CHAPTER II
REVIEW LITERATURES

2.1 Japanese encephalitis virus

2.1.1 General Background

Japanese encephalitis virus (JEV) is a mosquito-borne virus classified in family *Flaviviridae* genus *Flavivirus*. The virus consists of 5 genotypes, and it is transmitted to human by the bite of an infected mosquito. The major vector is *Culex tritaeniorhychus*. Infection by JEV leads to a wide spectrum of clinical presentation from a febrile headache to acute and possibly fetal encephalitis with 20-30% mortality rates (1, 2). Most of survivors have neuronal sequelae such as mental retardation, epilepsy, paralysis, deafness and blindness. At present, specific treatments and antiviral drug for JEV infection are not available. Early diagnosis and supportive treatment helps to reduce the mortality rate of JEV infection (2, 3).

2.1.1.1 Epidemiology

Japanese encephalitis virus was firstly isolated in Japan in 1935 but had been found in the area as early as 1870 (18). Japanese encephalitis (JE) affects more than 50,000 patients and 15,000 deaths annually (3). From there, JE had been spread to many countries especially in Eastern and Southeastern Asia such as China, Siberia, Korea, Japan, Taiwan, Guam, Saipan, Vietnam, Cambodia, Thailand, India, Nepal and Sri Lanka. Moreover, it has increasingly been found throughout most countries of East and Southeast Asia (Figure 2.1). The factors that support disease in this area comprise of the vector, the environmental conditions which are essential for mosquito breeding cycle such as rainfall, humidity, tropical temperatures, and the number of the amplifying host that are pigs and birds. Furthermore, JE spreading also related to rapid globalization and change in global climatic condition due to industrialization and deforestation (1-3, 19).

In Thailand, the first epidemic of JEV was reported in Chiang mai and nearby areas in 1969. Later on, there was a study of JEV genotype distribution in Thailand by collecting data in 7 provinces such as Chiang mai, Khon Khen, Nakhon Pathom, Ratchaburi, and Phuket. These provinces represent 4 parts of Thailand including North, North-east, Midland and South. Increasing of pig farming and trading is relevant to the dispersion of JEV (20).

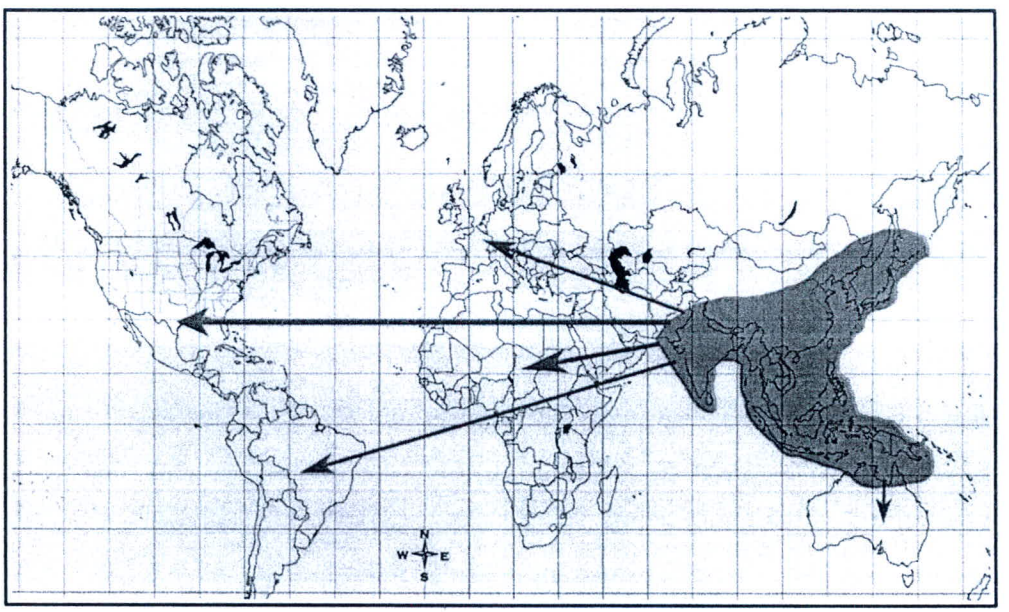

Figure 2.1 The global distribution of JEV (3)

This fiqure shows the global distribution of JEV. The red shaded areas indicate the present distribution of JEV, whereas the arrows indicate the areas where there is a high possibility of virus spread due to globalization and climatic change (3).

2.1.1.2 Clinical manifestations

After Japanese encephalitis virus (JEV) has invaded to human body by a mosquito's bite. The incubation period is 5-15 day. Clinical presentations in JE patients depend on the part of nervous system that is affected. Most of JEV infections are either asymptomatic or may result in a nonspecific flu-like illness follow by symptomatic such as reduced level of consciousness, seizure, headache, photophobia and vomiting. Some patients present with aseptic meningitis or acute flaccid paralysis. Severe form of JE patients is associated with respiratory paralysis and encephalitis. Survivors from JEV infection also have neurological sequelae such as epilepsy, paralysis and deafness. Furthermore, the extrapyramidal features include pill-rolling movement, opsoclonus myoclonus and bizarre facial grimacing, staring mask-like faces and lip smacking can be found in survivor patient (Figure 2.2) (2, 3, 21, 22).

2.1.1.3 Transmission cycle

JEV is transmitted to humans by a bite of infected mosquito vector, especially *Culex tritaeniorhynchus*. Pigs and warding birds are amplifying hosts. Humans are dead-end hosts in JEV transmission cycle because of low levels of viremia. Other domesticated animals such as dogs, sheep and rodent may become infected, however fail to develop a sufficient viremia for further viral amplification (Figure 2.3) (2, 3, 23).

2.1.1.4 Treatment

Up to date, no specific antiviral agent to mitigate the effects of JEV is available. JE therapy consists of supportive care and management of complications (3, 24).

Minocycline, a board spectrum synthetic antibiotic classified in tetracycline group, were suggested as an anti-JEV drug. Kumar et al. investigated that minocycline treatment can reduce neuronal apoptosis, microglia activation including caspase activity (25).

2.1.2 Classification and molecular biology

Japanese encephalitis virus belongs to the family *Flaviviridae*, genus *Flavivirus*. Apart from JEV, other members of the family can also cause encephalitis (Figure 2.4). West Nile Virus causes encephalitis and neuronal disorders in Africa, Southern and Central Europe, India, the Middle East and North America and St. Louis encephalitis virus is a major cause of viral encephalitis in North and South America (21, 26) .

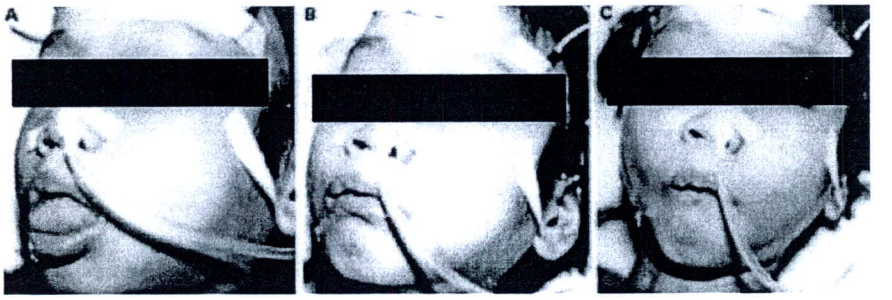

Figure 2.2 Facial grimacing in a Vietnamese boy with Japanese encephalitis (21).

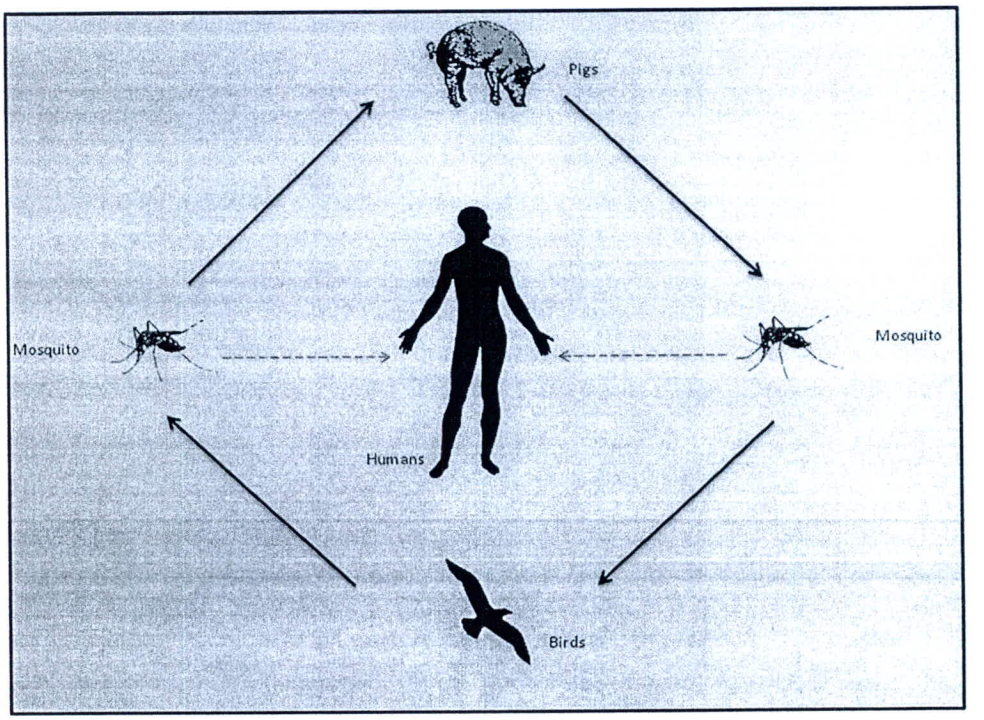

Figure 2.3 The transmission cycle of Japanese encephalitis virus (23)

This figure demonstrates the Japanese encephalitis virus transmission cycle. The virus transmitted in an enzootic cycle among birds, pigs and other vertebrate host through a bite of mosquitoes. Human is dead end host for the JEV (23).

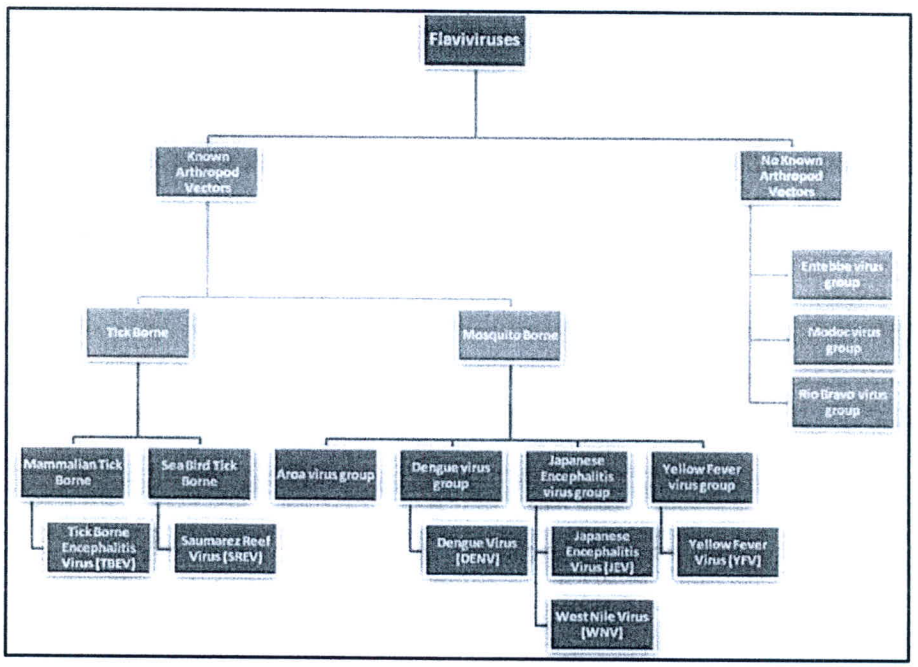

Figure 2.4 The classification of *Flavivirus* (23)

This figure show the classifications of *Flavivirus* . Japanese encephalitis virus can be classified as a mosquito-borne virus as same as West Nile virus, Dengue virus and Yellow fever virus (23).

There are at least 5 genotypes that are isolated from different geographic areas using nucleotide sequencing of C/PrM and E genes. Genotype I were isolated from Northern Thailand, Cambodia and Korea. Genotype II were isolated from Southern Thailand, Indonesia, Malaysia and Australia. Genotype III were isolated from many areas of Asia such as Japan, Korea, China, Taiwan, Philippines, India and Sri Lanka and Nepal. Genotype IV were isolated from Indonesia and Genotype V were isolated from some part of Malaysia and Singapore (21).

Japanese encephalitis virus is an enveloped virus, which consists of a 11 kb single stranded RNA viral genome. The RNA comprises of 5'- and 3'-untranslated regions (UTRs) and a single open reading frame encodes three structural proteins (Capsid protein, Precursor to the Membrane protein, Envelope protein) and seven nonstructural proteins)NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The E protein is a large structural protein that is important for virus entering into the host cell and it is a major target of host immune response (Figure 2.5) (22, 23, 27).

2.1.3 Life cycle and replication

Entry of JEV to host cells begins with attachment of virus particles onto the cell surface. JEV is internalized into the host call by receptor-mediated endocytosis and then virus is transported to early endosome that subsequently mature to late endosomes. The low pH environment in endosome triggers major conformation changes in the virion. This structure rearrangement mediates fusion of viral and cellular membranes. Lack of prM cleavage during maturation prevents conformation change of E protein during fusion. After the fusion the nucleocapsid is released into cytoplasm and translation of viral RNA is initiated (27).

Translation of viral RNA genome occurs in one open reading frame to from a viral polyprotein that is processed by host cell and viral protease. The structural proteins and non-structural protein are synthesized. Following the viral protein production, NS protein, the viral RNA genome and probably host factors are assembled to form the replication complex. Replication process occurs in association with membrane structure presumably by interaction involving hydrophobicity of some NS proteins. The replication of viral RNA occurs on intracellular membrane. Virus assembly occurs on the surface of rough endoplasmic reticulum (ER). The immature particles are transported to the secretory pathway of the trans-Golgi network (TGN), prM is cleaved by furin. Mature virions are then released into the cytoplasm by exocytosis (23, 28).

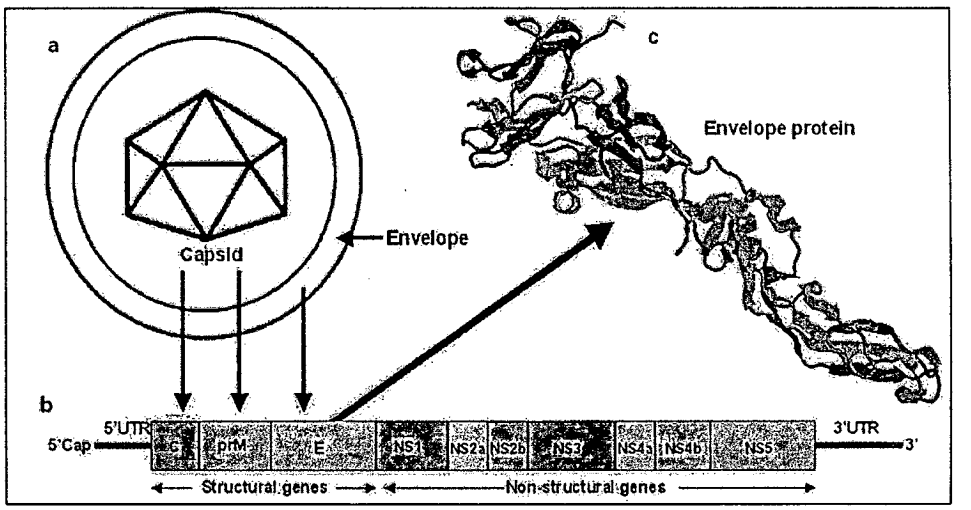

Figure 2.5 Schematic of Japanese encephalitis virus structure (19)

This figure demonstrates the schematic of Japanese encephalitis virus structure. (a) The JEV virion has a diameter of 50 nm. The envelope consists of E and M protein. The icosahedral nucleocapsid is composed of RNA viral genome and C protein. (b) Diagram of JEV genome indicates the orientation of genes encoding viral proteins. (c) Structure of E protein homodimers (19).

2.2 Microglia and JEV infection

Microglia are resident immune cells of the CNS and have an important role in host defense against invading microorganism. Microglia constitute 10-15% of cells in the CNS. Normally, microglia can be found in healthy mature CNS especially in hippocampus section. At the resting stage, microglia has a characteristic as ramified morphology (Figure 2.6).

Upon the activation by infection, trauma, neurogenerative disease or altered activity that is bothered brain homeostasis, microglia become amoeboid shape and migrate to the site of injury where they proliferate and release pro-inflammatory cytokines (28-30).

The mechanism by which JEV causes neurological disease remains unclear. Many studies have delineated important pathways being triggered in the process. RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) is one of an important chemokine that has been shown to be secreted by the microglia and astrocytes upon JEV infection. The expression of this chemokine recruited other immune cells to the area of infection (31). Ghoshal et al. showed that the number of activated microglia significantly elevated upon JEV infection. Activated microglial cells cause an up-regulation of reactive oxygen species and pro-inflammatory cytokines such as MCP-1, IFN-α, TNF-α, IL-8, and IL-6 (10, 32). Recent studies demonstrate that neuronal death occurs as a result of the inflammatory response, initiated during JEV infection (10, 25, 33). Moreover, JEV infection in animals as well as in vitro also results in the induction of pro-inflammatory cytokines, IL-1β and IL-18 (34). Previous study by Thongtan et al. reported that mouse microglial cells might serve as reservoir of JEV and JEV infection induced apoptotic cell death in microglia (12). Furthermore, Nazmi and colleagues investigated that JEV-infected peripheral macrophage can disseminate to blood brain barrier and possibly served as "Trojan Horses" to introduce virus to CNS and released pro-inflammatory cytokines which lead to apoptotic cell death of neurons (11).

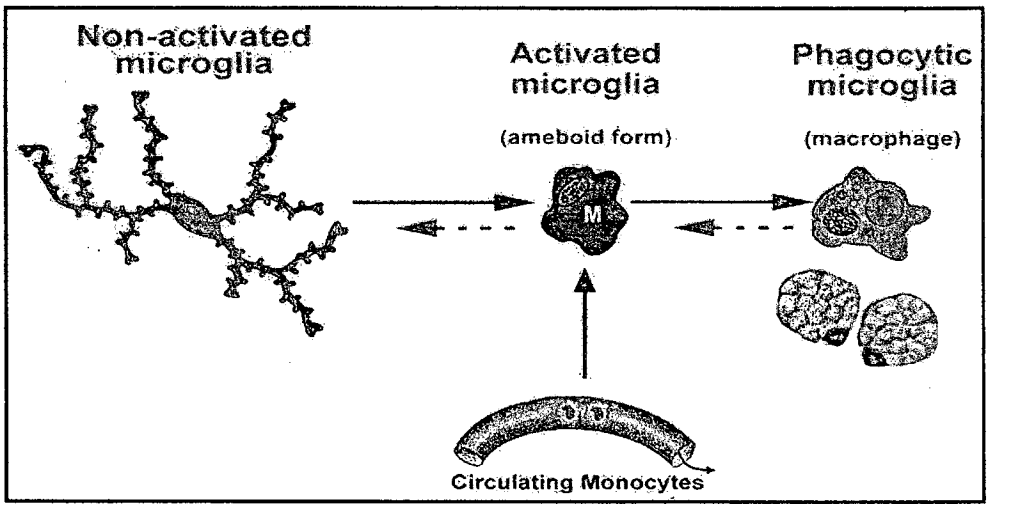

Figure 2.6 Morphology of microglia (35)

This figure shows the morphological change of microglia. At the resting stage, microglial cell has a characteristic as ramified morphology. With the activation, microglia convert to amoeboid forms and migrate to the site of injury, which show macrophage properties as a phagocytic cell.

(http://missinglink.ucsf.edu/lm/introductionneuropathology/Response%20_to_Injury/Microglia.htm)

2.3 Autophagy

Autophagy is the process of physiologically and immunologically controlled that sequesters and degrades cytoplasmic targets, including soluble macromolecular aggregates, lipids, and nucleotides from damaged proteins, cellular organelles, and intracellular pathogens such as, bacteria, protozoans and viruses (36). Autophagy is constantly maintained at the basal level and is up-regulated in response to stress conditions. There are at least three different types of autophagy classified by the route of delivery to lysosome, macroautophagy, microautophagy, and chaperone-mediated autophagy.

1. Macroautophagy, a major type of autophagy, cytoplasmic contents are delivered to lysosome with the formation of a double-membrane structure called the autophagosome which sequesters cytosolic material and delivers to the lysosome for degradation. Macroautophagy is conserved in mammals, and is mediated by a special organelle termed the autophagosome.
2. Microautophagy is the transfer of cytosolic components into the lysosome membrane itself by non-selectively engulfs a cytoplasm by invagination of the lysosomal membrane.
3. Chaperone-mediated autophagy (CMA) is characterized by their selective degradation. Cytosolic protein containing KFERQ –like motifs are recognized by a cytosolic Hsc70 that binds to lysosomal-associate membrane protein-2a (LAMP-2a) (37-39).

However, in most studies, the word "Autophagy" usually refers to Macroautophagy as its used here.

2.3.1 Molecular events of autophagy

Autophagy is a general process used for degradation. It is activated at the basal levels in most cell types in order to balance of organelles, protein and homeostasis of macromolecules. The process of autophagy involves a set of evolutionarily conserved gene products, known as Atg proteins including Atg 4, 5, 7, 8, 10, 12 and 16, which are required for the formation of isolation membrane and autophagosome(36, 40-42). The process of an autophagic pathway composes of four major stages (Figure 2.7).

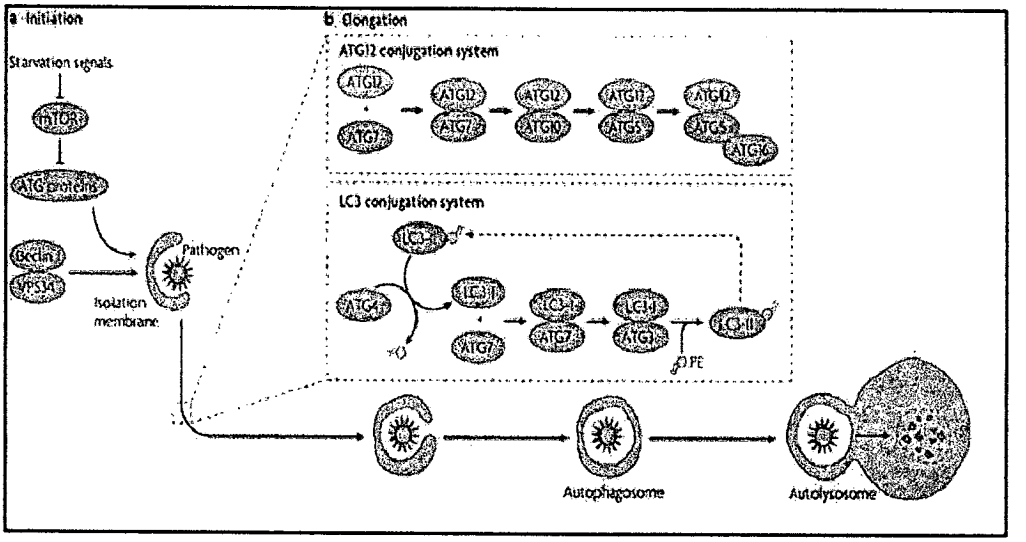

Figure 2.7 The Autophagy pathway (41)

(a) Initiation process of autophagy pathway is regulated by 2 regulatory molecules including mTor and Vps 34. (b) For elongation process or autophagosome formation needs two ubiquitin-like conjugation processes; Atg5-Atg12 conjugation system and lipid conjugation of Atg8 or LC3 conjugation system. For Atg12 conjugation system, Atg12 is activated by Atg7 which works as an E1-like ubiquitin activating enzyme, and then passed to Atg10 as an E2-like ubiquitin carrier protein to form linkage between Atg 12 and Atg 5. For LC3 conjugation system, LC3-I, cytosolic form is activated by Atg 7 and passed to E2- like enzyme Atg3 to form a covalent linkage between LC3 and phosphatidylethanolamine (PE), resulting in a conversion of LC3-I to membrane bounded form or LC3-II. PE molecules are eliminated from LC3-II by Atg4 for recycling (41).

2.3.1.1 Induction

As noted that, autophagy is regulated by set of Atg proteins. The autophagy regulatory molecules are the Ser/Thr kinase target of rapamycin (Tor kinase) and the class III phosphatidylinositol 3 kinase vesicular protein sorting 34 (Vps 34), the only PI3K that is evolutionarily conserved from yeast to mammals. Vps 34 binds to Atg6 or the mammalian homolog Beclin-1 to form a complex. In normal conditions with growth factors and abundant nutrients, mTOR activation is the major inhibitory signal that repress autophagy. Moreover, Bcl-2 can negatively regulate the activation of autophagy by interfering the Vps34-Bectin-1 complex. In contrast, during starvation, inhibition of Tor protein kinase leads to an induction of autophagy. Also, Bcl-2 is phosphorylated by Jnk1 such that it can no longer interact with Beclin-1. Some of biochemical agents that have an effect on Tor and Vps34 are usually used to regulate autophagy. Rapamycin acts as an autophagy induction by inhibit Tor kinase activity. On the other hand, wormannin and 3-methyladenine (3-MA) act as autophagy inhibitors by inhibiting class III PI3K kinase which is required for the early stages of autophagosome development (37, 41-43).

2.3.1.2 Acquisition of phagophore membrane

In this step, the cytoplasmic constituents are sequestered by unique membrane as known as phagophore. Membrane dynamic during this event is required ubiquitin-like conjugation systems which essential and also connected to elongation and autophagosome formation step. There are three enzymes which involved this process of conjugation: E-1 activating enzyme, E2-conjugating enzyme and E3-ligase enzyme. Firstly, Atg12 is activated by Atg7 which works as an E1-like ubiquitin activating enzyme and then transferred to E-2 like enzyme Atg10. Then, E2-like ubiquitin carried proteins to form covalently linkage between Atg 12 and specific lysine of Atg 5. After that, Atg5-Atg12 complexes bind to Atg16 to form Atg12-Atg5/Atg16 complex which is leading to induce a curve of the phagophore (17, 41, 44).

2.3.1.3 Elongation and autophagosome formation

This step is characterized by an increase in size of the phagophore, which wraps around the cytosolic component. The progression of autophagy also depends on another conjugating system: the formation of Atg8/PE conjugate. LC3 (microtubule-associated protein 1 light chain 3), a mammalian homolg of yeast Atg8, is normally expressed as a cytosolic protein and subsequently cleaved by Atg4 to form LC3-I. Then, LC3-I is activated by

E1-like enzyme Atg7 and transferred to E2-like enzyme Atg3 to form a covalent linkage between LC3-I and phosphatidylethanolamine. Lipidation of LC3-I results in the membrane bound form of LC3 (LC3-II) which located in autophagic membrane.

2.3.1.4 Lysosomal fusion and degradation

Upon this phase, the autophagosome directly fuses with the lysosomes or firstly fuses with endosome to form amphisomes, followed by lysosomal fusion to form autophagolysosome. After the fusion, macromolecules and sequestered material in autophagosome is released in the lysosomal lumen. Intra-lysosomal degradation occurs by lysosomal acid hydrolases to break the inner membrane of autolysosome (Figure 2.8) (17, 42, 47) (17, 41, 44).

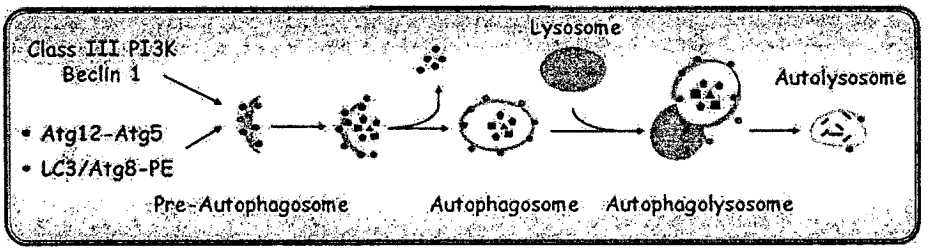

Figure 2.8 The autophagic process (17)

The autophagic process begins firstly with the nucleation of membrane structure called isolation membrane or phagophore. Secondly, **autophagosome formation**, cytosolic proteins and organelles are sequestered by a double membrane vesicle. Thirdly, **autophagosome fusion**, upon this phase, the autophagosome fuses with the lysosome to form autophagolysosome and finally **autolysosome breakdown** (17, 41, 43).

2.3.2 Dection of autophagic cell death

There are three principal techniques for demonstrating autophagy as follow.

2.3.2.1 Electron Microscopy

The electron microscopy is the conventional method to observe a double-membrane vesicle defined as an autophagosome at the ultrastructural level. In the past, electron microscopy has been used extensively to quantify the number of autophagosomes in a variety of cells and tissues. Autophagosome is recognized as a double membrane structure containing undigested cytoplasmic materials including organelles, while the autolysosome is a single membrane structure containing cytoplasmic components at various stages of degradation. These structures are often generalized as the autophagic vacuoles (Figure 2.9). To date, electron microscope is a very informative method for monitoring autophagy, however, it should be complemented by additional assay to ensure correct identification and quantification (45-47).

2.3.2.2 Fluorescence Microscopy

The assessment of autophagosome number by electron microscopy requires considerably specialized expertise, and is becoming increasingly replaced by light microscopy and biochemical methods that are more widely accessible to researchers in a variety of fields. As mentioned above, the mammalian autophagy protein, LC3 is a universal marker of autophagosomes. This protein is modified by a ubiquitin-like conjugation during autophagy induction (37, 38). Modification of LC3 from cytosolic from (LC3-I) to membrane bound form (LC3-II) can be monitored as punctuate dots. The number of punctuate LC3 can be visually count under fluorescence microscopy (42, 46, 48, 49).

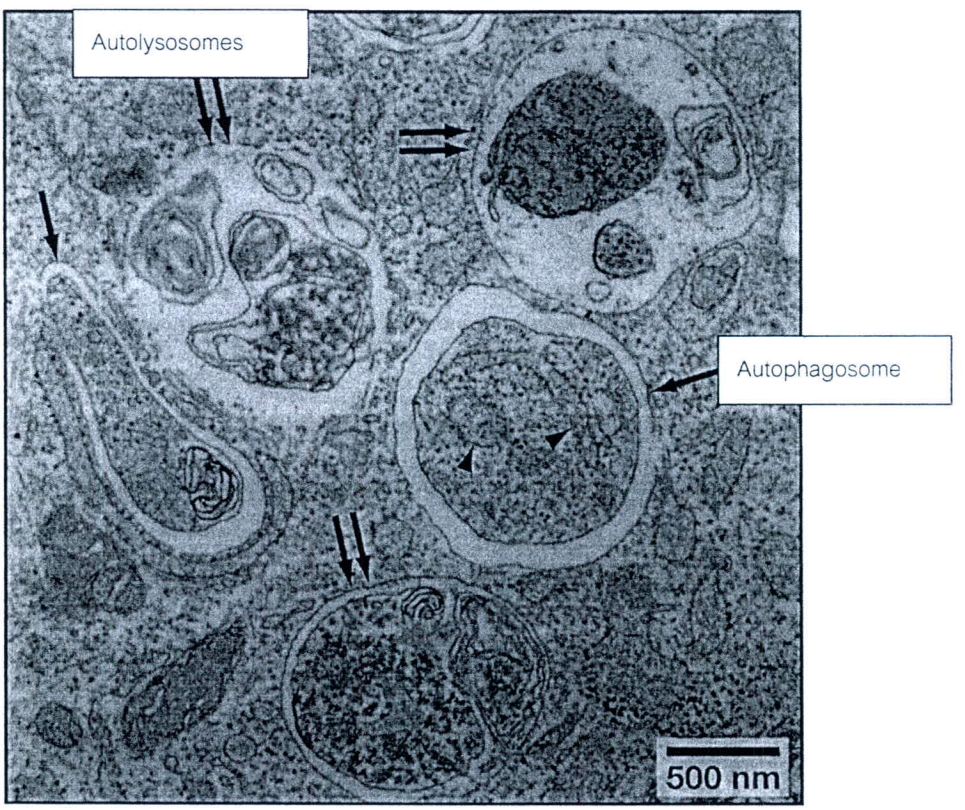

Figure 2.9 The Electron micrograph of autophagosome and autolysosome in starved mouse embryonic fibroblast cells (42)

This figure show morphological assessment of autophagosome and autolysosome in mouse embryonic fibroblast cells. The single arrows indicate autophagosome and double arrows indicate autolysosome which consist of degradation organelles (42).

Moreover, the co-localization of LC3 and protein markers which identify other cellular structure such as endosomes and lysosomes helps to identify stages of autophagic vacuole maturation. For example, co-localization of LC3 with LAMP-1 (lysosomal-associated membrane protein 1) indicates fusion of autophagosome with endosome or lysosome which result in amphisome and autophagolysosome respectively (50).

2.3.2.3 Immunoblotting

Besides its application in fluorescence microscopy assays, LC3 is also useful in biochemical assays to assess autophagy. The conversion from endogenous LC3-I to LC3-II can be detected by using immunoblotting with antibodies against LC3. Apparent molecular weight of LC-I on SDS-PAGE is 16 kD while that of LC3-II is 14 kD. The amount of LC3-II or the comparison of LC3-II/internal control ratio correlates with the amount of autophagosomes which reflects autophagic activity (45, 51).

2.3.3 Autophagy and virus infection

Autophagy displays an essential role of cellular homeostasis and also takes part in several pathologies including cancer, neurogenerative disease and infection (48, 49). N In cases of virus infection, the interaction between the autophagic machinery and the invading virus is proposed in three main outcomes: defense, avoidance and subversion (52, 53). For a cellular defense mechanism, autophagy is induced to limit the viral replication. An example of defense mechanism is supported by a study of tobacco mosaic virus (TMV). Silencing Beclin-1 or Atg6 gene in tobacco mosaic virus infected-tobacco plant leads to an increase in viral replication (17). In contrast, Herpes simplex virus types I (HSV-1) escape the fate of restriction by down regulating the pathway. It is well characterized that HSV-1 neurovirulence ICP34.5 protein, encoded by HSV-1, antagonized the function of dsRNA activated protein kinase (PKR) to suppress the induction of autophagy. Thus, HSV-1 has evolved strategies to counteract cellular antiviral function. This interaction is called avoidance (54). In addition, some viruses evolved to use autophagosomes for their replication such as poliovirus using autophagic membranes as a site for its viral replication. Inhibition of autophagy by siRNA-mediated silencing of Atg12 gene or Atg8 resulting in a reduction of viral production (17, 53).

Up to present, many Flaviviruses have been shown to subvert cellular autophagy for their benefit of viral replication (16, 54-57). Studies on Chikungunya virus (ChikV) by Krejbich-Trotot and colleagues showed induction of autophagy upon ChikV infection in HEK293. An

increase of Chikungunya virus production was seen during autophagy induction by Rapamycin, while inhibition of autophagy by using 3-MA as a pharmacological inhibitor or using RNA interference against the transcript of protein Beclin1 resulted in dramatically decreased of viral production. This study suggests that autophagy may play a promoting role in Chikungunya replication (55). For dengue virus (DENV), Panyasrivanit and colleagues showed that induction of autophagy upon DENV-2 infection in HepG2 cells is essential for viral replication (57). So, these evidences suggest subversion outcome of autophagy in ChikV and DENV. Recently, the involvement of autophagy in JEV infection was shown by Li and colleagues using human NT-2 cells. NT-2 cells were infected with JEV strain RP-9 and RP-ms. Induction of autophagy by rapamycin, biochemical autophagic inducer, increased viral production whereas down regulation of autophagy, either by using 3-methyladenine or by knockdown autophagy essential gene, Atg5 and Beclin-1, reduced viral yields suggesting a proviral role of autophagy in JEV replication (56).

In this research, JEV strains Beijing-1 and human microglial cell line were used as a model for the study of viral susceptibility and autophagy induction. Even though this viral strain is not endemic in Thailand, it induces high level of neutralizing antibodies. The induced antibodies are protective against heterologous JEV strains such that the Beijing-1 strain was used for the production of the first-generation, mouse brain-derived JE vaccine. This vaccine effectively decreases the incidence of JE in Thailand. Viral susceptibility was demonstrated by immunocytochemistry. Transmission electron microscope, indirect immunofluorescence staining and western blotting of LC3 are performed for detection of autophagy.