Updated Aug. 5, 2019

This report should be submitted within 2 weeks after you return to Japan.Please do not change the formatting

（Abroad・Domestic）Internship report form（Student） (Year/Month/Day)

Name	Sittinee　Kulprasertsri
Laboratory	Comparative pathology
Year (Grade)	DC3
Internship institution	The monitoring and surveillance center for zoonotic diseases in wildlife and exotic animal (MoZWE) and FAO reference center for zoonotic and wildlife diseases.
Internship period	Internship period:　1st October– 25th November, 2019 (Departure Date from Sapporo: 2/10/2019, Arrival Date in Sapporo: 25/11/2019)
Purpose	- To acquire knowledge and skill for developing an Induced Neural Stem Cells (iNSCs) from avian origin - To gain an experience of disease epidemiology and surveillance by using computer based analytical method - To observe the work flow of laboratory management system in MoZWE - To establish the good laboratory network between staff and organization in MoZWE both domestically and internationally

- The reason why you chose this institute

I have been interested in induced pluripotent stem cell (iPSCs) since I was DC1 phD student in Hokkaido university. I wanted to generate avian iPSCs to use in my research project and also one of my laboratory members want to develop a horse iPSCs from equine fibroblast cell, however we couldn't succeed to do that. So I discuss with my friend, she is a phD student at Mahidol University. She is a member of porcine iPSCs development in faculty of veterinary Science, Mahidol university. Her supervisor is Professer Sasitorn Rungarunlert, she engages in an embryonic stem cells, murine and porcine iPSCs development. At this moment, this research team is able to induce porcine iPSCs to other cells including neuronal cells which I am now interested in. So I contacted her and she would be glad to supervise me in this internship program, even though the duration is quite short to learn the whole process for applying to avian iPSCs but at least I will know important techniques for generating iPSCs for future projects.

There are 2 units related to my study in Mahidol university; the monitoring and surveillance center for zoonotic diseases in wildlife and exotic animal (MoZWE), plus FAO reference center for zoonotic and wildlife diseases. Since my research is also one of zoonotic

disease which is *Flavivirus.* I have been studying on Japanese encephalitis virus (JEV) and Duck Tembusu virus (DTMUV) for three years. Both of them are members of mosquito-borne flavivirus and their vectors are mosquitos. These 2 viruses circulate between vector and host that particularly originate in wild animals.

- Result of the activity（about 800 words、provide photos, tables and figures that clearly show the activities during the period）

For generating porcine iPS cell line namely VSMUi001-D cell line

I had learned the procedure of preparing porcine iPSCs from pig embryonic fibroblast (PEFs) culture and process to maintain the emerging colonies.

A cross breed piglet at embryonic day 28 (E28) are used for PEFs preparation. Cells are transfected by the viral vector containing iPSCs factors to reprogram porcine fibroblast cell. In this group, retroviral vector containing five human reprogramming transcription factors (OCTS, SOX2, KLF4, c-MYC and LIN28) were used based on a published protocol (Esteban et al., 2009). In present day, we have many vectors for generating iPSCs such as Lentivirus, Retrovirus and Sendai virus. However, it's said that retrovirus system is one of the easiest way with high percentage of success in murine and porcine iPSCs. In my opinion, it's quite time consuming to generate iPSCs. Because you need to prepare something on time before you do a next process. For example, you need to preparing primary mouse fibroblast (MEFs) as feeder cell when you start to generate porcine embryonic colony. Before using the primary mouse fibroblast cell as feeder cell, this cell should be 100% confluent in plate and then this cell is inactivated by special media with mitomycin-C after that MEFs will become inactive MEF (iMEFs)and we will use those cells for feeder cells. Importantly, we cannot use the older iMEFs over 4 days after this cell show confluent. Otherwise, the pig-induced pluripotent stem cell (piPSCs)-like colonies cannot grow well (Fig 1).

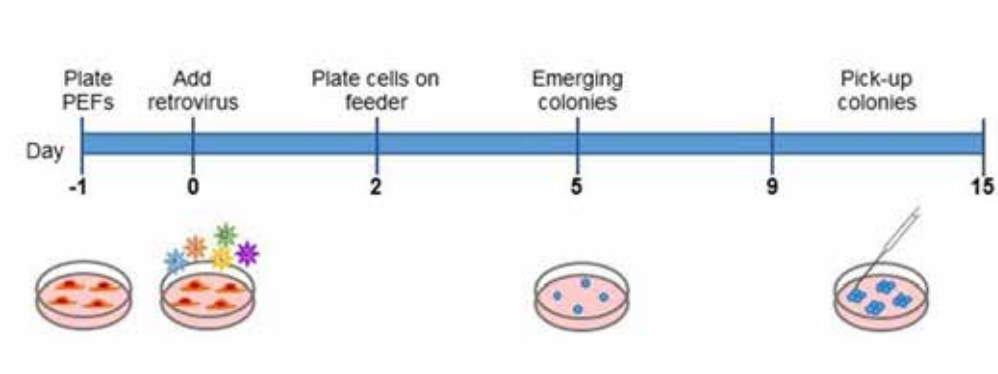


Fig 1: experimental procedure for generating the pig-induced pluripotent stem cells (piPSC)

After PECs infected by retrovirus for 2 days, the PEFs were digested by 0.25% trypsin and regrowth on iMEFs. Five days after reprogramming, piPSCs-like colonies were observed. In addition, they showed me the character of piPSCs emerging colony. Different animals show different morphology of emerging colony. In case of porcine emerging colony, they display the typical character like a mouse embryonic stem cells (mESCs). Porcine emerging colonies include compact, dome shape, and clear edge colonies which different from PECs (Fig. 2 and 3).

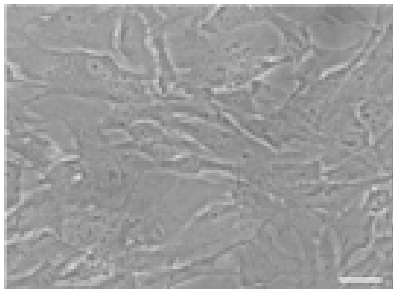


Fig 2: Primary porcine embryonic fibroblast cell

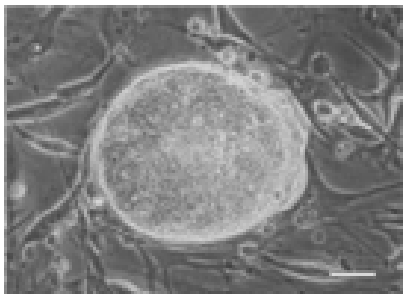


Fig 3: Pig induced pluripotent stem cell (piPSC)-like colony

After 9-15 days post re-programming, ESC-like colonies were first passaged by mechanical techniques then enriched by special media. We need to confirm the stem cell properties in further experiment. At this time, I had learned how to pick-up piPSC colony by using Pasteur pipette but with modification. Pasteur pipette was burnt while being pulled out the tip of glass pipette in opposite direction until the tip of glass pipette is extended and sharp like needle. Then, colony was cut under light microscope by two modified glass pipettes. Only one colony to be cut surrounding edge like square.

After transducing PECs by iPS reprogramming vector, more than one colonies of stem cell-like will appear. So, each colony need to be checked for morphology. For stem cell property confirmation, many technique can be used such as alkaline phosphatase staining, RT-PCR for checking stem cell gene expression and immunochemistry by expression of pluripotent markers including OCT, SOX2, SSEA-1, and teratoma formation. Porcine iPS colonies were used for checking teratoma differentiation by injecting these colonies into nude mice. The teratoma which was induced by iPS colonies composed of three germ layers including ectoderm (a keratinized squamous epithelium), a mesoderm (skeletal muscle) and endoderm (respiratory-like epithelium). These cells need to be cultured until 40 passages for checking the self-renewal activity.

By removing some growth factors such as LIF and bFGF from stem cell like colonies, the embryoid bodies (EBs) were spontaneously differentiated. Depend on the target cell, for

example, if we want to differentiate iPS to neuronal like cell, we will add a retinoic acid. Finally, for making stock of iPS colony, we can keep them in -80°C freezer or liquid nitrogen. However, I did not perform this step but my friend said some cells will die or cannot grow well after freezing and thawing.

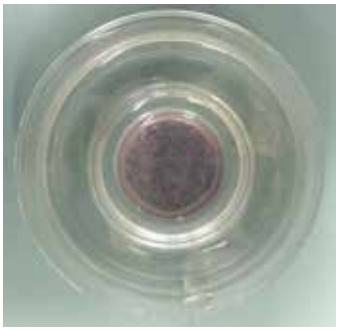


Fig 4: Alkaline phosphatase staining plate

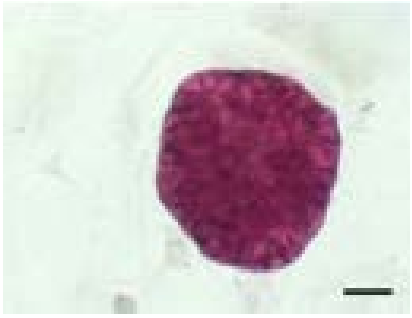


Fig 5: magnified colony from Fig 4.

For disease surveillance

I have a chance to trap vectors in the field by using NZI trap (Fig 6) and CDC light trap (Fig 7). NZI traps were used for collecting a Tabanid in the grass field near the cattle, buffalo and horse stables. We set the NZI trap in the grass field where the cattle, buffalo and horse freely feed on in the day time. We set these traps in the field because the tabanids will normally hide in the field until the twilight, they will suck blood from host in the night time. For NZI trap, we collected the insect sample twice a day, in the morning around 6:00-8:00 AM and in the evening around 17:00-18:00 PM. After that, all of insects will be collected into the plastic bag and they were kept in -20 C freezer overnight. Then, the MoZWE staff taught me how to identify the species of tabanids by morphology such as the presence of ocelli, .

Fig 6: Nzi trap

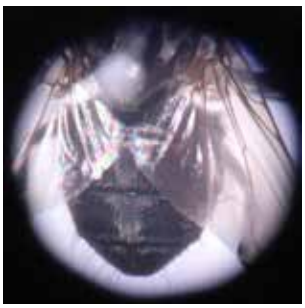
Fig 7: CDC light trap

CDC light trap were used for collecting sand fly around the old building that had many of dark space and near fresh water supply. We set this type of trap in the evening around 17:30-18:00 PM, and collect the sample in the morning of the next day. These traps are also used for trapping mosquitoes. However, we need to add dry ice inside the trap for inducing mosquitoes because dry ice emits carbon dioxide gas. For collecting sand fly from trap, we use mouth suction for suckling all insects from the bottom of the trap (Fig 8). We need to collect all insects first because sandflies are very tiny. then we will only select sand flies by examining under stereoscope. After that we put a sandfly on the glass slide, we used a normal pin to separate head from body part. Mounting solution was directly dropped on the body and head of sand fly, this solution will use for decalcifying an integument of sand fly within 2-3 day. After that, sandflies are identified regarding sex and species by the presence terminalia and the shape of cibarium. (Fig 9 and 10)

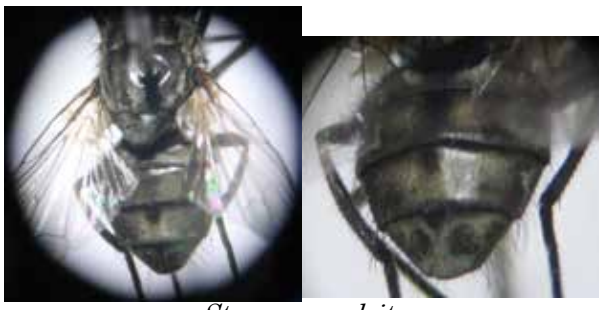
Fig 8: Collect sample from CDC light trap

Fig 9: Male sand fly

Fig 10: Female sand fly



Stomoxys bengalensis



Stomoxys calcitran

Atylotus lotus

Atylotus cryptotaxis

Tabanus megalops

Fig 11: The sample of insect trapping from Kasetsart university, Thailand

- What do you think the positive impact of the activity will have on your further career path?

I think this activity will have good impact in educational relationship between Kasetsart university, Mahidol university and Hokkaido university. Dr. Sasitorn Rungarunlert glad to advise students who are interested in iPS generation. During internship period, I had introduced my research in Hokkaido university to Dr. Sasitorn group, she has also asked me

to join the research with her research team by using a porcine-induced neuronal-like stem cells for studying JEV neuropathology in the future. This project will help us search for more information in viral-host interaction, neuropathology and disease mechanisms. This will be useful identify and develop for therapeutic purposes.

-Advice for your junior fellows

Never give up on something you really want. However, things may seem impossible

There is always a way.

Approval of supervisor	Institution・Official title・Name

※1 Send the electronic file to the Leading School section, International Affairs Office

※2 Attach a copy certificate of the content of internship activity that is prepared by the counterpart at the internship institution (anyform with a signature of the counterpart).

※3 The Steering Committee of the Leading Program will first confirm the content of this report and report will be forwarded to the Educational Affairs Committee for credits evaluation.

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