

FUNDAMENTAL STUDY ON THE PROLIFERATION OF FELINE CALICIVIRUS ;

Relationship Between It's Replication and Ultra-  
microstructure in the Host Cells.

Motonobu HARA  
( Azabu Univercity, School  
of Veterinary Medicine )

1981

## Introduction

Calicivirus was classified in picornavirus group until 1963 years. The history of calicivirus had a very interesting background from the finding of vesicular exanthema of swine virus to nowadays, mainly on the taxonomy of the virus group. Vesicular exanthema of swine virus (VEV) was first found in USA and continued 1936 to 1957 years. It was wonder why the infectious disease disappeared suddenly at 1956 years and be forgotten afterthere. Once more it followed that San miguel sea lion virus was isolated from sea lion in abortion case at 1973 years by Smith in California USA and they revealed that the viral physicochemical and morphological characteristics could not be divided entirely with VEV such as naked RNA virus , 37 nm in size and hollow capsomere. From their further experiment to attempt the pathogenicity it was very surprised that the agent caused vesicles to pig and monkey by means of intradermal inoculation, and produced antibody in pig, fox and man. Those pathological features will give rise to a future problem in man. On the other side, the first isolation of feline calicivirus was carried out by Fastier at 1957 years. In those days the agent was

called as feline picornavirus until 1971 years. Now feline calicivirus is also well known as causative agent of upper respiratory infection of cats in Japan, which was reported by Konishi and Akimoto. The group of feline calicivirus, VEV and San miguel sea lion virus has proposed a question on the possible member of Family Picornaviridae in disagreement with morphology possessing 37 nm in size and hollow capsomere. The comparative studies on VEV, SMSV, feline calicivirus and picornavirus suggested that calicivirus might be coincided with alphavirus belonging to Family Togaviridae rather than Picornaviridae, especially in the view of detection of two single-stranded RNAs in calicivirus infected cells in despite of one single-stranded RNA in poliovirus infected cells. As described above, recently the most interesting problem was proposed to new Family Caliciviridae by following reason, for examples, calicivirus is naked RNA virus similar to picornavirus, nevertheless, producing two single-stranded RNAs similar to alphavirus which proves a typical membranous virus. Thus, for the newest family, it is necessary that the mechanism of viral replication will be examined by means of molecular biology or morphology. The relationship between calicivirus and picornavirus must be investigated, but also picornavirus has the longest history

and the mechanism of the replication has been studied remarkably at molecular level, on the other hand, calicivirus lacks in those studies on viral replication at all. In despite of successful development at molecular level on the replication of poliovirus, the difficulty of morphological examination remained because of too small and simple naked virion presenting 20-30 nm in size. It may be indicated only technical limit by means of electron microscope. The periodic studies on the replication of picornavirus have not been reported so many, Dales (1965) provided most exactly the morphological studies, Peterson (1970) in feline calicivirus and Zee (1967) in VEV reported respectively, while they remained insufficiently. Thus, author attempted to supplement a weak point of picornavirus by calicivirus with larger size of 37 nm and prove further evidence to mechanism of calicivirus replication.

## Materials and methods

Viruses : FIV-1 strain of feline calicivirus was isolated by Dr. Akimoto and derived from his kindness. The strain was passaged 17-19 times through the primary tissue-cultured cells of feline kidney.

Cell cultures : Feline kidney cells of kittens were prepared at the age of one to six weeks by trypsinization method. Eagle MEM media for growth of cells were supplemented with 10 % calf serum, 1 % TPB (Tri-phosphate broth), 100 µg/ml Kanamycin. The medium for viral growth consisted of Eagle, 1 % TPB, 0.05 % Yeast and 0.1 % glucose without calf serum.

Immunofluorescent antibody techniques : The conjugation with hyperimmunized serum and fluorescein isothiocyanate was performed by following procedures in Figs.1 and 2.

Electron microscopical procedures : Infected cells were harvested at each time and spun down, then mixed with agar and fixed as next schedule.

### 1. Fixative procedure

- 1) Excise the cells and put into the drop of the Karnovsky's fixative as quickly as possible. The agar mixed cells were cut out with a razor blade into a small piece.
- 2) These pieces are picked up with a spatula and placed

in a bottle containing 1 to several ml of fixative for 2 hours at room temperature.

- 3) Rinse the tissue several times with cacodylate buffer.
- 4) Postfix with 1 % solution of OsO<sub>4</sub> in cacodylate buffer for 1 - 2 hours at room temperature.
- 5) Pour off the fixative and add the aqueous solution of uranyl acetate (0.5-2 %), soaking for 2 hours.

## 2. Dehydration and embedding procedure

- 1) After fixation and post-treatment, dehydration of blocks can be performed through the increasing concentrations of ethyl alcohol.
- 2) 50, 70, 80, 95 % solution of ethyl alcohol ( 15 minutes each at 4°C ).
- 3) 15 min. in 95 % ethanol and 95 % acetone ( 1 : 1 ).
- 4) 15 min. in 95 % acetone.
- 5) 10 min. in 99 % acetone.
- 6) 20 min. in absolute acetone ( 3 times ).
- 7) 60 min. in acetone and Epon mixture ( 3 : 1 ).
- 8) 60 min. in acetone and Epon mixture ( 2 : 1 ).
- 9) Overnight in acetone and Epon mixture ( 1 : 1 ).
- 10) 60 min. in acetone and Epon mixture ( 1 : 2 ).
- 11) 60 min. in fresh Epon mixture.
- 12) Embed in Epon mixture in gelatin capsules.
- 13) Polymerization at 37°C ( 1 day ), at 45°C ( 1 day ), and finally at 60°C ( one and a half day ).

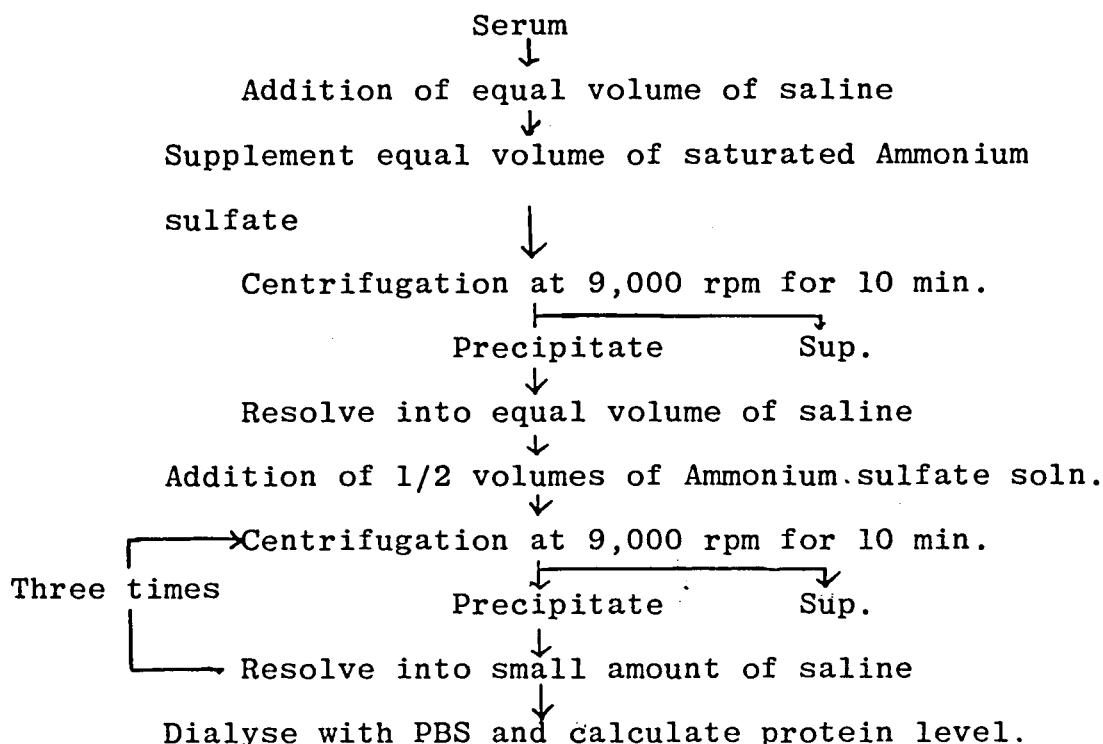


Fig. 1. Purification of  $\gamma$  - Globulin.

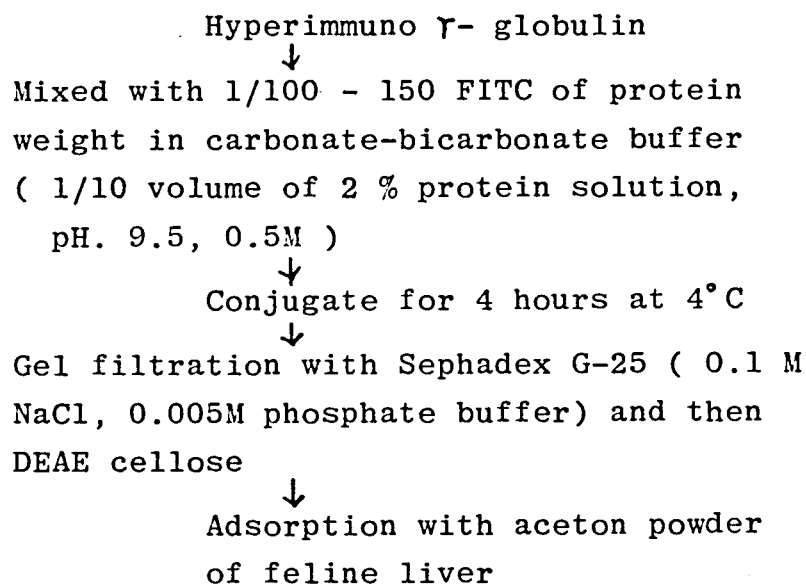


Fig. 2. Formation of label antibody with FITC.

## Results and discussion

### 1. Growth curve of feline calicivirus and appearance of viral antigen

The appearance of infectious viruses in feline calicivirus infected cells and releasing into aqueous phase was determined respectively. The infectious viruses were not detected at 2 hours after infection. Then the titer of producing viruses at 4 hours in the cells was calculated as  $10^{5.25}$  TCID<sub>50</sub> per ml and reached logarithmically to the plateau at 16 hours, soon decreased afterwards. The releasing viruses into medium propagated in parallel with intracellular viruses at rate of rather low titers. The result expressed one step growth and under the experimental condition the eclipse of calicivirus was considerable to estimate 2 hours after infection.

The experiment on the growth of feline calicivirus resulted in a very rapid viral production similar to another general small RNA viruses such as poliovirus reported by Mandel (1962) or Howes (1957), while reovirus with double strand RNA and naked double protein layers was estimated 7 hours as long eclipse. The next all experiments on feline calicivirus were performed under the condition as described above.



The localization of specific fluorescence to viral antigen and the periodic variation was next followed. The immunofluorescent antibody technic prepared by two procedures was tested to determine a site of specific antigens of feline calicivirus. The first procedure was derived by conjugation of fluorescein isothiocyanate with hyperimmunized rabbit antiserum to viral antigen and another with cat antiserum. Both antisera could be removed nonspecific fluorescence by adsorption with acetone powder of cat liver. The later method provided more successful result. The effects of MOI were tested at 4 grades consisted of 3, 0.3, 0.03, and 0.003 PFU/cell respectively. Percent of infected cells was calculated as bright cells visible in dark field per all number of HE stained cells counted at 400 folds. At 18 hours after infection those fluorescent cells reached to 74 %, but practically it might be provided 80 % over because the affected cells were detached from the glass wall with rounding form. The specific fluorescence was localized only in cytoplasm, but not detected in nucleus. Ozaki (1968) reported crystalline array including empty viral particles in the nucleus. On the other hand, the present picture was not recognized in the case of calicivirus infected cells. Progressive observation followed slight brightness appeared in the cytoplasm at 4 hours and strengthened the brightness

with the times to 8 hours. At the stage recognizable of viral antigen, the new progeny viruses were produced already, therefore, enough amount of protein induced by viral infection was accumulated in cytoplasm. After 4 hours the brightness had a tendency to be found around the vacuole and changed the shape for granular or filamentous arrangement. Those results corresponded with the knowledge reported previously by Buckley (1957) who appeared at 5 hours after picornavirus infection.

On the synthesis of virus specific protein, the additional or removal tests of Puromycin well known as inhibitor of protein synthesis were attempted even though indirectly. The viral growth was suppressed extremely in the concentration of 10  $\mu$ g per ml but not 1  $\mu$ g. Therefore the maintenance medium supplemented with 10  $\mu$ g per ml was applied to the following tests and those of which visualized by electron microscopical observation. The synthesis of viral protein happened between 2 to 3 hours and continued mildly to 6 hours after infection. Especially the cytoplasmic membranes such as rough and smooth endoplasmic reticulum or vacuoles disappeared as observed by electron microscope.

Usually the protein synthesis of picornavirus occurred between 2.5 and 4.5 hours. In the view, the both picorna- and calicivirus showed very similar result making the

viral protein at quite early stage. Levintow (1962) and Scharff (1963) reported that the addition of Puromycin at the stage of eclipse suppressed the RNA synthesis and viral maturation. They supported the necessity of protein synthesis before RNA replication.

There was no effect of Actinomycin D known as DNA dependent RNA polymerase inhibitor. Although Actinomycin D of 0.5 µg per ml caused a mild toxicity to normal cultured cells, feline calicivirus could grow quite well as same as no exposure. It was indicated that RNA synthesis from DNA in host cells was not needed for the viral replication at all. Also the effect of HBB (2- $\alpha$ (hydroxybenzyl)-benzimidazole) working as suppressor at the level of picornavirus RNA synthesis has reported that the reagent inhibited the growth of many enteroviruses, whereas not done in calicivirus. Further comparison of two viruses consisting of feline calicivirus and coxackie-4B was performed by addition of HBB of 219 µg per ml. Coxackie-4B was suppressed completely, but feline calicivirus could propagate constantly. It may be considerable that calicivirus has clear difference at the level of replication or transcription of viral RNA, otherwise, RNA structure itself.

## 2. Ultramicroscopical events of the nucleus of infected cells

As described above, the examination by means of immunofluorescent antibody technique could not account for various events developing in nucleus of infected cells except for finding of viral antigen exclusively in cytoplasm. On the other hand, observation by electron microscope provided very characteristic morphological changes in nucleus. The morphogenesis of nucleus varied with cytoplasmic alteration. The outer and inner unit membranes of some nuclei took place a fine irregular complicated roughness and separated each other with narrow opening space at 3 hours after infection, then between 5 and 8 hours the outer nuclear membrane separated gradually from inner membrane and formed the extrusions and deep invagination, furthermore spreading into cytoplasm and formed islands. Those expanding membranes could not be divided in the developing cytoplasmic membranes. But the present structural changes of nucleus did not play a positive role on the viral growth. Hinz (1962) in poliovirus and Dales (1962) in mengovirus reported similar results too.

The accumulation of chromatin adjacent to nuclear membrane and polylobulation of nucleus were observed. The rate of altered cells with accumulation of chromatin increased  $4.4 \pm 2.7$  (M  $\pm$  S) to  $36.4 \pm 8.5$  % between 3

and 5 hours, then decreased to  $2.7 \pm 5.4$  %. Obviously the degree of morphological nuclear alteration increased with the advance of viral production and followed to very severe polylobulation of nucleus later, but these severe nuclear alteration might be caused by degeneration owing to viral growth because viruses could be produced in infected cells without nuclear damage.

### 3. Morphological structures at early stage of the cytoplasm of virus infected cells

There was not recognizable change in cytoplasm until 2 hours, at 3 hours the small granular structures were visualized with very high electron density and bound titely with cytoplasmic membranes forming the specifical regions which divided from those of normal cells. Such granular shape took a several size, one presented 43.4 nm and characterized by core like structure with very high dense in the center, surrounding low dense materials. Another detectable granular structures in cytoplasm were estimated in each size such as 18.4 nm in free ribosomes, 20.0 nm in ribosomes derived from rough endoplasmic reticulum, 20.6 nm in ribosomes from complex membranous body (CMB) and 27.1 nm - 32.6 nm in viral particles as calculated by electron microscopical observation of thin sections. The identified essential materials seemd to be of largest size and attached to

mitochondria. Sections at 5 hours after infection showed further development of the viral precursor complex like structure described above and few numbers of viral particles. The present structure had very significant features as precursor at early stage of viral infection, thus it was designated as viral precursor complex (VPC). The precursor complex increased 48.1 % to 76.5 % during 3 to 5 hours and took place exclusively throughout the viral replication cycle from the termination of eclipse to the appearance of progeny viruses. While CPE(roundind of cells) was observed by right microscope at 5 hours, at the same time many small vesicles diffused each other and making more larger precursor complex with accumulating dense materials around the vesicles to 8 hours. Intermediate structure , which was formed from precursor complex, was detected less frequently at later stage, it seemed to be Nucleoplasma proposed in NDV and poliovirus previously.

Further relationship between the viral precursor complex and cytoplasmic membrane was tested. The morphological observation revealed certainly that the cytoplasmic membrane bound titely with the precursor complex and developed with the viral infection. Furthermore the relationship between the cytoplasmic membrane and viral production was determined by using Iodoacetic acid which

suppresses especially glycolysis of membrane, and Anphotelicin B which opens a pin hole to cell membranes and causes hemolysis as exposed to red blood cells. To attempt those experiments, each of the drugs was tested on the toxicity to feline kidney cells or red blood cells and the yield of infectious viruses by addition of several concentrations. Iodo acetic acid suppressed completely the viral yield under concentration of 0.01 mg per ml, but also having the middle toxicity. Whereas, the viral yield was suppressed at middle rate in 0.001 mg/ml, but being no toxicity. From this result, the proper concentration of Iodo acetic acid was employed 0.01 mg per ml and inhibited the production of infectious viruses at so early stage as 30 minutes. The effect of Ido acetic acid terminated at 5 hours after infection, therefore, it is suggested that the cytoplasmic membrane works during a period from uncoating to appearance of the new progeny viruses. Generally RNA of picornavirus bound to cytoplasmic membrane after the uncoating so that it may be possible that the cytoplasmic membrane proves a space to assembly of the viral particles after RNA and protein synthesis. While Anphotelicin B could not show so clear results, which the reagent caused strong hemolysis by addition of 1 mg per ml and yield of viruses could not be suppressed so enough by addition of 0.1 mg per ml.

#### 4. Morphological structures at later stage of cytoplasm of virus infected cells

Peterson et al (1970) reported the structure designated as "complex membranous body"(CMB) in feline calicivirus infected cells, but they could not conclude about the function because the experimental condition was not synchronized exactly so that each periodic picture did not appear as the constant morphological sequence with time course. Also the present structure of CMB was reported in picornavirus. The both findings of calicivirus and picornavirus seemed to be formed by the endoplasmic reticulum itself, but also rich number of ribosomes accumulating on the surface of expanding cytoplasmic membrane increased the higher electron density compared with normal cells. The remarkable development which connected with very high dense and rich ribosomes has a doubt enough to guess a region regarded as the virus producing factory. But it appeared at first 8 hours in this experiment system and no more advancing. Therefore CMB was considered as degenerating production rather than the viral matrix. Those altered membrane or degraded ribosomes are more apt to aggregate with each other and keeping the original morphology after broken up.

The appearance of viral particles was recognized



in the cytoplasm between 5 to 8 hours after infection. The general shape of the viral particles by the observation in thin section presented the dense core in center and layered around the core with low dense fringe. The viruses arranged in several types ; crystalline array packed in lattice ( $27.1 \pm 3.9$  nm in size), linear array bound with fine fibers ( $28.9 \pm 1.5$  nm), pre-crystalline array was detected most frequently, of which particle size exhibited larger calculation of  $32.6 \pm 3.7$  nm and near to  $37.2 \pm 3$  nm as the size of negative staining. Someone attached clearly along with membrane or being similar to a bunch of grapes. The core less empty capsids were found at very low frequency.

To determine the morphological sequence of viral replication in the infected cells, each observed cell was divided into six morphological types with two features due to nuclear and cytoplasmic events. The cells belonging to type 1 to 6 had following features in table 1. At the end of viral adsorption, the cells of type 2 predominated mostly at the rate of  $85.3 \pm 5.4$  %, the two types 4 and 5 at 5 hours reached to 76.5 % and the type 6 increased 55.7 % in exchange for decrease in 18.4 % of the types 4 and 5 at 8 hours. At that time the cell type 6 exhibited rather low value because it has been already destroyed practically. The

quantitative analysis explained further significantly for the viral precursor complex to be virus formation factory. Levinthal (1969) demonstrated the vesicles bound with the specific ferritin-antibodies in poliovirus infected cells, but his present picture was very simple structure without such high dense granular materials. Is that so, in comparison of another reports, the morphogenesis of small RNA viruses may be divided in three patterns consisting of the first poliovirus, rhinovirus and echovirus, the second mengovirus and the third calicivirus.

Table.1. Characteristics of infected cells with time course.

Cell alteration	Cell types					
	1	2	3	4	5	6
Chromatin accumulation of nucleus	-	-	+	-	+	
Cytoplasmic alteration						
M : Membrane						
VPC : Viral precursor complex	poor	rich	rich	rich	rich	rich
CMB : Cytoplasmic membranous body	M	M	M	VPC	VPC	VP
VP : Viral particle						CMB

##### 5. Viral replication and organelle

Mitochondria did not advance so severe morphologically with the times, also  $\text{NaN}_3$  suppressing the cytochrome oxidase reaction did not inhibit the viral growth at all.

Some mitochondria showed a visualizable morphological degeneration at 15 hours, and during 8 to 15 hours sometimes very large aggregates of degrading ribosomes were observed.

On the releasing mechanism of progeny viruses, there was not a special mechanism throughout all viruses which appeared in pictures. The present paper did not describe about the mechanism of adsorption and how to enter into cells or what factors concerned to CPE and kinetics of viral molecular components. Especially Cordell-Stewart (1971) suggested very interesting double strand RNA inducing CPE. In future, it must be studied on the metabolism directed by the viral infection.

#### 6. The third dimensional structure of feline calicivirus infected cells.

The virion and precursor complex as observed by second dimensional section method were determined further by solid state. The infected cells, which harvested for a period of 10-12 hours, were spun down and melted by Ammonium acetic acid, after 30 minutes stained by 2 % phospho-tangstate at 4°C. One picture showed scattered viral particles individually, another aggregated each other. The most remarkable features were several bunches of strings formed with subunit like small

chains and covered with those rich subunit like substances on the membranes. Also those structures contained some numbers of viral particles localized one side or elsewhere. Some viral particles combined with one suck with similar size of virion. Another picture of the sucks may explain a pre-capsid making up one virion. The structure of the virion was provided by rotation techniques of negative staining or section. In this result, feline calicivirus proposed 3 and 5 times helical symmetry and making up two layers with hollow capsomeres. The inner capsid and core combined with more closely rather than outer capsid because the outer capsids formed core less empty capsids as observed by section or negative staining. Zwillenberg and Bürki (1966) first reported about double layer capsids similar to reovirus. Peterson and Studdert (1970) suggested further detail model in the point of core and hollow capsomere formation by means of rotation techniques. There was not further new structural knowlege on the virion in this study compared with previous reports, whereas provided further evidence concerning with membrane as virus producing factory, in which synthesizing viral RNA and assembling virion, and it might be possible to demonstrate more simple morphological development of the infected cells rather than another picornvirus.

Generally the RNA viruses have proper RNA such as

simple strand + RNA in picornaviridae and togaviridae,  
- RNA in rhabdoviridae and double strand RNAs in reoviridae, or another case in retroviridae which forms RNA dependent DNA. In the criteria, calicivirus belonged to picornaviridae previously, but recently has been supporting the similarity to alphavirus in the family Togaviridae by reason of two pieces of single strand RNAs in infected cells. On the other hand, picornaviridae has 4 polypeptides in the virion. Those properties except for the difference of virion will propose the possibility that calicivirus may prove another morphological advance compared with picornaviridae if observed by electron microscope. While Caliguiri (1973) suggested that viral RNA polymerase correlated with smooth endoplasmic reticulum, he (1970 and 1971) reported that viral RNA was replicated on the smooth endoplasmic and protein synthesized on the rough endoplasmic reticulum. Horne revealed a large body containing viral subunits and viral particles on the membranous structure at late stage of poliovirus infected cells, which was very similar to membranes presented by negative staining of feline calicivirus. Dales (1963) and Mayor (1962) reported small bodies(SB) and viral bleb(VB) , suggesting that both membranous structures involved several number of virus-like particles. Still now the molecular and morphological studies were not

summarized significantly, but coincided with the relationship of membrane throughout viral replication. Perhaps it will not be so easy to encounter further new findings by means of electron microscopical study. From those reasons, calicivirus was compared with picornaviridae in this study and both viruses would be separated each other.

## Summary

The periodic advance of feline calicivirus replication in the infected cells was determined by several methods such as immunofluorescent antibody technique, inhibition tests of protein synthesis or glycolysis of cell membrane and electron microscope. In comparison with picornavirus, especially the morphogenesis of feline calicivirus proved the further evidence that the viral precursor complex(VPC) correlated with a virus producing factory. in which performed the viral RNA synthesis and assembly of progeny virus. The presented VPC was divided in SB or VB designated in the case of poliovirus and DM in mengovirus. From those results, it was suggested that calicivirus must be clasified in family Caliciviridae.