VIROLOGY RESEARCH DIVISION

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During the period of 2016, the Virology Research Division was involved in three main research areas: namely viral diarrhoea, arbovirology and viruses causing acute respiratory infections. The research projects were mostly involved in disease surveillance of viral infections for timely prevention of disease outbreaks. Also, some of the studies were aimed to monitor the emergence of new viral strains or subtypes to provide base-line data for the formulation of effective candidate vaccines and for elucidating the contribution of viral genetics to the changing patterns of disease.

RESEARCH PROJECTS

1. COMMUNICABLE DISEASES

1.1. DIARRHOEA/ DYSENTERY

1.1.1. Surveillance of rotavirus diarrhoea in Yangon Children Hospital (2016)

Rotavirus gastroenteritis (RVGE) is the leading cause of severe diarrhoea affecting young children worldwide. To reduce the burden of RVGE, prevention by vaccination is the most effective strategy. In consideration of rotavirus vaccine (RV), not only epidemiological information like prevalence, age and gender distribution, seasonal variation and severity of disease is required, but also identification of currently circulating genotypes are crucial for evaluation of the effectiveness of a vaccine. Therefore, this study was carried out to determine the epidemiology of rotavirus diarrhoea and genotype distribution among less than 5 years old children attending the three medical wards of Yangon Children Hospital. Stool specimens not less than 3 ml were collected from diarrhoeic children as soon as possible after hospital admission and transported daily to the laboratory in the Virology Research Division. From January to December 2016, a total of 820 stool samples were collected and clinical features were recorded in the Case Report Form. All the samples collected were tested for the presence of rotavirus antigen by a commercial enzyme immunoassay kit (ProSpecT^{TM} Rotavirus from OXOID, UK). Among 820 samples tested, rotavirus was detected in 376 (46%) of cases. Rotavirus positivity was the highest in 6-11 months age group (50%) and

during the cooler, drier months of the year (January-February and November-December). The majority (78%) of the rotavirus positive cases were severe diarrhoea according to the Vesikari clinical severity scoring system. From rotavirus positive samples, 55 samples were selected and analyzed by RT-PCR for genotyping. Regarding G genotype, G9 accounted for 33% (18/55), followed by UT 23% (13/55), G1 18% (10/55), G2 and G3 7% each (4/55), G12 and G1+9 4% each (2/55), G2+9 and G3+9 2% each (1/55). As for P typing, 46/55 (84%) was P[8], 7/55 (13%) was UT and 2/55 (3%) was P[6]. The most common combination was G9P[8] followed by UTP[8] and G1P[8]. Continuous surveillance of RVGE is an important platform which will provide vital information for considering the target population and timing of vaccination plan in the future by policy makers and vaccine programmers and will also be enable monitoring the effectiveness and long-term impact of vaccination on rotavirus epidemiology and on circulating rotavirus strains.

Percent distribution of G genotypes by year (2012-2016)

	G1	G2	G3	G9	G8	G12	G1+ G12	G9+ G12	G1+ G9	G2+ G9	G3+ G9	Untyp -able
2012	24.0	13.0	1.0	7.0	0.0	42.0	1.0	2.0	0.0	0.0	0.0	10.0
2013	42.0	29.0	0.0	22.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	6.0
2014	18.0	7.0	0.0	63.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.0
2015	0.8	2.4	0.0	81.6	0.0	0.0	0.0	0.0	0.8	1.6	0.0	12.8
2016	18.0	7.0	7.0	33.0	0.0	4.0	0.0	0.0	4.0	2.0	2.0	23.0

Percent distribution of P genotypes by year (2012-2016)

	P[4]	P[6]	P[8]	P[9]	P[4]+ P[6]	P[4]+ P[8]	P[6]+ P[8]	P[9]+ P[8]	Untypable
2012	13.0	15.0	54.0	1.0	0.0	0.0	5.0	1.0	12.0
2013	29.0	6.0	54.0	0.0	0.0	0.0	0.0	0.0	11.0
2014	2.0	2.0	70.0	0.0	0.0	2.0	0.0	0.0	24.0
2015	0.8	4.8	58.4	0.0	0.0	0.8	3.2	0.0	32.0
2016	0.0	3.0	84.0	0.0	0.0	0.0	0.0	0.0	13.0

1.1.2. Hospital-based surveillance of intussusception and possible association with rotavirus infection among children in Yangon (2016)

The rotavirus vaccine “RotaShield” was licensed in 1998 and withdrawn in 1999 because of the demonstrable association between receipt of this vaccine and intussusceptions. Since then, the important first step for making decisions regarding conducting trials of introducing rotavirus vaccine in developing countries is to understand the epidemiology and baseline risk of intussusception in these settings. With this concept, surveillance for intussusception among children less than 2 years of age admitted to the surgical wards of Yangon Children Hospital was conducted. In the year 2016, among 2782 less than two years old children admitted to the surgical wards of Yangon Children Hospital, 86 cases were diagnosed as intussusception. Age range of patients was from 4 to 12 months and presented with bloody mucus diarrhoea, vomiting and fever. Diagnosis for intussusception was done by Ultrasound or Barium X Ray or both. Most cases were Ileocolic type. Out of 86 intussusception cases, stool samples were collected from 19 cases and tested for the presence

of rotavirus by a commercial ELISA kit (ProSpecT^{TM} Rotavirus, Oxoid, UK). Rotavirus was not detected in all cases. This data will provide base-line data for investigators and programmers in balancing the risk and benefit in post vaccination period in the future.

Intussusception cases admitted to YCH from 2012 to 2016

	2012	2013	2014	2015	2016
Number of <2 year admission	1711	679	3146	5396	2782
Number of Intussusception cases	44	36	32	73	86
Number of stool collected	9	-	7	12	19
Rota Ag Test Positive by ELISA	0	-	0	0	0

1.1.3. Detection of norovirus in children less than five years of age admitted to Yangon Children Hospital for diarrhoea

Norovirus is the second most common cause of viral diarrhoea in children under age five around the world, next to rotavirus. The data on norovirus in many low and middle income countries is still insufficient to describe the epidemiology of the disease and circulating strains across different regions although such information is critical when considering vaccine design and vaccine implementation strategies. In Myanmar, there is no study on detection of norovirus and no baseline data yet although norovirus is on the rising trend and of increasing global interest. Therefore, this study aimed to investigate the proportion of diarrhoea caused by norovirus in children less than five years of age in hospital setting for the first time. A total of 142 stool samples which has been screened for rotavirus by ELISA and negative were tested for the presence of norovirus by IDEIA^{TM} Norovirus EIA kit. Norovirus was detected in 9/142 (6.3%). Among 9 positive samples, 7 (78%) was male and 2 (22%) was female. Regarding affected age, all positive cases were older than 6 months and under 2 years of age; 5 (56%) of 6-11 months age group and 4 (44%) of 12-23 months age group and it is in accordance with other reports. Although norovirus was reported to be more prevalent in winter months, in this study, norovirus positive cases were detected in February (1 case) March (1 case), April (2 cases), July (1 case), October (1 case) November (1 case) and December (2 cases). All cases (100%) were presented with diarrhoea, vomiting (8, 89%) and fever (4, 44%), some degree of dehydration (4, 44%) and only 3 (33%) needed rehydration by IV. According to the Vesikari clinical severity scoring system, 6 (67%) were severe category and 3 (33%) were moderate category. The mean hospital stay is 2.2 days (SD ±0.83) and there was no mortality among positive cases. This information is the first and baseline data of norovirus in under five years old children population hospitalized for diarrhoea.

1.2. DENGUE HAEMORRHAGIC FEVER

1.2.1. Dengue virus serotypes among dengue haemorrhagic fever patients in Yangon Children Hospital (2016)

This study was carried out to determine the prevailing dengue virus serotypes in children with dengue infection admitted to the medical wards of Yangon Children Hospital. From January to December 2016, a total of 668 serum samples were collected from clinically diagnosed dengue patients and serological confirmation was done by Immuno-chromatographic test (SD BIOLINE Dengue Duo NS1 Ag and IgG/IgM). Out of 568 serum

samples tested, 337 (59%) were serologically confirmed dengue infection. Among them, 129 (38%) was found to be secondary dengue infection and 208 (62%) was primary infection. Regarding gender distribution, 169 (50%) was male and 168 (50%) was female. Dengue virus infection was most prevalent in 5- 9 years age group (33%) followed by 9-12 years age group (29%), 3-5 years age group (15%), >12 years age group (10%), 1-3 years age group (8%) and least prevalent in infants (5%). Dengue cases were admitted to the hospital year round as previous years. However, the peak occurrence of dengue cases was changed from June-July to November –December. Regarding severity, majority of the cases were presented with DHF I (56%) followed by DHF II (25%), DSS (DHF III + IV) 8%, infant dengue (3%) and the diagnosis on discharge of the rest 8% was changed to other than dengue. Some positive samples with early fever days (<5 days) and some acute seronegative samples were subjected to virus isolation by tissue culture in C6/36 mosquito cell lines with 24 well culture plates and further typed by indirect Immunofluorescent Antibody Technique with serotype specific monoclonal antibodies to the four dengue viruses. Of the 35 samples subjected to isolation, dengue virus was isolated from 5 samples, accounting for 14% isolation rate. Among the isolated viruses, 4 samples (80%) were DENV1 and 1 (20%) was DENV4. The DENV 4 was from DHF I case, two DENV1 were from DHF I and two were from DHF II. Continuous and comprehensive virological surveillance is crucial for identification of currently circulating strains and timely detection of new viral strains, which is essential for the prediction of occurrence of outbreaks and severity of the disease and provides beneficial information for preventive and control interventions.

Distribution of Dengue virus serotypes from 2012-2016

Year	Total isolates / RT-PCR	DENV-1	DENV-2	DENV-3	DENV-4	Mixed (DENV-1+4)
2012	7	2	1	0	3	1
2013	36	34	0	0	2	0
2014	30	14	5	2	9	0
2015	20	14	2	1	0	3
2016	35	4	0	0	1	0

1.1. ACUTE RESPIRATORY INFECTIONS

1.3.1. Influenza virus

1.3.1.1. Molecular characterization of influenza virus circulating among children in Yangon Children Hospital (2016)

Influenza causes human epidemics and occasionally causes pandemics for centuries. Influenza A virus (seasonal subtype: H3N2 and H1N1) and influenza B virus (Victoria Lineage and Yamagata lineage) usually circulate among people worldwide. Occasionally avian influenza A H5N1 virus infects humans causing local outbreaks all over the world. Determining the predominant subtype of influenza A virus or predominant linage of influenza B virus helps the assessment of influenza outbreak. This study was conducted with the aim to determine the predominant subtype of influenza A virus or predominant linage of influenza B virus among children attending Yangon Children Hospital. A total of 153 children with influenza-like illness were recruited at Out Patient and Emergency Department of Yangon

Children Hospital from January to December 2016. Nasopharyngeal swabs were taken from them and viral RNAs were extracted from the specimens by QIAamp® Viral RNA Mini Kits. Matrix genes of influenza A virus and influenza B viruses were detected by conventional multiplex PCR. Influenza A virus positive samples proceeded to subtyping by conventional multiplex PCR using specific primers of HA gene of influenza A (H1N1) virus, influenza A (H1N1)pdm09 virus, influenza A (H3N2) virus and influenza A (H5N1) virus. Victoria lineage and Yamagata Lineage of influenza B virus were identified by conventional multiplex PCR using primers of HA genes of these two lineages. Influenza virus was found to be responsible for 22 out of 153 children with ILI (14.4%). The positivity of influenza virus in 2016 was higher than that in previous 3 years. Of 22 influenza cases, 15 cases (68.2%) were males and 7 cases (31.8%) were female. The ratio of male to female was 2.1:1. Twelve cases (54.5%) of influenza positive children were under 5 years of age, 10 cases (45.5%) were between 5 to 9 years and no case in age group 10 to 12 years. Majority of influenza cases were detected in rainy season especially in June and July. Fever, cough and rhinorrhoea were found as the main presenting features of influenza cases that accounted for 100%, 100% and 81.8% respectively. Among 22 influenza virus positive cases, 12 cases (54.6%) revealed matrix gene of influenza A virus and 10 cases (45.4%) revealed that of influenza B virus. Prevalence of influenza B virus increased from 0% in 2013-2015 to 40% in 2016. Of 12 cases of influenza A virus, 11 cases (83.4%) were found to be of influenza A (H3N2) virus, one case (8.3%) was of influenza A (H1N1)pdm09 virus and one case (8.3%) was unsubtyped. There was no case of influenza A (H1N1) virus or influenza A (H5N1) virus. So, influenza A (H3N2) virus could be regarded as predominant subtype of influenza A virus among children in 2016. All influenza B virus (100%) detected in 2016 were of Victoria lineage and could be regarded as the predominant lineage of influenza B virus among the children.

Prevalence of types of influenza virus, subtypes of influenza A virus and lineages of influenza B virus among children attending Yangon Children Hospital from 2013-2016

ILI cases	2013	2014	2015	2016
	(n=100)	(n=98)	(n=67)	(n=153)
• Influenza virus positive	6 (6.0%)	7 (7.1%)	6 (8.9%)	22 (14.0%)
• Influenza virus negative	94 (94.0%)	91 (2.9%)	61 (91.1%)	131 (86.0%)
Influenza virus positive cases	(n=6)	(n=7)	(n=6)	(n=22)
• Influenza A virus	6 (100.0%)	7 (100.0%)	6 (100.0%)	12 (54.6%)
• Influenza B virus	0 (0.0%)	0 (0.0%)	0 (0.0%)	10 (45.4%)
Influenza A virus positive cases	(n=6)	(n=7)	(n=6)	(n=12)
• Influenza A (H3N2) virus	5 (83.3%)	6 (85.7%)	4 (66.7%)	10 (83.4%)
• Influenza A (H1N1)pdm09 virus	1 (16.7%)	1 (14.3%)	2 (33.3%)	1 (8.3%)
• Influenza A (H1N1) virus	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
• Influenza A (H5N1) virus	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
• Unsubtyped	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (8.3%)
Influenza B virus positive case	(n=0)	(n=0)	(n=0)	(n=10)
• B/Victoria Lineage	0 (0.0%)	0 (0.0%)	0 (0.0%)	10 (100.0%)
• B/Yamagata Lineage	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

1.3.2. Measles Virus, Rubella Virus and Parvovirus B19

1.3.2.1. Detection of measles, rubella and parvovirus B19 in children with fever and rash attending Out Patient Department of Yangon Children Hospital

This study aims to determine the occurrence of measles, rubella and parvovirus B19 infections among children presenting with fever and rash. During 2016, 90 serum and oral fluid samples were collected from children with fever and rash who admitted to Yangon Children Hospital. Of 90 cases with fever and rash, males and females were distributed equally (i.e 45 each). Regarding the age, there were 13 cases in under 1year age group, 36 cases in 1-4 years age group, 29 cases in 5-9 years age group and 12 cases in 10-12 years age group. Among them, 78 cases (86.6%) received MR vaccination and 12 cases (13.4%) did not receive the vaccination. Anti-measles and anti-rubella IgM were tested on 90 serum samples by Enzygnost anti-measles and anti-rubella IgM ELISA kits. Measles IgM was detected in 26 cases (28.8%) in which male and female were equally distributed (i.e 13 each). Their age distribution was 4 cases in under 1 year age group, 16 cases in 1-4 years age group, 3 cases each in 5-9 years and 10-12 years age groups respectively. Twenty two cases (84%) received MR vaccination and 4 cases (16%) did not receive the vaccination. Age distribution of non-vaccinated children was 3 cases in under 1 year and 1 case in 1-4 years age group. Regarding Rubella IgM detected cases, 10 cases (11%) were positive for rubella IgM. There were 4 males and 6 females. Their age ranges were 3 cases in 1-4 years, 4 cases in 5-9 years, 3 cases in 10-12 years and there was no case in under 1 year age group.

1.3.3. Human Rhinovirus

1.3.3.1. Molecular detection of human rhinoviruses in children with influenza-like illness attending Yangon Children Hospital

Influenza-like illness (ILI) is caused not only by influenza virus but other viruses including human rhinovirus (HRV). HRV usually causes common cold and exaggerates asthmatic attack and otitis media in children. The aim of this study was to determine the prevalence and clinical severity of HRV among children with influenza-like illness. It was a cross-sectional study conducted at Out Patient and Emergency Department of Yangon Children Hospital (YCH). Nasopharyngeal swab samples were obtained from a total of 153 children with ILI from January to December 2016. Viral RNA was extracted by QIAamp® RNA Mini kit. Non-coding region of HRV gene was detected by conventional RT-PCR using Qiagen One Step RT-PCR kits. Of 153 cases, HRV was detected in 42 cases (27.5%). Males were slightly more affected than females with the ratio of male to female 1.2:1. The maximum number of HRV cases was found in the children aged less than 5 years that accounted for 71.4%. During the study period, HRV positive cases were detected in rainy season and winter season peaking in June, November and December. Fever, cough and rhinorrhoea were observed as the main symptoms of HRV infections that were responsible for 100%, 100% and 81% of HRV cases respectively. Gastrointestinal symptoms such as diarrhoea and vomiting were observed in 7.1% and 2.4% of HRV positive cases and fast breathing was observed in 2 HRV cases (4.8%). There was no HRV positive case that presented with tightness of chest. Most of the HRV affected children presented with low grade fever (mean=100.7°C, SD ± 0.85). Clinical diagnosis of HRV cases included acute viral infection (AVI), acute respiratory infection (ARI), pneumonia and dengue haemorrhagic fever grade 1 (DHF I) accounting for 83.3%, 7.1%, 4.8% and 4.8% respectively. This study provided base line information about ILI cases due to human rhinovirus that would be useful for the assessment of HRV outbreak and management of children with influenza-like illness.

1.4. ZIKA Virus

1.4.1 Molecular detection of Zika virus in pregnant women presenting with fever and rash attending Central Women Hospital

Zika virus (ZIKV) infection is an emerging mosquito-borne disease caused by Zika virus. The commonly reported symptoms include maculopapular rash (90-96%), fever (65-70%), non-purulent conjunctivitis (55-63%) and a few percentages with headache, muscle and joint pain. The association between Zika virus infection and congenital microcephaly and other birth defects is a serious issue which alerts for detection of ZIKV in pregnant women with suspected symptom and continuous monitoring throughout pregnancy with serial prenatal ultrasound scans. In Myanmar, ZIKV has never been reported and there is no available data on ZIKV infection due to lack of awareness and detection techniques although Myanmar is one of the countries where the vector of ZIKV infection, the *Aedes* mosquitoes, has long been existed and ZIKV was detected in the neighbor country like Thailand since 2012. In order to work along with the WHO recommendation and to get early diagnosis of Zika virus infection in pregnant women presenting with fever and rash, a protocol was developed and submitted to the Protocol Review Committee on 7.3.2016 and to Ethics Review Committee on 14.3.2016. The study was started in April after obtaining approval from ERC. In April, one sample was collected from a 36 year old Primigravida Nulliparous woman (37 weeks of gestation) presented with fever and rash. That sample was subjected to virus isolation in C6/36 mosquito cell line and the TCF after day 14 were analyzed by molecular method for Zika virus and showed negative result. Since then, no more eligible cases were admitted to CWH. Therefore a request letter was sent in September to the Director General of Public Health Department to participate in this study by informing the investigators when eligible cases presented to Township Health Departments are seen.

1.4.2. Isolation of Zika virus from mosquito samples in Yangon Region

Mosquito samples caught from the house and surroundings of a pregnant woman (who was said to be confirmed Zika virus infection) were sent from VBDC in October. Virus isolation was done according to the protocol for virus isolation from mosquitoes. Briefly, mosquitoes were pooled and put in 1.5 ml eppendorf tube, 0.5 ml of Media was added and homogenized by using sterile homogenizer. The homogenized samples were centrifuged and the supernatants were collected and filtered by 0.45 μm syringe filter. The supernatants were used for isolation of virus on C6/36 mosquito cell lines. The Tissue Culture Fluid on day 14 were used for RNA extraction followed by molecular detection for Zika virus by RT-PCR. Both 2 batches of mosquito samples were negative. Continuous monitoring of the suspected cases should be carried out to provide evidence of ZIKV infection in Myanmar and to support logistic thinking of the association between occurrence of microcephaly and ZIKV infection.

SERVICES PROVIDED

ACADEMIC

Sr.	Name	Course	Responsibility
1.	Dr. Theingi Win Myat	MMedSc(Microbiology)	Teaching
		Dip Med Microbiology	Teaching
		MMedTech (Med Lab Tech)	Teaching
		BMedTech (Med Lab Tech)	Teaching
2.	Dr. Htin Lin	MMedSc (Microbiology)	Teaching
		Dip Med Microbiology	Teaching
		MMedTech (Med Lab Tech)	Teaching
3.	Dr. Win Kay Khine	MMedSc (Microbiology)	Teaching
		Dip Med Microbiology	Teaching
		MMedTech (Med Lab Tech)	Teaching

LABORATORY

Sr.	Subject	Tested samples
1.	Performing platelet counts for patients admitted to YCH with suspected DHF and other bleeding disorders.	668 samples
2.	Performing Western Blot tests for confirmation of HIV-1 antibody	109 samples

VIROLOGY RESEARCH DIVISION (POL)

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	…	Dr. Thet Htoo Aung MBBS (UMM)
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	…	Daw Yin Yin Khine BSc (Physics) (Meikhtila University)
	…	Daw Thida Aung BSc (Chemistry) (Meikhtila University)
Research Assistant (4)	…	Daw Khin Moh Moh Win

Virology Research Division has been actively engaged in conducting a number of research projects mainly in the area of communicable diseases. The division collaborates with other Research Divisions of DMR (POL) as well as other institutions to carry out research projects.

RESEARCH PROJECTS

1. COMMUNICABLE DISEASES

1.1 VIRAL INFECTION

1.1.1 Clinical, virologic and epidemiological characteristics of dengue outbreak in Myanmar (2015)

Dengue (DEN) is an endemic disease in Southeast Asian countries including Myanmar. In this study, hospital-based surveillance was conducted in 2015 when the largest dengue epidemic occurred in Myanmar. The aims of the study were to characterize the clinical manifestations and viremia pattern of DEN patients and to understand the molecular epidemiology of dengue viruses (DENV) in the two regions of this country. Acute phase serum samples were collected from 332 clinically diagnosed DEN patients in Upper and Lower Myanmar from July-August, 2015. Of the 280 DENV-confirmed patients, 121(43.2%) and 111(39.6%) had primary and secondary infections, respectively. A high number of cases with severe DEN had primary infection (24.5%; 12/49). Patients with primary infection or negative for DENV IgM antibody demonstrated significantly higher viremia levels using plaque assays by FcγRIIA-expressing BHK and non FcγRIIA expressing BHK cells. However, the mean viremia levels were not significantly different among the different severity groups (DEN with and without warning sign and severe DEN) but remained high up to day 5 in patients with severe DEN and with warning sign. A total of 106 DENV strains were isolated (76 DENV-1 genotype 1, 24 DENV-2 Asian 1, 1 DENV-3 genotype III, and 5 DENV-4 genotype 1) including two cases with dual serotype infection. Serotype (except DENV-3) and genotype distributions were similar in both areas. Phylogenetic analyses of the envelope gene of the epidemic strains revealed close similarity with the strains previously isolated in Myanmar and neighboring countries.

High percentage of primary infection was noted among the patients and DENV-1 dominated the epidemic in 2015. Viremia levels among patients with primary infection were notably high for a longer period and this supported the spread of the virus by the mosquito vector during epidemic.

1.1.2 Genetic characterization of *Culex theiliriflavi virus* (CTFV) isolated in Myanmar

Entomological surveillance of arbovirus is important for detection of potentially hazardous pathogen circulating in society. Total 8,357 mosquitoes (mainly *Culex* species, *Culex tritaeniorhynchus, Culex vishuni, Culex fusocephalus*) were collected from Mandalay Region during 2013-2014 and virus isolation was done using mosquito cell line. Nineteen CTFV viral strains were isolated from mosquito pools but failed to get the isolation of Japanese Encephalitis virus. According to the phylogenetic analysis based on the polyproteins of the virus, the isolated viral strains were closely similar to the strains circulating in Portugal. There was 91% nucleotide similarity and 95.62% amino acids similarity (the whole ORF) between isolates from Myanmar and CTFV from Portugal. In conclusion, insect-specific flavivirus (ISFs) were found worldwide and the isolate from Myanmar were closely similar to the CTFV isolated in Portugal, Europe. Although *Culex* mosquitoes are main vector for spreading Japanese Encephalitis virus but only ISF virus can isolate in this study. There is still need to find out the effect (suppress or enhance) of ISFs on pathogenic flaviviruses among mosquitoes.