC-1446, from two asymptomatic foreign labors. And now, we get the new clinical strain, called TCDC-2126, from another asymptomatic foreign labor who had been treated by standard metronidazole and paromomycin therapy. That meant the TCDC-2126 strain might have higher probability of drug tolerance in the field. We compared TCDC-2126 with TCDC-1198, the two clinical strains belonged to two different six tRNA-STR genotypes (fig. 1). The *AIG1* gene had been indicated as an important pathogenic factor of *E. histolytica* (3,4). We will examine the *AIG1* gene of TCDC-2126 in the future. In the same culture condition, the TCDC-2126 grew faster and had better adhesion on the surface of culture tube than TCDC-1198.

2. Time series analysis of drug cytotoxicity of clinical and standard strain

    To do the time series analysis of drug cytotoxicity, we chose the 80 μM metronidazole and paromomycin as a curve model. After the metronidazole treatment for 24 hours, HM1 and HM1 with *C. fasciculata* cell number were 25.5% and 17.4% left in compared to the control group respectively (fig. 2). In the same time, the morphology of amoebic parasites was significantly changed, it became round-up and lysis in the culture medium. But TCDC-2126 strain showed higher survival rate than any others within 24 hours (fig. 2). In paromomycin induction examine, TCDC-1198 and TCDC-2126 displayed better survival rate than HM1 (fig. 3). These data indicated TCDC-1198 and TCDC-2126 had higher tolerance than HM1 in paromomycin, but only TCDC-2126 was more tolerance than HM1 in metronidazole treatment (fig. 2 and 3).

3. Proteome analysis of paromomycin induction

    To figure out parasiticidal mechanism of paromomycin, we use mass spectrometry to analysis total protein through drug induction. Based on time series analysis of paromomycin cytotoxicity curve, we chose the 80 μM paromomycin as an examine dosage and two time points (8 and 15 hours) to sample collection (fig. 4). When drug induction for 8 hours, we could see metabolism changes of cell, adhesion molecule reduction, and maybe involve endoplasmic system or organelle changes (table 1). After 15 hours treatment, we also could see the same thing (table 2). However, we only observed ubiquitin enzyme system expression changes in late stage (15 hours). These results indicated that there have more stories, not just necrosis-like phenomenon.

4. Organelle observation through mitosome morphology

    To observe cell organelle changes, we use immunofluorescence assay to see the mitosome morphology. According to Fumika et al. (2009) report (6), we chose ATP sulfurylase (AS) as mitosome marker. When paromomycin induction for 8 hours, we could see that mitosome morphology changes of HM1. Their mitosome morphology changed from large spot to small or background cloudy or

aggregation form. However, TCDC-1198 also have been slightly transformed; TCDC-2126 could not see anything difference (fig. 5). To straighten out organelle changes of paromomycin induction, we will test the different time points and other organelles.

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. The new clinical strains TCDC-2126 had been isolated and cultured from one asymptomatic foreign labors that had been treated by standard therapy. The *AIG1* genes had been indicated to be an important virulence factor (3,4), we will test it soon. The same dose of paromomycin and metronidazole culture condition had been used to understand the drug tolerance between HM1 strain and clinical isolates (fig. 2, fig. 3). These results showed that two clinical strains in compared with standard strain had higher drug tolerance (fig. 2, fig. 3). 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 continue 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 someday.

The proteome data showed that mechanism of paromomycin was a complex mystery, not just necrosis-like phenomenon. We observed that cell had metabolism changes, adhesion molecule reduction, and maybe involve endoplasmic system or organelle damage (table 1, table 2). That means paromomycin have unkown signal pathway or target, not just a protein synthesis inhibitor (7). The act of paromomycin was irreversible reaction that maybe could bind to protein through covalent bond for enzyme invalidation. We would continue to find out the binding target of paromomycin that could demonstrate mechanism of paromomycin and their signal pathway. It will help to development of new drug for parasite disease.

Acknowledgment:

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

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Publication list for this work:

NIL

Table 1. Part of data of paromomycin induction proteome for 8 hours

C8-P8	Protein Name	Protein-ID	Mass(Da)	t-Test-p	Fold
Metabolism	Glyceraldehyde-3-phosphate dehydrogenase	S0AX47	35956	8E-05	-9.8
	Alcohol dehydrogenase	M7VZZ3	42545	0.03058	-4.28
	Glyceraldehyde-3-phosphate dehydrogenase	S0AYU4	36067	0.01474	-1.55
	Phosphoglycerate kinase	S0B0M3	44850	0.0111	-1.5
	UDP-glucose 4-epimerase	C4M746	37838	0.01783	1.52
	Pyruvate phosphate dikinase	Q24801	97899	0.0334	3.07
	Pyruvate:ferredoxin (Flavodoxin) oxidoreductase	M3U3Q3	127636	0.00029	3.14
	Malate dehydrogenase	S0B2B8	40417	0.02047	5.59
	NAD+dependent alcohol dehydrogenase	M2S0B1	91254	0.00729	7
	Glycerol-3-phosphate dehydrogenase	M7W8R7	116226	0.00152	10.37
	Malic enzyme	S0AZR0	53276	0.04302	100
Adhesion molecular	Galactose specific adhesin 170 kDa subunit	M7WQ34	41937	0.00051	-6.88
	Gal/GalNAc lectin heavy subunit	C4LTM0	144328	0.00703	-4.84
	Galactose-specific adhesin 170kD subunit	Q24835	145177	0.03688	-2.2
	Gal/GalNAc lectin Igl1	Q964D2	119513	0.00429	-2.2
	Gal/GalNAc lectin heavy subunit region E	M7X0L8	50575	0.02494	-1.66
Cytoplasm	Hybrid-cluster protein	Q8WR14	60323	0.00212	-1.7
	Fe-S cluster assembly protein NifU	Q8MU40	38921	0.01889	1.91
	Vesicular transport protein Yip1	S0AV18	25766	0.0049	-7.35
	Vesicle-associated membrane protein	M3TQ48	9312	0.01398	-3.17
	Beta prime-COP	Q1EQ29	91246	0.00777	1.67
	Adaptor protein (AP) family protein	Q1EQ27	80916	0.00478	2.34
	Clathrin heavy chain	N9UXX0	192972	0.00013	2.73
	Vacuolar protein sorting 26	Q53UB0	47978	0.01957	7.13
	Actin-related protein 2/3 complex subunit 1A	S0AYJ7	40469	0.00363	9.79
	Actin	S0B0J4	46950	0.00662	15.41

Table 2. Part of data of paromomycin induction proteome for 15 hours

C15-P15	Protein Name	Protein-ID	Mass(Da)	t-Test-p	Fold
Metabolism	UDP-glucose 4-epimerase	S0AYJ6	37779	0.04209	-4.17
	Malic enzyme	S0B0U0	53392	0.01172	-2.74
	Deoxyribose-phosphate aldolase	S0AVD5	23208	0.03864	-2.61
	Phosphoglycerate kinase	S0B0M3	44850	0.00253	-1.29
	Pyruvate phosphate dikinase	Q24801	97899	0.04236	2.22
	Fructose-1,6-bisphosphate aldolase	S0AWU7	36156	0.0014	3.51
	NAD+-dependent alcohol dehydrogenase	N9UXI6	36334	0.00056	5.19
	Enolase	S0AYJ0	47198	0.00964	5.75
	Pyrophosphate-dependent phosphofructokinase	S0AWD4	60162	0.00521	6.01
	Pyruvate:ferredoxin (Flavodoxin) oxidoreductase	M3U3Q3	127636	0.02399	6.22
	Alcohol dehydrogenase	C4M230	95574	0.00017	7.36
	Pyruvate,orthophosphate dikinase	M7X1R2	93599	0.0007	12.47
Adhesion molecular	Galactose-specific adhesin 170kD subunit	Q24835	145177	0.00328	-5.14
	Galactose-inhibitable lectin 170 kDa subunit	B1N3C7	145253	0.00442	-4.2
	Gal/GalNAc lectin Igl1	Q964D2	119513	1E-04	-3.69
	Galactose-specific adhesin 170kD subunit	C4M8N3	145088	0.01818	-3.04
	Gal/GalNAc lectin heavy subunit	C4LTM0	144328	0.00023	-2.71
	Galactose-specific adhesin 170kD subunit	B1N559	142580	0.00554	-2.56
Cytoplasm	Molybdenum cofactor sulfurase	M3TYA6	60486	0.00038	-3.08
	Vesicle-associated membrane protein	M3TQ48	9312	0.00811	-3.04
	Hybrid-cluster protein	Q8WR14	60323	0.00018	-2.58
	Fe-S cluster assembly protein NifU	Q8MU40	38921	0.01735	-2.27
	Clathrin heavy chain	N9UXX0	192972	0.00247	2.03
	Ubiquitin	M2QCI6	8698	0.04422	2.41
	Ubiquitin-activating enzyme	C4M7T9	111879	0.04889	2.46
	Actin binding protein	M2RZP6	10045	0.04417	2.88
	Plasma membrane calcium-transporting ATPase	C4M3X0	119130	0.04523	3.08
	Ubiquitin-conjugating enzyme family protein	C4M310	17361	0.01652	10.24
	vacuolar proton-transporting ATPase subunit	Q24802	67069	0.00673	11.03
	Myosin-2 heavy chain, non muscle	M7WF67	239484	0.04609	11.31
	Actin	P11426	42030	0.0175	12.52
	Actin-related protein 2/3 complex subunit 5	B1N2Q5	14846	0.00443	100
	Circumsporozoite protein	M7W2Y4	19674	0.02088	100
	Coatomer subunit beta	B1N3Z7	45910	0.00167	100
	Dynamin family protein	M3SAI6	74597	0.01292	100

Fig. 1 Six tRNA-STR genotyping analysis of *Entamoeba histolytica* clinical strain

Fig. 1: These were part of tRNA-STR loci sequence data.

Fig. 2 Time series analysis of metronidazole cytotoxicity of *Entamoeba histolytica*

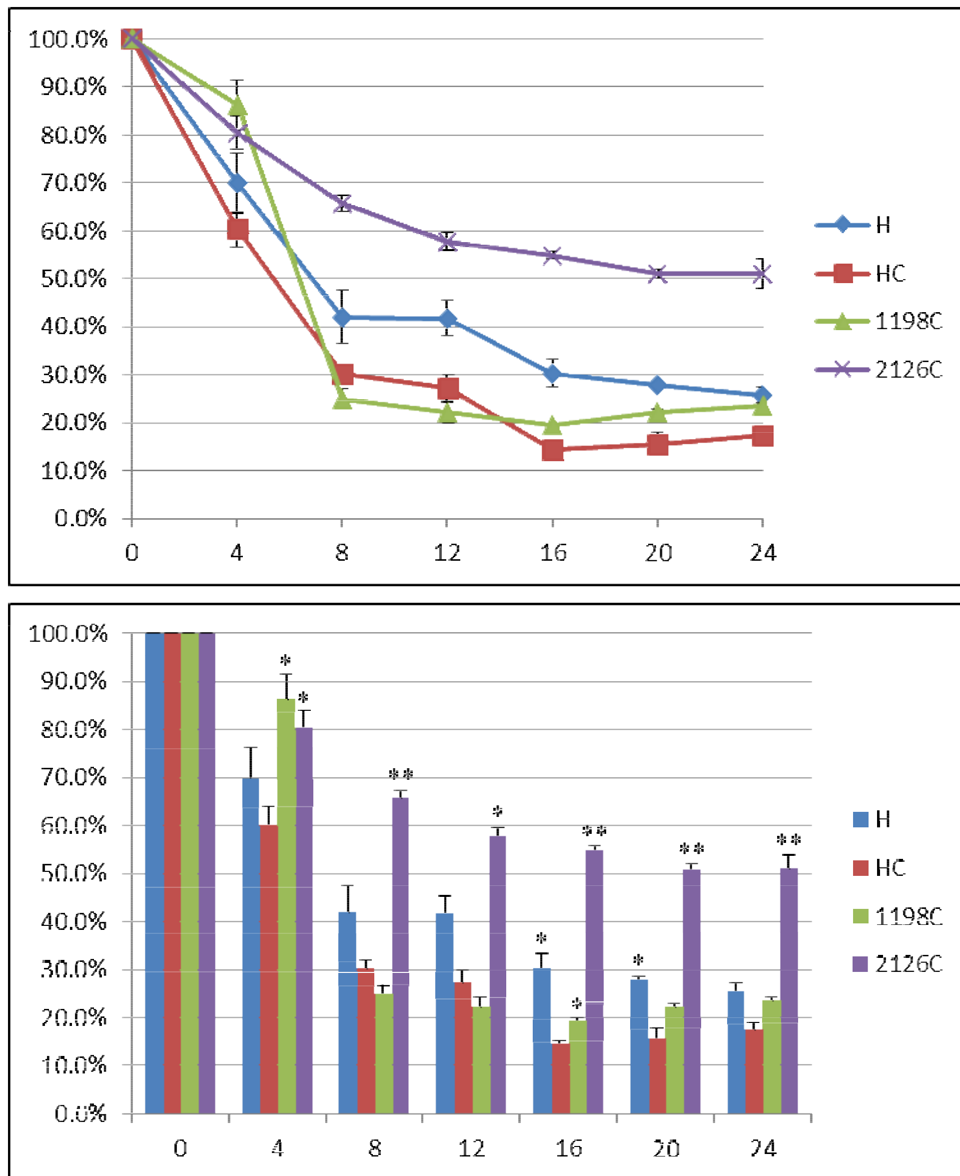

Fig. 2:

This experiment was cell seeding about $5x10^5$ for overnight with Nocodazole (100 nM), which was synchronization drug. Before metronidazole treatment, we changed from the LYI-S-2 medium with Nocodazole to fresh. After incubated with 80 μM metronidazole for indicated time point, we calculated the total cell number. The clinical strains co-culture with *Crithidia fasciculate* (1198C and 2126C), HM1 with *C. fasciculate* (HC), HM1 without *C. fasciculate* (H). Normalization was each strain compared with control group of himself. *: Compared with HC, $p < 0.05$; **: Compared with HC, $p < 0.01$

Fig. 3 Time series analysis of paromomycin cytotoxicity of *Entamoeba histolytica*

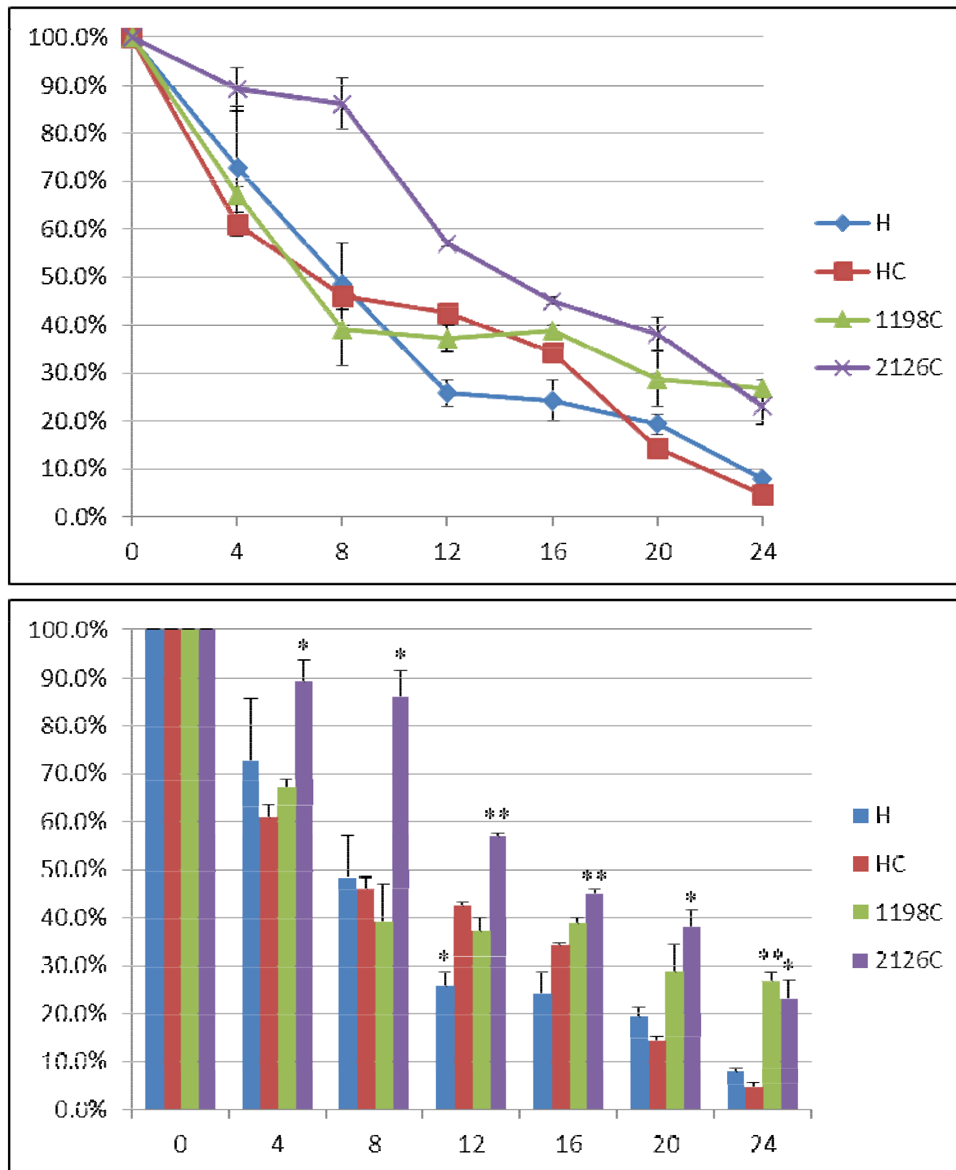

Fig. 3:

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 fasciculate* (1198C and 2126C), HM1 with *C. fasciculate* (HC), HM1 without *C. fasciculate* (H). Normalization was each strain compared with control group of himself. *: Compared with HC, $p< 0.05$; **: Compared with HC, $p< 0.01$

Fig. 4 Total protein coomassie blue staining

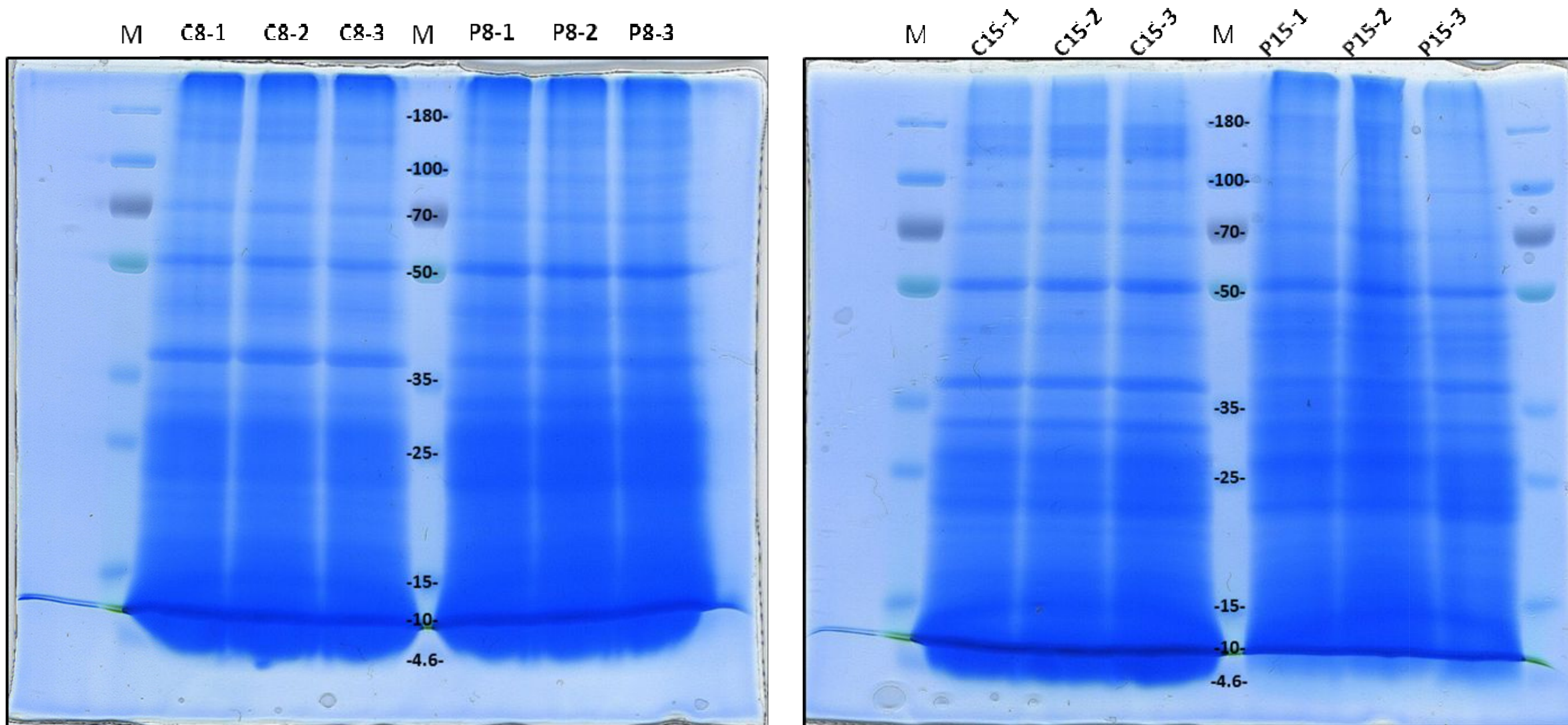

Fig. 4:

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 80 μM paromomycin for indicated time point (8 or 15 hrs), we collect the total cell lysis by lysis buffer (50 mM Tris-HCl (pH 6.8)、1% SDS with protease inhibitor and phosphatase inhibitor). 100 μg/well protein loading, 10 % SDS-PAGE. C (control); P (paromomycin treatment).

Fig. 5 Indirect Immunofluorescence Assay for mitosome morphorgy.

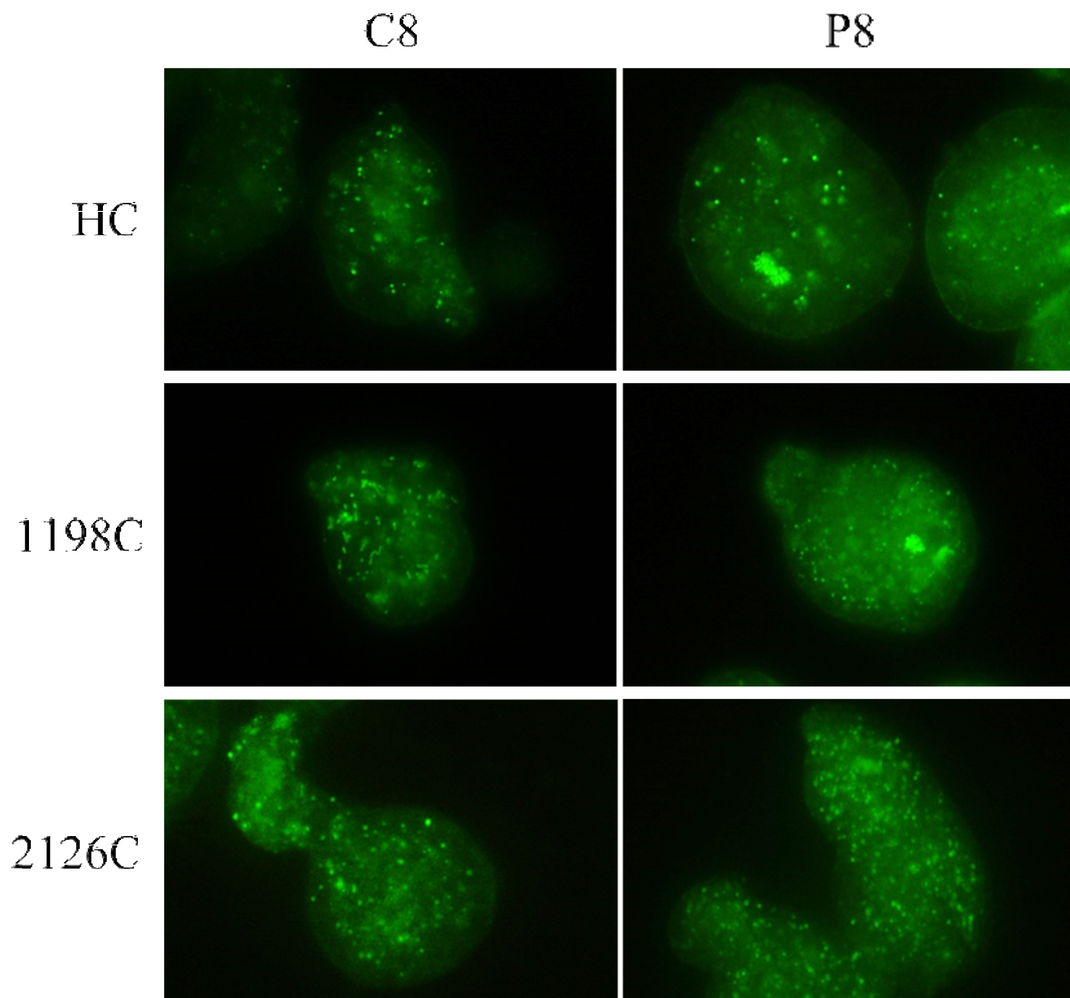

Fig. 5:

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