infrequently in young children [9, 12]. There are few reports of immunocompetent children presenting with respiratory symptoms. Here, we describe a case of bronchitis caused by *B. holmesii* in a young child with asthma.

Case report

A 2-year-old girl was brought to Kishiwada Tokushukai Hospital for evaluation of 2 days of productive cough and 1 day of moderate fever (37.6 °C). She had an asthmatic response during naptime that day. The patient had a history of asthma hospitalization for 4 days within 1 month and had been treated with montelukast, a leukotriene receptor antagonist (4 mg once daily at bedtime). She had previously received three doses of acellular pertussis vaccine.

Physical examination revealed mild wheezes and coarse crackles in her lung sounds, and chest radiography demonstrated bronchitic markings. Bronchitis was initially diagnosed, and a bronchodilator, procaterol (30 μg/day), and a macrolide antibiotic, clarithromycin (15 mg/kg/day), were administered as outpatient treatment (Fig. 1). Salbutamol (1.5 mg) was also administered using a nebulizer. On day 1, the patient's body temperature returned to normal, but the productive cough persisted. The patient returned to the physician on day 2, and blood and antibody tests were performed. Her white blood cell count was 6.6×10^9/l with 56.9 % lymphocytes, and her C-reactive protein level was 15.5 mg/l (reference, <3.0 mg/l). *Mycoplasma pneumoniae* antibody titer assessed by the particle agglutination test (SERODIA MYCO II; Fujirebio, Tokyo, Japan) was 80, indicating that she was antibody negative for *M. pneumoniae* infection. A rapid serodiagnosis kit (ImmunoCard Mycoplasma kit; Meridian Bioscience, Cincinnati, OH, USA), however, yielded a positive result for *M. pneumoniae* infection. The patient's condition was diagnosed as mycoplasmal bronchitis on the basis of her symptoms and the rapid serodiagnosis performed on day 4. The patient experienced an uneventful recovery after a 7-day course of treatment with procaterol and clarithromycin. The duration of fever and productive cough were 2 days and 4–5 days, respectively.

Microbiological investigation was performed on the aspirated sputum collected on day 0 using nucleic acid amplification and culture tests. The nucleic acid amplification test loop-mediated isothermal amplification (LAMP) was performed on the sputum DNA sample for both *M. pneumoniae* and *B. pertussis* on day 4 [13, 14]; however, the LAMP tests were negative for the organisms. The sputum culture showed the presence of a slow-growing, gram-negative bacillus in pure culture on *Bordetella* CFDN agar plates (Nikken Bio Medical Laboratory, Kyoto, Japan) after culture day 7, and the organism was identified as *B. holmesii* by 16S rRNA and *recA* gene sequencing on day 18 (Supplement 1). The bacteria were identified after the patient's recovery because of difficulties in culturing the organism. As a result of these findings, the patient's condition was finally diagnosed as *B. holmesii* bronchitis. Of interest, *B. holmesii* and other organisms were not recovered at 24 h on 5 % sheep blood agar cultured in a CO_2-enriched environment.

In the present case, the *B. holmesii* isolate that was collected from initial culture on *Bordetella* CFDN agar was divided into two subpopulations on third-generation cephalosporin cefotaxime *E* test on Bordet–Gengou agar; isolate-1 formed microcolonies inside the entire inhibition zone, and

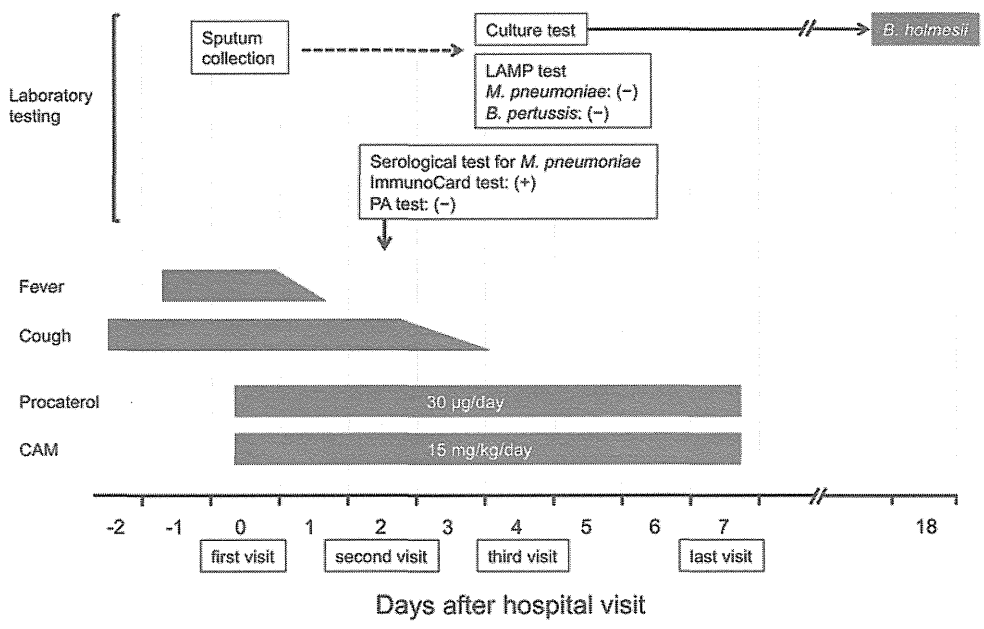

Fig. 1 Clinical course and laboratory findings of a 2-year-old girl with bronchitis caused by *Bordetella holmesii*. Sputum was collected at the first visit (day 0). LAMP assay and sputum culture were performed on day 4. The sputum culture produced a slow-growing, gram-negative bacillus in pure culture on day 11, and the organism was identified as *B. holmesii* on day 18. Salbutamol was administered by nebulizer at a dose of 1.5 mg on days 0 and 2. *M. pneumoniae*, *Mycoplasma pneumoniae*. *LAMP* loop-mediated isothermal amplification, *PA* particle agglutination, *CAM* clarithromycin

Table 1 In vitro susceptibility of *Bordetella holmesii* isolates

Antimicrobial agent	MIC (μg/ml)		
	Isolate-1	Isolate-2	Reference strain[a]
Ampicillin	2	0.25	0.5
Pipercillin	0.064	<0.016	0.032
Cefotaxime	16	2	4
Ceftriaxone	8	1	1
Imipenem	1	1	2
Erythromycin	0.5	0.064	0.5
Clarithromycin	2	0.25	2
Azithromycin	0.25	0.064	0.5
Gentamicin	0.25	0.25	0.125
Minocycline	0.5	0.25	0.25
Norfloxacin	1	0.5	0.5
Ciprofloxacin	0.25	0.125	0.125
Levofloxacin	0.25	0.125	0.064

Minimal inhibitory concentrations (MICs) were determined by *E* test on Bordet–Gengou agar

[a] *Bordetella holmesii* ATCC51541

isolate-2 was obtained from outside the inhibition zone. As shown in Table 1, isolate-1 had higher minimum inhibitory concentrations (MICs) for cefotaxime (16 μg/ml) and ceftriaxone (8 μg/ml) on *E* test, and isolate-2 had lower MICs for the antibiotics (cefotaxime, 2 μg/ml; ceftriaxone, 1 μg/ml). These two isolates also had different MICs for ampicillin, pipercillin, erythromycin, clarithromycin, and azithromycin, similar to cefotaxime and ceftriaxone.

Discussion

Bordetella holmesii infections were recently found to be associated with meningitis, septic arthritis, and pericarditis [11, 15, 16], in addition to bacteremia, endocarditis, and pneumonia. This infection leads to a broad spectrum of clinical manifestations in immunocompromised patients; however, there are few case reports of respiratory illness [7, 17]. Our patient with asthma developed a moderate fever and productive cough as symptoms of bronchitis resulting from *B. holmesii* infection, and she recovered after she was treated with procaterol and clarithromycin. The possibility of mixed infection with other respiratory viruses cannot be excluded because of the lack of laboratory testing for the viruses; however, this case report adds to the evidence on clinical manifestations of *B. holmesii* infection for immunocompetent children.

Respiratory manifestations of *B. holmesii* have been previously described as pertussis-like symptoms in adolescents and adults. Most patients frequently showed persistent cough, paroxysmal cough, post-tussive vomiting, and whoop [8, 9]. *B. holmesii* was also reported to be associated with severe manifestations, weight loss, exertional dyspnea, and a worsening breathlessness without cough in an immunocompetent adolescent [17]. The patient with asthma described here presented with productive cough but not pertussis-like respiratory symptoms. It remains unclear whether the productive cough is caused by *B. holmesii*-induced asthma exacerbations. In a previous study of 23 patients with *B. holmesii* infections, 35 % had occasional asthma or allergies [9]. These data suggest that asthma may be a risk factor for *B. holmesii* infection. Further analyses are needed to examine the relationship between asthma and *B. holmesii* infection.

Previous in vitro susceptibility data suggest that carbapenems and fluoroquinolones may have clinical utility for treating *B. holmesii* infections [3, 15, 16, 18]. The two *B. holmesii* isolates identified in the present patient also had lower MICs as compared to imipenem and fluoroquinolones (norfloxacin, ciprofloxacin, and levofloxacin). Of interest, the two isolates had different MICs for third-generation cephalosporins; one had higher MICs of cefotaxime (16 μg/ml) and ceftriaxone (8 μg/ml), and the other had lower MICs than these antibiotics (cefotaxime, 2 μg/ml; ceftriaxone, 1 μg/ml). In previous reports, *B. holmesii* isolates showed wide ranges of MICs of the cephalosporins (1 to >32 μg/ml for cefotaxime and 1.5 to >32 μg/ml for ceftriaxone [15, 16, 18, 19]. Some *B. holmesii* isolates had higher MICs to the cephalosporins. In the present patient, we observed that *B. holmesii* isolate could be divided into two subpopulations that have different MICs to cephalosporins. To compare these results with those of previous studies, standardized susceptibility testing is required.

Our patient's condition was initially misdiagnosed as mycoplasmal bronchitis on the basis of her clinical symptoms and rapid serodiagnosis of mycoplasmal infection. This misdiagnosis may have been caused by a low specificity (<80 %) of the ImmunoCard IgM assay [20] and difficulties in culturing and identifying *B. holmesii*. Another possible cause of the misdiagnosis was the lack of physician awareness of *B. holmesii* infection in children. In recent years, *B. holmesii* has come to be known as a type of pathogenic bacteria with protean clinical manifestations in adolescents and adults. Our case report demonstrates that *B. holmesii* can be a true pathogen for bronchitis in children with asthma as well.

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Conflict of interest The authors have no conflict of interests to declare.

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Genetic relationship of vector mosquitoes and the vector-borne pathogens between Taiwan and Japan

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Summary:

This project of this year is to study the genetic relationship of vector mosquitoes and vector-borne pathogens between Taiwan and Japan to clarify the taxonomic status of *Culex annulus* and gene flow of *Cx. vishnui* subgroup. The results showed that *Cx. annulus* and *Cx. vishnui* is the same species which share the common gene pool between Taiwan and Japan, even extend to mainland China. The same situation was also applied to the population of *Cx. tritaeniorhynchus* between Taiwan and Japan. Additionally, we also presented our surveillance results of dengue cases in Taiwan in 2012. A total of 207 confirmed imported dengue cases were identified. The travelers were arriving from 11 countries in Southeast Asia, Western Pacific, and the Indian subcontinent. From the acute phase serum samples of imported dengue cases, 44 DENV-1, 35 DENV-2, 17 DENV-3, and 22 DENV-4 strains were identified by PCR. For local dengue outbreaks in Taiwan, a total of 1,271 dengue cases were laboratory confirmed with 36 cases of dengue hemorrhagic fever. Epidemiological study showed that 7 different DENV strains were circulated in Taiwan, including 3 DENV-1 strains circulating in Tainan City, Kaohsiung City, Taoyuan County, New Taipei City, 2 DENV-2 strains circulating in Kaohsiung City, Tainan City, Pingtung County, 1 DENV-3 strain circulating in Kaohsiung City; and 1 DENV-4 strain circulating in Kaohsiung City. Phylogenetic analyses of E gene sequences of DENVs isolated from indigenous and imported dengue cases showed that most epidemic DENVs circulated in Taiwan in 2012 were introduced from Southeast Asia. In addition, a DENV-1 strain introduced from the Central America caused overwinter outbreak in Tainan City during 2011-2012.

Purpose:

To study the genetic relationship of vector mosquitoes and vector-borne pathogens between Taiwan and Japan can provide basic information on the risk of vector-borne infectious diseases. Understanding the epidemiological situations of the diseases and the phenotypic and genotypic characteristics of viruses and vectors contributes to the development of new strategies for control and prevention. This year was focus on Japanese vectors, including *Culex tritaeniorhynchus* Giles, *Cx. annulus* Theobald, and *Cx. fuscocephala* Theobald, which belonged to *Cx. vishnui* subgroup. This subgroup also include *Cx. Pseudovishnui, Cx. perplexus, Cx. Alienus*, and *Cx. Incognitus*. These mosquitoes showed

similar phenotype and breeding habits, result in confusing us about mosquito classification. As a result, we applied the molecular biology methods to further classify the mosquito species. Additionally, dengue virus was also studied.

Method:

1. Mosquito study

(1)Mosquito collection and identification

We collect the at least 10 larva or adult mosquitoes (*Cx tritaeniorhynchus* Giles, *Cx. annulus* Theobald, *Cx. psudovishnu*i) in northern, southern, middle, eastern Taiwan and Philippine. We also apply the *Cx tritaeniorhynchus* Giles, *Cx. annulus* Theobald, *Cx. psudovishnu*i DNA sequence or mosquitoes from National Institute of Infectious Diseases for further study. The phenotype identification was performed by an experience-rich expert by stereo dissecting microscope follow Lien's method.

(2)Mosquito DNA extraction

First, pipet 20μl QUIAGEN protease into the bottom of a 1.5ml microcentrifuge, and added 200μl sample to the microcentrifuge tube. To add 200μl buffer AL to sample and mixed by pulse-vortexing for 15 seconds, than incubated at 65℃ for 10 min. Briefly centrifuge the tube and removed drops from the inside of the lid. Added 200 μl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid. Carefully apply the mixture to the QIAamp Mini spin column without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 500 μl Buffer AW1 without wetting the rim. To close the cap and centrifuge at 8000 rpm for 1 min and place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate. Finally, place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 μl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 8000 rpm for 1 min.

(3)PCR

After collection the mosquitoes DNA sample, we next performed polymerase chain reaction. To add 10 X buffer (Invitrogen) 2.5μl, 1μl forward primer (4 mM), 1μl reverse primer (4 mM), 2μl dNTP (2.5mM), 1μl MgCl2 (50 mM), 0.2μL Taq enzyme

(Invitrogen), 1μl DNA (10-100ng/μl), and 16.3μL distilledwater to a final volume 25μl. We use the specific primer to amplify the target sequence that we want. 18SF (GTAAGCTTCCTTTGTACACACCGCCCG), 28SR1 (GGGGTAGTCACACATTATTTG), 18SF4 (GGCTGGTCAGTCTATATCGC), 58SR1 (TTGCGGATGACCAGTCG). Two sets of primers including Flavivirus-specific primers (FL-F1: 5'-GCCATATGGTACATGTGGCTGGGAGC-3'; FL-R3: 5'-GTKATTCTT GTGTCCCAWCCGGCTGTGTCATC-3; FL-R4: GTGATGCGRGTGTCCCAGCCR GCKGTGTCATC-3') and JE virus-specific primers (10F : 5'-CTGGGA ATGGGCAAT CGTG-3', 325R:5'-TGTCAATGCTTCCCTTCCC-3') were used for real-time RT-PCR. Following the PCR program to amplify sequence: Activation, 3min at 95℃(1 cycle), Denaturation, 30sec at 95℃(40 cycle), Annealing, 30sec at 52℃(40 cycle), Extension, 1min at 72℃(40 cycle), and final Extension, 4min at 72℃ (1 cycle).

(4) DNA sequence analysis and phylogenetic analyses

We analysis DNA sequence base on 18S rRNA, partial sequence of internal transcribed spacers2(ITS2), 5.8S rRNA gene, and full internal transcribed spacers 1(ITS1) sequence in mosquito ribosomal DNA spacer. Multiple sequence alignment was performed by using MEGA version 5. Genetic distances were calculated by using Kimura 2-parameter distance algorithm with 1,000 bootstrap replicates. We used neighbor-Joining method to generate the phylogenetic trees.

2. Dengue viruses

(1)Clinical samples and laboratory diagnosis

Human serum samples from clinically suspected DENV and other arbovirus infections were submitted to Research and Diagnostic Center, Centers for Disease Control, Taiwan (Taiwan CDC), Department of Health, for laboratory diagnosis. A confirmed dengue case was defined as febrile illness associated with a positive real-time reverse transcription (RT)-PCR test, isolation of DENV, or a ≥4-fold increase in DENV-specific IgM and IgG antibodies. A multiplex one-step real-time RT-PCR was developed to simultaneously detect and differentiate various flaviviruses and alphaviruses in the acute-phase serum samples using group-specific and virus-specific primers. In addition, a flavivirus/alphavirus-specific capture ELISA was developed to detect and differentiate various flavivirus/alphavirus infections.

(2)Virus isolation and identification

DENVs were isolated from the acute phase serum samples of confirmed cases. The virus isolation was performed using mosquito cell line (clone C6/36 of Aedes albopictus cells). For each acute phase serum, 4 μl of serum sample was diluted in 200 μl cultured medium

（RPMI , Gibco/BRL, Life Technologies, containing 1% FCS）and added to a 96-well microtiter plate, 50 μl/well in quadruplicate. Then, 105 cells/100 μl/well of C6/36 cell line were added into the microtiter plate and incubated at 30℃ for 2-7 days. Cells were harvested and virus isolates were identified by the indirect fluorescent antibody test with virus group-specific and serotype-specific monoclonal antibodies.

(3)Primers used for RT-PCR and nucleotide sequencing of DENV

The diagnostic tests for flavivirus infection from fevered patients on the basis of the results of one-step SYBR Green I-based real-time RT-PCR and envelope/membrane-specific capture IgM and IgG ELISA had been described previously. To screen viremic fever patients with alphavirus infection, a multiplex one-step SYBR Green I-based real-time RT-PCR was developed. A cocktail consisted with three sets of primers were mixed and used for RT-PCR screening. The alphavirus-specific primer set （AL-2: 5'-AAG CTY CGC GTC CTT TAC CAA AG-3' and AL-3: 5'-GTG GTG TCA AAC CCT ATC CA-3'） targeted a consensus region of the nonstructural protein 1 (nsp1) genes to detect all alphaviruses. Positive results were then confirmed by gene sequence analysis, virus isolation, and serological test.

(4)Preparation of viral RNA, RT-PCR amplification and nucleotide sequencing

Viral RNAs were extracted from either acute phase serum samples or culture supernatant of C6/36 cell line infected with each of the isolated DENV or CHIKV strains using the QIAamp viral RNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Nucleotide sequences of partial NS5 gene fragment of DENV were determined directly from acute phase serum samples using RT-PCR product of one-step SYBR Green I quantitative RT-PCR . Partial NS5 gene sequencing was routinely performed to detect and differentiate serotype and genotype of the newly identified DENVs. For full-length structure gene sequencing, extracted viral RNA from culture supernatant of C6/36 cell line infected with each of the isolated DENV strains was used as a template for cDNA synthesis, which subsequently was used for PCR amplification. Two overlapping PCR products spanning the full-length structure gene were purified from agarose gels and directly sequenced in both directions using ABI Prism automated DNA sequencing kit and ABI Prism 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's protocol. Overlapping nucleotide sequences were combined for analysis and edited with the Laser software package (DNASTAR Inc, Madison, WI).

(5)Phylogenetic analysis

Phylogenetic analyses were conducted using PHYLIP (version 3.6) or MEGA version 5. Genetic distances were calculated by using Kimura 2-parameter distance algorithm with

1,000 bootstrap replicates. Neighbor-Joining method was used to generate the phylogenetic trees.

Result:

1. Mosquito study

We collected the mosquito samples in Taiwan, all mosquitoes carried to laboratory for further analysis. In Taiwan, we also collected mosquitoes in different areas, especially; we go to Wu-Wei Gang waterfowl, the protected areas in Yilan county. We detected the flavivirus and Japanese encephalitis virus in these collected mosquitoes, a total of 497 *Cx. tritaeniorhynchus* females adults were grouped by date into 11 pools, and were analyzed for both flavivirus and JE virus infections by the real-time PCR. We didn't detect any flavivirus or JEV signal by real time PCR in these mosquitoes (Table 1).

After collection of mosquito samples, we created the *Cx. annulus/ Cx. vishnui* gene map. We aligned the 18S rRNA and 5.8S rRNA partial gene sequence and full length of internal transcribed spacers 1, and then did phylogenetic analysis. We analyzed 83 *Cx. annulus* DNA sequences, and one sequence from China, eight sequences from Japan. The multiple sequences alignment showed there was 96-100% similarity in these sequences. The phylogenetic analysis indicated that the Japan *Cx. vishnui* can't form a specific group, but grouped to other mosquito sequences collected from Taiwan. Four *Cx.. vishnui* sequences (CV-JP244-1, CV-JP205-1, CV-JP622 and CV-JP494-1) grouped to other 31 *Cx. annulus* sequences from Taipei city, Kaohsiung city, Yunlin county, Hualien county, and Tainan. Two sequences (CV-JP56R and CV-JP54) grouped to other 12 *Cx. annulus* sequences from different Taiwan areas, and other two *Cx. vishnui* sequences (CT11and CT-JP50-1) grouped to 17 *Cx. annulus* sequences from Taiwan, and one from China. (Fig.1)

We also analyzed the *Culex tritaeniorhynchus* gene map by 18S rRNA and internal transcribed spacers 2 (ITS2) partial sequence, internal transcribed spacers 1(ITS1) and full length of 5.8S rRNA. The mosquito samples also collected in Taiwan, and receive the related gene from Japan. After sequences alignment, there was 96-100% similarity between all the sequences. The phylogenetic tree showed the sequences from Japan can't grouped as a specific cluster, eight samples from Japan (CT-JP12-1, CT-22, CT-JP49-1, CT-JP50-2, CT-JP6-2, CT-JP9-1, CT-JP51-1 and CT-JP9-2) were grouped to twenty four sequences from Taiwan; other four samples from Japan (CT-JP15-2, CT-JP8-2, CT-JP8-1, and CV-JP15-1) also grouped to eleven sequences from Taiwan. (Fig.2)

2. The Epidemiology of Dengue

(1)Imported dengue cases in Taiwan, 2012

A total of 207 laboratory confirmed imported dengue cases were identified in Taiwan in 2012. Table 1 showed the summary of countries of origin and the DENV serotypes of

imported cases. The imported cases were arriving from 11 countries. Most of these imported cases were infected in the Philippines, Indonesia, Vietnam, and Thailand. DENV-1 and DENV-2 are the predominant serotypes of imported DENV strains. Among them, 42 DENV-1, 23 DENV-2, 12 DENV-3, and 19 DENV-4 strains were isolated. The nucleotide sequences of complete E gene sequences of DENV strains were determined and deposited in Taiwan Pathogenic Microorganism Genome Database for molecular epidemiological analysis.

(2) Multiple dengue epidemics in Taiwan, 2012

For local dengue outbreaks in Taiwan, a total of 1271 dengue patients were laboratory confirmed with 36 cases of DHF in 2012. The complete E gene sequences of DENV strains isolated from representative indigenous dengue cases were determined. Representative indigenous cases were selected based on the information of epidemiological investigation and preliminary results of partial NS5 gene sequences showing different infection time, infection place, and DENV serotypes and genotypes of these patients. Sequence analyses of DENV isolates obtained from acute phase serum samples of indigenous cases showed that 7 different DENV strains (3 DENV-1, 2 DENV-2, 1 DENV-3, and 1 DENV-4) were circulated in Tainan City (744 cases), Kaohsiung City (507 cases), Taoyuan County (7 cases), New Taipei Cities (5 cases), and Penghu County (2 cases).

(3) Nucleotide sequencing and phylogenetic analysis

Phylogenetic analyses of E gene sequences of DENVs isolated from indigenous and imported dengue cases showed that epidemic DENVs circulating in Taiwan in 2012 were likely introduced from the Americas, the Philippines and Cambodia (DENV-1); Indonesia and Thailand (DENV-2); Thailand (DENV-3) and the Philippines (DENV-4). Figure 3 shows the phylogenetic tree of complete E gene sequences of DENV-1 strains. Phylogenetic analysis showed that genotype I of DENV-1 contains virus strains isolated from imported cases from Indonesia, Malaysia, Myanmar, Cambodia and Vietnam. Genotype II of DENV-1 contains virus strains from the Philippines. Genotype III of DENV-1 contains virus strains from India and Malaysia. A DENV-1 strain, D1/Taiwan/700TN1109a/2011, is the major epidemic strain circulating in Tainan City during 2011-2012. Notably, this strain belonged to genotype III and is closely related to virus strains from Haidi in Central America. Figure 4 showed the phylogenetic tree derived from complete E gene sequences of DENV-2. Phylogenetic analysis showed that DENV-2 strains isolated from imported cases from Vietnam, Cambodia, and Thailand in 2012 belonged to the Asian genotype 1. DENV-2 strains isolated from imported cases from the Philippines, Indonesia, India and Bangladesh belonged to the Cosmopolitan genotype. A DENV-2 strain, D2/Taiwan/KH/2012, which belonged to the Cosmopolitan genotype, is the major epidemic strain circulating in Kaohsiung City in 2012. This strain is closely related to virus strains from Indonesia.

Discussion:

This study proof that the *Cx. vishnui* mosquito in Japan and *Cx. annulus* in Taiwan was the same species, from the phylogenetic analysis, we know that there was no geographic specific cluster. In Taiwan, previously study showed the phenotype of *Cx. vishnui* was different with *Cx. annulus*, so finally, this species mosquito was named the *Cx. annulus*. The molecular biology method clearly indicated *Cx. vishnui* and *Cx. annulus* was the same species and share common gene pool.

The increase in international trade and tourism facilitated the spread of disease vectors and pathogens. Our results showed the constant importation of various DENV strains into currently non-endemic areas, including Japan and Taiwan. Molecular epidemiologic studies analyzing DENV strains isolated from imported and indigenous cases showed that different serotypes, genotypes, and/or strains were responsible for outbreaks that occurred each year, and the epidemic strains disappeared with the ending of each local outbreak in Taiwan. Notably, our study showed that epidemic strains circulating in Taiwan were all introduced from Western Pacific and Southeast Asia, except an epidemic strain that circulating in Tainan City during 2011-2012 belonged to genotype III of DENV-1, which is closely related to virus from the Central America. The results demonstrated that local mosquitoes in Taiwan are competent vectors for transmission of a variety of DENV strains circulating in Southeast Asian, Western Pacific as well as Central America. More effective and efficient control program of dengue, including various surveillance systems, network of rapid diagnostic laboratories, and rapid response carried out by central and local health departments would be needed to control dengue and maintain Taiwan as a non-endemic country.

Virus detected	Total pools tested	Positive pools	No. of *Culex tritaeniorhynchus* tested	
			May	September
JE virus	11	0	39	458
Flavivirus	11	0	39	458

Table 1. Virus infection in field-caught *Culex tritaeniorhynchs* adults collected in Wu-Wei Gang waterfowl protected areas in Yilan.

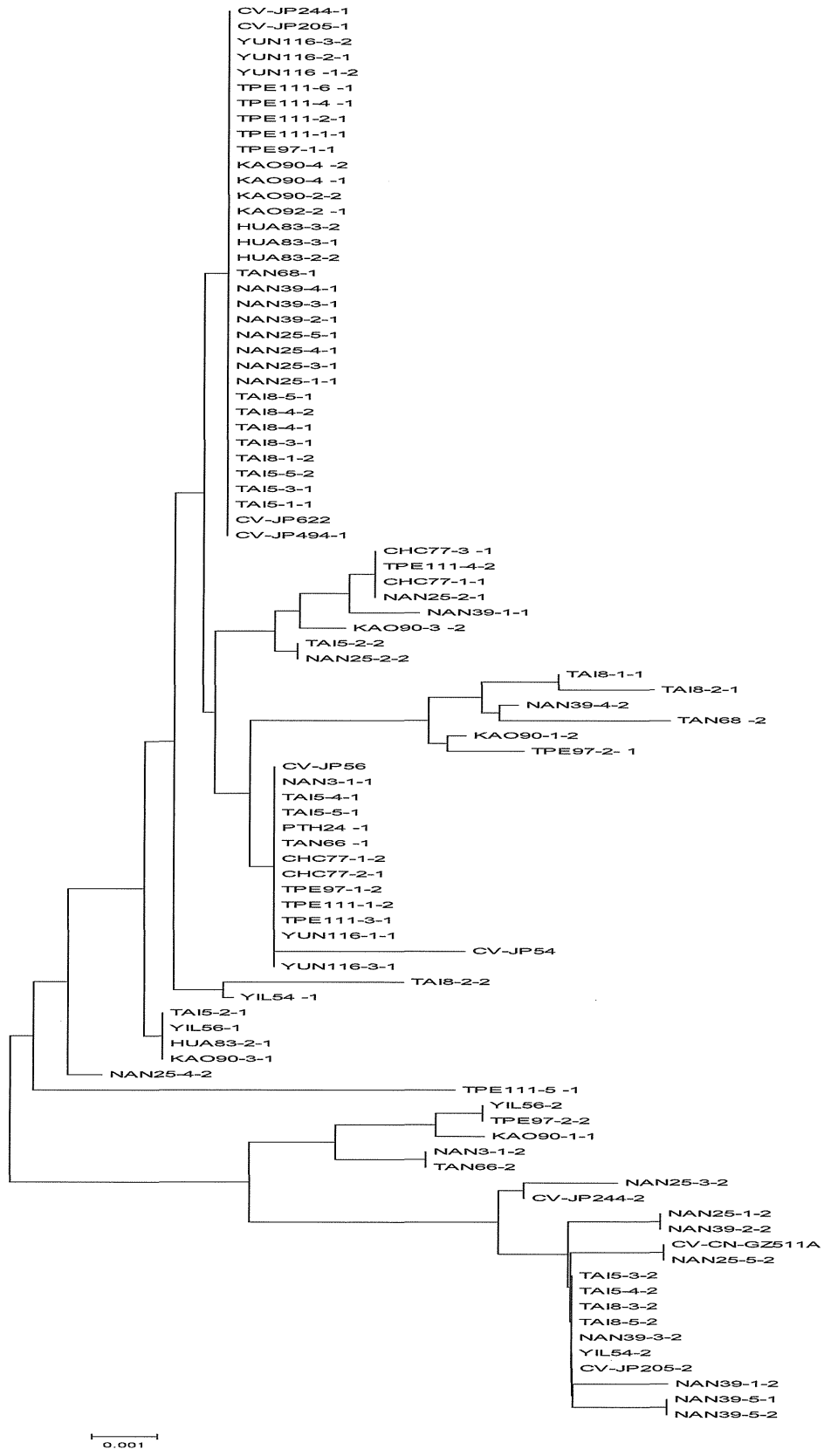

Fig.1 The phylogenetic analysis of *Cx. annulus* (Taiwan) and *Cx. vishnui* (Japan). All the sequence didn't form the specific group, but grouped with other sequence originated from Taiwan.

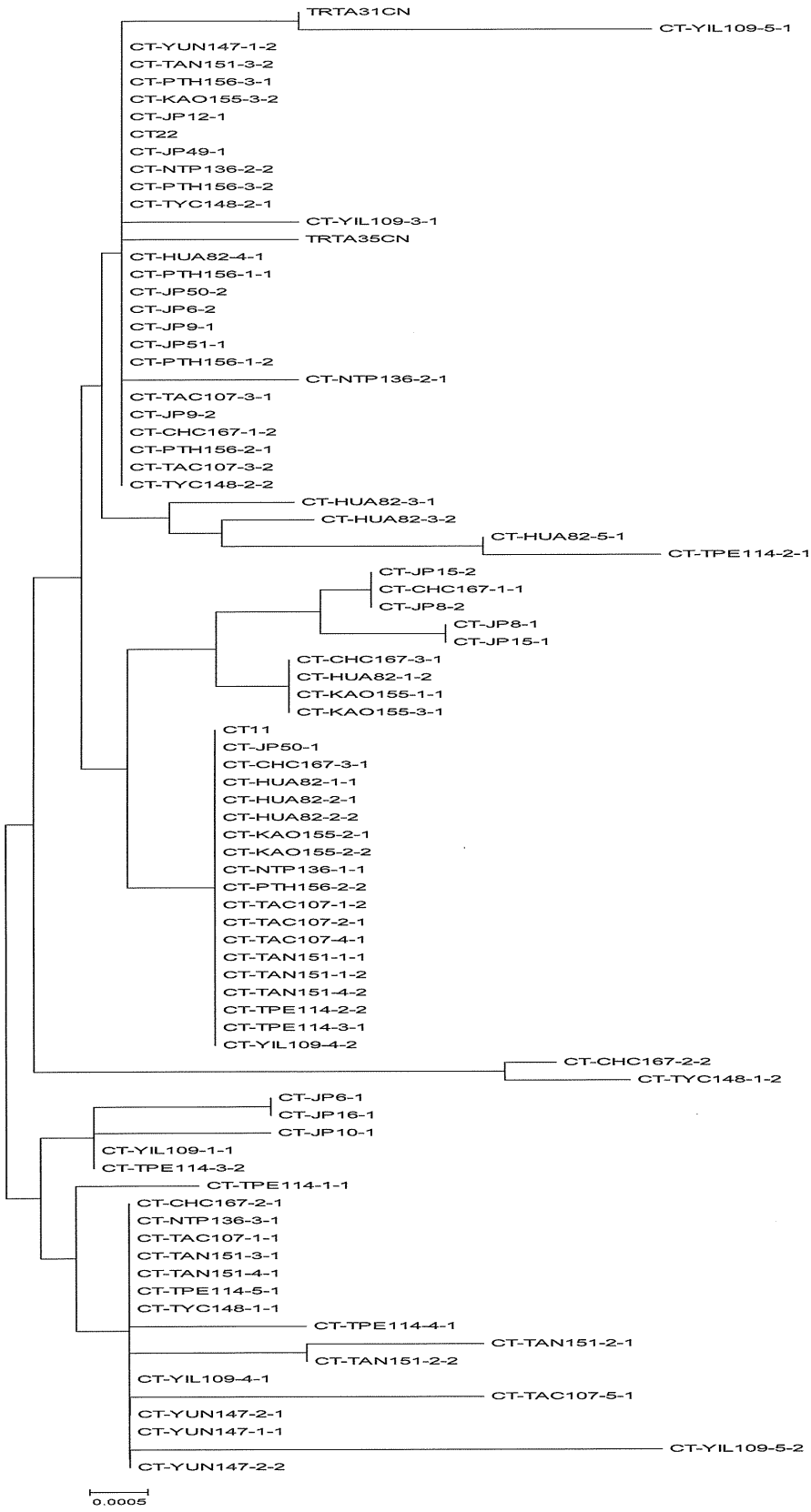

Fig.2 The phylogenetic analysis of *Culex tritaeniorhynchus* from Japan and Taiwan. All the sequence didn't form the specific group, but grouped with other sequence originated from Taiwan.

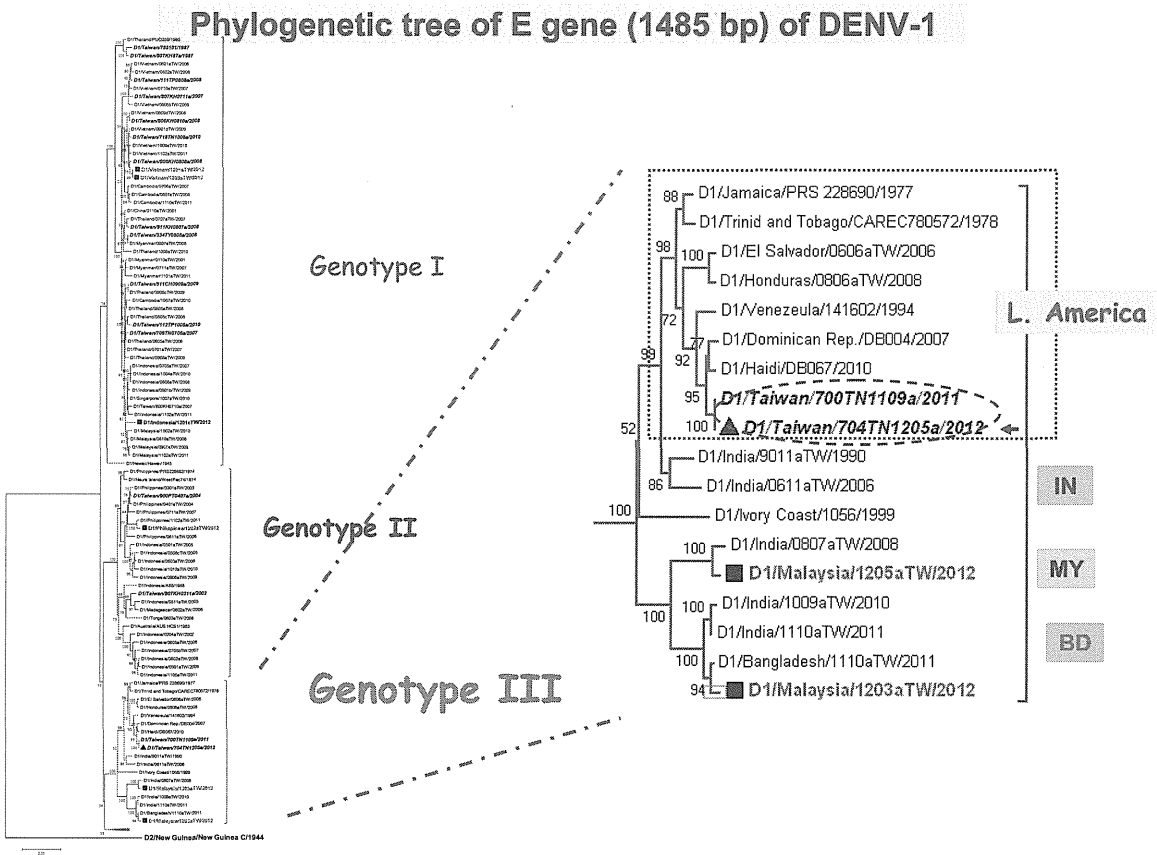

Figure 3. Phylogenetic trees derived from full-length E gene sequences of DENV-1. DENV-1 strains isolated from imported and indigenous cases in Taiwan, 2012, are designated in full squares and full triangles, respectively. The trees were constructed by the neighbor-joining method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation

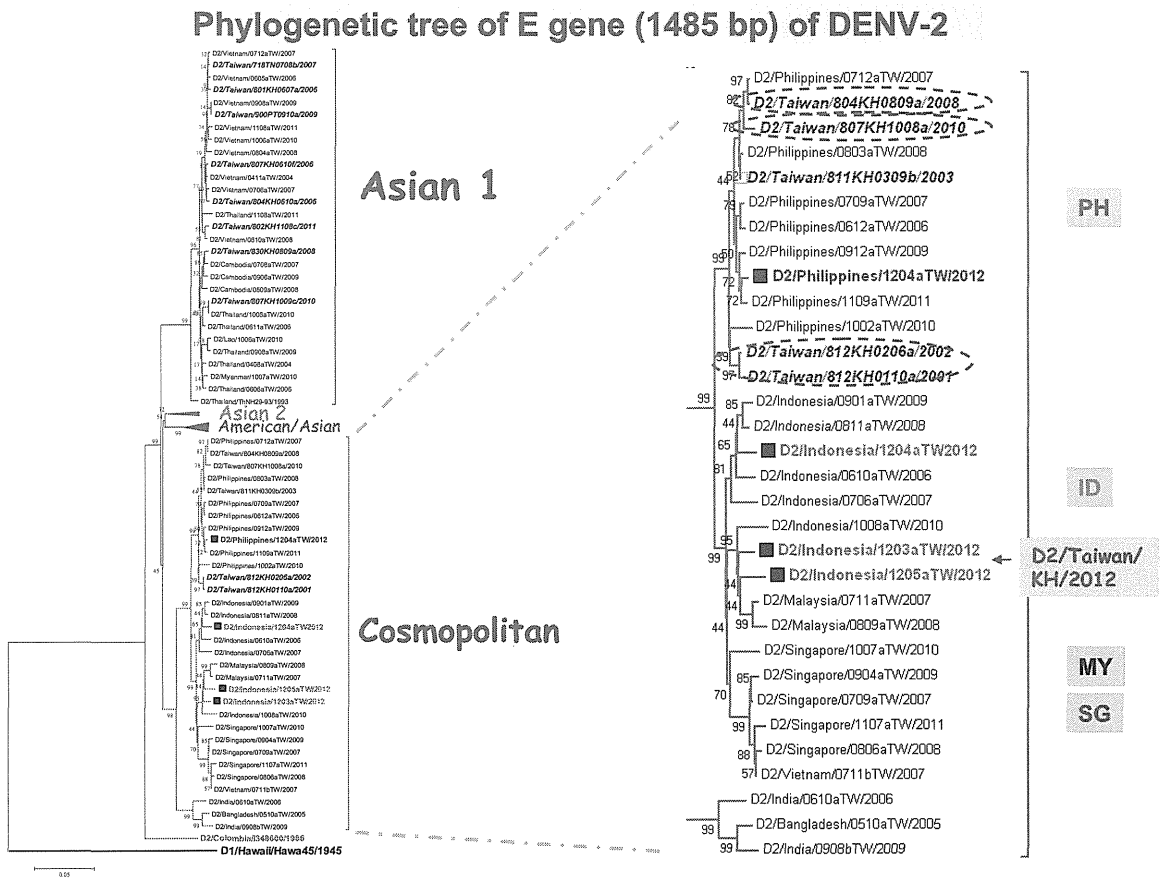

Figure 4. Phylogenetic trees derived from full-length E gene sequences of DENV-2. DENV-2 strains isolated from imported cases in Taiwan, 2012, are designated in full squares. The trees were constructed by the neighbor-joining method. Viruses were identified using the nomenclature of serotype/country/strain/year of isolation

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Publication list for this work:

NIL

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°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°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°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℃