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THE ISOLATION OF BOVINE EPHEMERAL FEVER VIRUS IN TAIWAN IN 1984

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At the epizootic of Bovine Ephemeral Fever (BEF) in dairy cattle in 1984, the isolation of the causative virus was first attempted in Taiwan. From the febrile cattle (3 Holsteins and 1 Hybrid) in southern Taiwan, 4 strains of BEF virus were isolated by intracerebral inoculations in mice.

In the studies on the physical characteristics of isolated virus, the microorganism showed susceptibility to various chemicals and treatments. With the studies on;

1. **Cross neutralization test between the isolated virus and the standard BEF virus,**
2. **The findings of the specific fluorescent antigen in BHK 21 cell culture after the inoculation of isolated virus,**
3. **Morphological observation of the isolated virus as a Rhabdoviridae virus by electron microscope,**
4. **Inoculation of the isolated virus reproduced the symptoms of BEF, i.e. depression, anorexia, pyrexia, excessive salivation and nasal discharge, tachypnea and articular pain caused lameness, the isolated virus was identified as BEF virus.**

The occurance of Bovine Ephemeral Fever (BEF) has been reported in African countries, India, Indonesia, Australia, Near East countries and Japan since early 20th century.

In Taiwan, the occurance of BEF in 1963 and 1967 was suspected, because no isolation of virus was made and only clinical signs and partial serological test had been made. The situation of BEF in Taiwan was, therefore, largely obscure.

Up to the date, BEF virus was isolated only in the following 6 countries in the world, namely South Africa, Nigeria, Kenya, Iran, Australia and Japan.[1,3,9,10,11,14,15,16,19] The author attempted to isolate BEF virus in Taiwan at the outbreak of BEF in 1984. Using the method described by Van der Westhuizen et al.[19] and Sasaki et al.,[14] 4 strains of BEF virus was successfully isolated from the blood of febrile cattle. Consequently, physical characteristics, specific fluorescence in culture cells and the electron microscopic morphology of the isolates were studied. Finally inoculating the isolated virus to a calf, the clinical symptoms of BEF were successfully reproduced.

MATERIALS AND METHODS

1. Mice

One day old suckling DDY hybrid mice available at the market were used

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for the isolation and passages of the virus.

2. Viruses

(1) Bovine Ephemeral Fever Virus:

The YHL strain, the standard BEF virus, which was given to the Institute by the National Institute of Animal Health, Japan in 1979, and the isolated Taiwan Livestock Research Institute (TLRI) and Liu Yin strains were used.

(2) Bovine Entero Virus (BEV):

The entero virus isolated by Dr. M. F. Jong from the nasal mucous fluid of a imported cow in Taiwan in 1973 was used as reference virus.

(3) Infectious Bovine Rhinotracheitis Virus (IBRV):

Another reference virus, Infectious Bovine Rhinotracheitis Virus C strain, which was given to this Institute by Auburn Veterinary College, U.S.A. in 1973 was used.

3. Antisera

The rabbit anti BEFV sera were manufactured. The YHL, TLRI and Liu Yin strains of BEFV were each innoculated in BHK 21 (Baby Hamster Kidney Origin) cell line and each culture fluid was centrifugated with low verocity when the titer of virus in the culture reached $10^{5.5-6.0}$ $TCID_{50}$/ml. The supernatant was then centrifugated (73,000 Xg) for 90 minutes. The precipitate was in turn diluted with Phosphate Buffer Solution (BPS) to 1/100 of its original volume. Equal part of this virus solution and Freund's complete adjuvant were mixed and 1 ml of this mixture was innoculated intramuscularly 3 times, with an interval of 2 weeks each, to the rabbits. The rabbits were bled 3 weeks after the last innoculation and the sera were stored at -20°C.

4. Isolation of Virus

An amount of 0.1-0.3 ml of defibrinated febrile cattle blood was innoculated intracerebrally into one day old suckling mice. Within the observation period of 21 days, the brains of dead or clinical mice were made into 10% emulsion for the blind passage.

5. Determination of Virus Titer

A volume of 0.025 ml each of the 10 fold sequentially diluted (Maintenance media, Gibco, containing 8% calf serum) virus culture fluid was added to 4 wells, in which BHK 21 or HmLu-1 cells were grown. After observing cytopathic effect (CPE) in 5 days, $TCID_{50}$ was culculated.

6. Physical Characteristics of the Isolated Virus

(1) The influence of ethyl ether and chloroform:

A mixture of 0.2 ml of ethyl ether and 0.8 ml of virus fluid was left at room temperature (25°C) for 60 minutes. After a centrifugation of 20 minutes, the ether in the tube was removed by negative pressure and then the titer of the virus was measured.

Likewise, the influence of chloroform to the isolated virus was investigated with the mixture of 0.1 ml of chloroform and 1.9 ml of virus culture fluid.

(2) The influence of sodium deoxycholate:

One ml of 0.2% sodium deoxycholate, which was diluted with PBS (pH 7.4), was added to 1 ml of virus culture fuid. After 60 minutes treatment of the mixture at 37°C, the virus titer was tested.

(3) The influence of trypsin:

One ml each of 1.25% and 0.25% trypsin (Difco 1:250) was mixed with 1 ml each of virus culture fluid. The mixtures were treated at 37°C for 60 minutes. To remove the activity of trypsin in the mixture, 8 ml of cooled bovine serum (inactivated at 56°C for 30 min., serum neutralization antibody to isolated virus negative) were added to each mixture and then the titer measured. As for the reference test, PBS instead of trypsin was used.

(4) The resistance to pH:

A volume of 1.8 ml each of pH 3.0 and pH 8.0 McIlvaine buffer solution and PBS (pH 7.2) was added to 0.2 ml each of virus culture fluid and left for 60 minutes before the titer was tested.

(5) Freeze-thaw influence:

One ml virus culture fluid each was put in 10 tubes. These tubes were alternatively put in dry ice - acetone and running water to repeat the process of freeze-thaw up to 10 times. The titer of the virus in each tube was then tested.

(6) The influence of 1M $MgCl_2$:

To 1.0 ml each of 10 fold diluted virus culture fluid, 1.0 ml each of 2M $MgCl_2$, distilled water and MEM was added and left for 1 hour at 50°C. Then the titer was tested.

IBRV and BEV were likewise treated as the erference.

(7) The influence of 56°C:

One ml each of virus culture fluid was taken in the tubes and put in 56°C water bath. At 5, 10, 20, 30, 60, 90 and 120 minutes, 1 tube at a time was transfered to ice water standing and the titer tested.

7. The Measurement of the Growth of Isolated Virus in BHK 21 Cells

The isolated TLRI strain (3 passages in mice and 7 passages in BHK 21 cell line) with a titer of $10^{5.0}TCID_{50}$/ml was used.

To the BHK 21 cells grown in petri dishes, 0.2 ml each of above mentioned virus culture fluid was added and adsorbed for 1 hour at 37°C on the 3rd day. Then the dishes were rinsed 3 times with YLE (yeast, lactoalbmin and Eagle's solution) solution and 4 ml each of MEM were added.

Periodically, 2 petri dishes were taken and the content mixed and centrifugated at 3,500 rpm for 20 min. The virus in the supernatant was taken as liquid phase virus and its titer tested.

As to the cell phase virus, the virus culture fluid was first emptyed and washed 3 times with YLE. Then 4 ml of YLE were added and repeated 3 times the freeze-thaw process alternatively at -80°C (ultra low freezer) and 15°C (running water). The content was then put in a tube for centrifugation at 3,500 rpm for 20 min. The supernatant was used as cell phase virus titer measurement.

8. Preparation of Fluorescent Labeled Antibody

The convalescent serum of the cattle (cattle No. 073-232), from which the Liu Yin strain was isolated, with a serum neutralization titer of 256 was used. After 2 fold dilution with PBS, equal amount of saturated ammonium sulfate was slowly added and left at room temperature for 30 min. And then it was centrifugated at 4,000 rpm for 5 min., discarding supernatant, and an amount of PBS double of its original serum was added to solve the precipitates. After dialysing the precipitates 3 times in 50% ammonium sulfate solution, an amount of PBS 1/2 to the original volume

of serum was added.

After removing out ammonium sulfate by putting dialysing tube (Visking) in running water and then in PBS, the fluid was centrifugated at 3,000 rpm for 5 min. The protein content of the supernatant was tested with proteometer.

Fluorescent isocyanate (BBL, USA) of 1/50 volume to the total protein was solved in 4 ml of 0.5 M sodium carbonate buffer solution (pH 9.0) and added to above mentioned supernatant and mixed with magnetic stirrer at 37°C for 1 hour. To remove unbind pigment, the fluid was let through the cephadex G-25 column equilibrated with 0.005 M K_2HPO_4 solution.

The filtered fluid was further adsorbed on DEAE Cellrose equilibrated with 0.005 M K_2HPO_4 to remove unspecific fluorescence. The cellrose was washed with 0.005 M K_2HPO_4 added 0.1 M NaCl and the washed fluid was used as labeled antibody. A volume of 0.1% of NaN_3 was added to the labeled antibody fluid and stored in freezer.

9. Staining Method of Fluorescent Labeled Antibody

The isolated virus was innoculated in cover slip media. The cover slips were hourly taken and washed 3 times with PBS and dried in cool wind and then fixed with acetone (-20°C) for 10 min. Fluorescent labeled antibody fluid was put on cover slip and after let it stood at 37°C for 30 min. the cover slip was washed 3 times with PBS. Direct observation of specific fluorescent antigen antibody reactions was made.

10. Electron Microscope (EM) Observation

Isolated virus, Liu Yin strain cultured in BHK 21 cells at 34°C for 48 hours was used. The culture fluid was first condensed with 3,000 rpm centrifugation for 20 min.. The super natant was ultra centrifugated with 90,000 rpm for 15 min.. Negative stain of the precipitate with 2% phosphotangstate was prepared for EM observation.

Ultra thin section of infected cells was made. Cultured cells were washed 3 times with PBS and fixed with 2% glutaldehyde at room temperature for 1 hour. The scrathed down cell pellets, were further fixed with 1% OsO_4 solution and dehydrated in serially ascending concentration of ethanol. The pellets were then buryed in Epon 812 for ultra thin section with glass (diamond) knife. The section was double stained with uranil acetate and plumbic acetate for EM (Hitach 600) examination.

11. Reproduction Test of BEF

The virus of TLRI strain isolated in May 1984 was used as inoculant after 3 passages in mice and 1 passage in BHK cell line. The calf was born in non epidemic period and artificially reared for 3 month (sero negative). The above mentioned virus fluid ($10^{6.0}TCID_{50}$/ml) was inoculated to the calf intravenously. Post inoculation, the calf was clasely observed for 10 days and the body temperature, respiration and pulse rate were recorded.

RESULTS

1. Virus Isolation

To each one day old suckling mouse 0.3 ml of the defibrinated blood of febrile cattle sent from the various part of Taiwan in April - May 1984 was inoculated intracerebrally. Subsequently, the mice showed

Table 1. The Condition of 1-day-old Mice in the Process of the Isolation of Liu Yin Strain

No. of Passage in Mice	Nortality (%)	Ave. No. Day to Death
1	6/10* (60.0)	11±2
2	5/6 (83.3)	9±1
3	8/8 (100.0)	4±1
4	12/12 (100.0)	4±1
5	10/10 (100.0)	4±1

*Denominator: Number of mice tested
Numerator : Number of mice died

clinical signs between 8 to 13 days. The mice showed clinical signs, i.e. depression, anorexia, paralysis of hind quarter, etc and died 2 to 4 days later (Fig. 1).

At the second passage, the mice died between 6 to 14 days and the 3rd passage, 3 to 5 days. The condition of mice in the process of the isolation of Liu Yin strain is shown in Table 1. The mortality in the first passage was 60%. It increased to 83% in the second passage and to 100% at the 3rd. Approximately taking the same process, 3 other strains, namely Kaohsiung strain, TLRI strain and Chiayi strain were isolated.

2. The Cell Culture of Isolated Virus

Adaptation of the isolated 4 strains of BEFV to BHK 21 cell and HmLu-1 cell was tried. At the 3rd day of inoculation, the cells started showing cytopathic effect (CPE). Rounding of the cells became more eminent with the lapse of time (Fig. 2) and finally they falled from the tube wall.

3. The Physical Characteristics of Isolated Virus

(1) The resistance to chemicals:

Ethyl ether and chloroform inactivated the TLRI and Liu Yin strains of virus completely after a treatment at 25°C for 60 min. IBRV was also inactivated under the same treatment, but BEV was resistant (Table 2).

(2) The influence of deoxycholate:

To 1 ml of BHK 21 cultured fluid, 1 ml of 0.2% deoxycholate or PBS was added and mixed. The virus titer was tested after the mixture was treated at 37°C for 1 hour. Both TLRI and Liu Yin strains were completely inactivated, and so was IBRV, but BEV was remained stable (Table 3).

(3) The influence of trypsin:

Both 1.25% and 0.25% PBS diluted trypsin solutions inactivated the 2 strains of isolates under 37°C for 60 min. IBRV was inactivated with 1.25% trypsin, it

Table 2. The Influence of Ethyl Ether and Chloroform to the Isolated Virus

Virus	Titer $\log_{10}TCID_{50}/ml$		
	Ethyl Ether	Chloroform	Control
Isolate 1*	≤0.5	≤0.5	5.5
Isolate 2**	≤0.5	≤0.5	6.0
IBR Virus	≤0. 5	≤0.5	7.5
Bovine Entero Virus	6.5	6.5	7.0

*TLRI Strain
**Liu Yin Strain

Fig. 1.
The conditions of mice on the 6th day after the intracerebral inoculation of the defibrinated cattle blood, which lead to the isolation of Liu Yin strain.

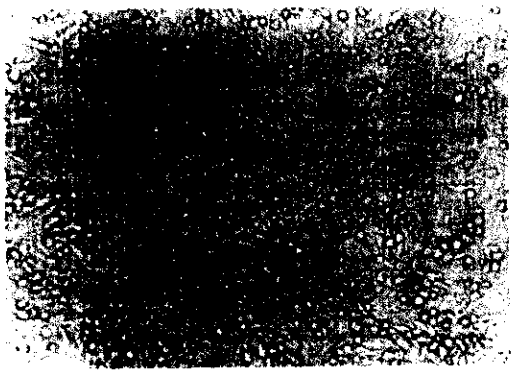

Fig. 2.
Cytopathic Effect in BHK 21 cells after the inoculation of isolated BEFV, Liu Yin strain.

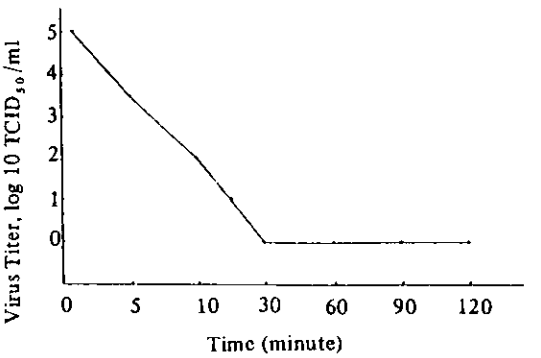

Fig. 3.
The Resistance of the Isolated Virus to Heating.

Fig. 4.
Specific-fluorescent antigen in BHK 21 cell membrane (24 hours post inoculation of isolated virus, X200).

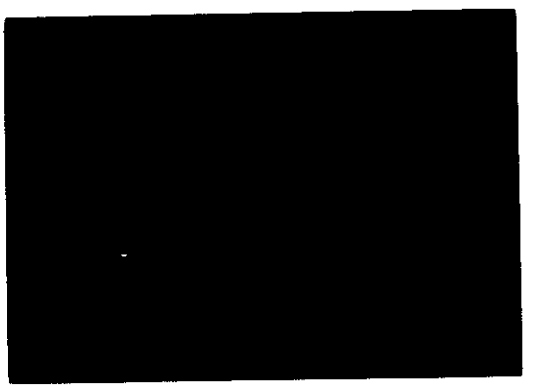

Fig. 5.
Specific fluorescent antigen in BHK 21 cell plasma (48 hours post inoculation of isolated virus, X200).

Table 3. The Influence of Sodium Deoxycholate to the Isolated Virus.

Virus	Titer $\log_{10}$ $TCID_{50}$/0.1 ml	
	0.1% Sodium Deoxycholate	PBS
Isolate (TLRI)	+ 0.5	4.5
Isolate (Liu Yin)	⩽ 0.5	5.0
Bovine Entero Virus	5.75	6.0
IBR Virus	⩽ 0.5	6.0

Table 4. The Influence of Trypsin to the Isolated Virus.

Virus	Trypsin concentration		PBS (pH 7.4)
	1.25%	0.25%	
Isolate (TLRI)	⩽ 0.5*	⩽ 0.5	5.0
Isolate (Liu Yin)	⩽ 0.5	⩽ 0.5	5.5
Bovine Entero Virus	5.5	5.5	5.5
IBR Virus	⩽ 0.5	1.5	6.0

*$\log_{10}$ $TCID_{50}$/0.1ml

Table 5. The Influence of pH to the Isolated Virus.

Virus	Mc Ilvaine Buffer Saline		pH 7.4 (PBS)
	3.0	8.0	
Isolate (TLRI)	⩽ 0.5*	4.5	4.5
Isolate (Liu Yin)	⩽ 0.5	4.5	4.5
Bovine Entero Virus	6.0	6.0	6.0
IBR Virus	⩽ 0.5	4.0	4.5

*$\log_{10}$ $TCID_{50}$/0.1 ml.

showed only the lowered titer of $10^{4.5}$ $TCID_{50}$/ml under 0.25% trypsin treatment. BEV was resistant to trypsin (Table 4).

(4) The influence of pH:

The two strains of isolated BEFV showed resistance to pH 8.0, but they were inactivated at pH 3.0 when 0.2 ml each of the culture fluid was added to 1.8 ml of pH 3.0 and pH 8.0 buffer solutions and left at room temperature for 60 min. BEV was stable, but IBRV was inactivated with pH 3.0 treatment (Table 5).

(5) The influence of freeze-thaw process:

The virus titer of BHK 21 cell culture after 1, 2, 3, 5 and 10 repeatings of freeze-thaw process in dry-ice-acetone and running water (20°C), measured $10^{5.5}$, $10^{5.5}$, $10^{5.25}$, $10^{5.25}$ and $10^{5.25}$ $TCID_{50}$/ml respectively. Untreated virus had a titer of $10^{5.5}$ $TCID_{50}$/ml (Table 6).

(6) The presence of 1 M $MgCl_2$:

The BHK 21 cell culture of isolated virus was diluted 10 fold with distilled water. To each ml of the diluted fluid, 1 ml each of 2 M $MgCl_2$ solution or MEM or distilled water was added respectively and treated at 50°C for 60 min. The results showed that all treatments inactivated the two strains of the isolates. It also inactivated IBRV, but not BEV (Table 7).

(7) The influence of heat:

Liu Yin strain with a titer of $10^{5.0}$ $TCID_{50}$/ml was treated at 56°C for 5, 10, 30, 60, 90 and 120 min. The heat lowered the titer of 1.5 and 30 minutes or longer inactivated the virus (Fig. 3).

4. The Cross Neutralization Test

As shown in Table 8, the neutralization test between the isolated virus strains and the standard BEFV, YHL strain crossed well.

5. The Specific Fluorescent Antigen

The specific fluorescent antigen was first observed at 12 hours after the inoculation of isolated virus to BHK 21 cell. It was first seen on cell membrane (Fig. 4) at 12 hours PI and then in cell plasma 24 hours PI (Fig. 5).

6. Electron microscopic observation

Ultracentrifugated and negatively stained isolated Liu Yin strain under electron microscope appeared as a bullet shaped and the bottom concaved particle (Fig. 6). The size was approximately 80 x 140 nm and its cross striations curved (Fig. 7).

In ultra thin section, the EM revealed the budding-s of virus from the nuclear membrane (Fig. 8). The particles were always seen in around the cytoplasmic vesicles. Beside bullet shaped, round shaped particles were also seen.

7. The Growth of Isolated Virus

The existence of cell phase virus was first detectable at 3 post inoculation hours (PI) and the multiplication of virus at 10 hours PI. The titer reached $10^{4.5}TCID_{50}$/ml at 12-20 hours PI and reached to the peak of $10^{5.5}TCID_{50}$/ml at 22 hours PI. The titer rapidly dropped after 32 hours PI.

The liquid phase virus appeared later than cell phase virus and first detected at 14 hours PI. It reached peak titer of $10^{5.5}$ $TCID_{50}$/ml at 20 hours PI and maintained it through up to 48 hours PI. The titer showed a marked decline after 72 hours

Table 6. The Influence of Freeze-Thaw Process.

No. of Freeze-Thaw	Titer (log_{10} $TCID_{50}$/ml)
0	5.5
1	5.5
2	5.5
3	5.25
5	5.25
10	5.25

Table 7. The Influence of Heat under 1 M $MgCl_2$.

Virus	Titer log_{10} $TCID_{50}$/ml			
	Non treated	50°C 1 hours		
		1M $MgCl_2$	H_2O	MEM
Isolate (TLRI)	6.0	⩽0.5	⩽0.5	⩽0.5
Isolate (Liu Yin)	6.5	⩽0.5	⩽0.5	⩽0.5
Bovine Enterovirus	7.0	7.0	6.5	6.5
IBR Virus	5.0	⩽0.5	1.0	1.25

Table 8. The Cross Neuralization Test Between Isolated Virus and Standard Bovine Ephemeral Fever Virus.

Antiserum		Isolated Virus		Bovine Ephemeral Fever Virus YHL Strain
		TLRI Strain	Liu Yin Strain	
Isolated Virus	TLRI	128*	64	64
	Liu Yin	128	128	128
Bovine Ephemeral Fever Virus, YHL Strain		128	64	128

* : Neutralization Titer.

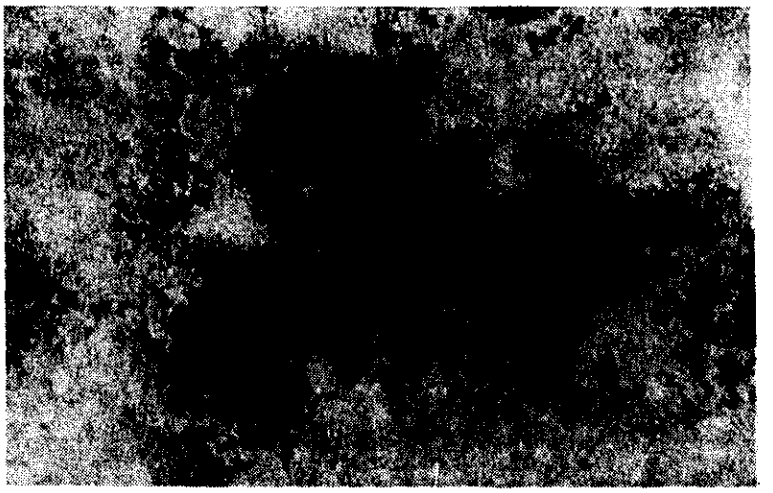

Fig. 6.
Electron microscopic photograph of the particle of isolated virus, BEFV, Liu Yin strain (Negative stain, X150,000).

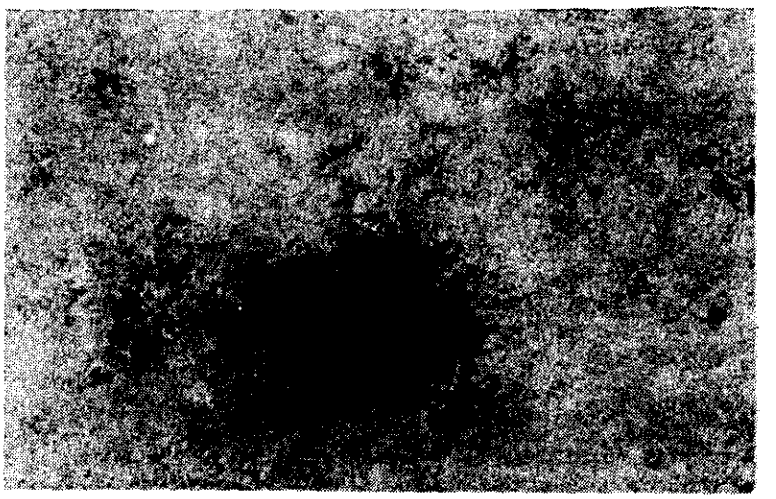

Fig. 7.
Curved cross striation of the isolated virus, Liu Yin strain (negative stain, X150,000).

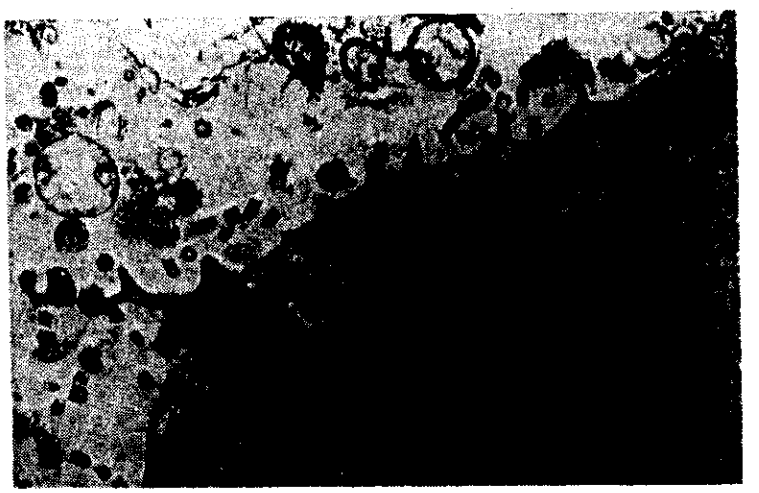

Fig. 8.
Buddings of the bullet shaped isolated virus, Liu Yin strain from the cell membrane (double negative stain, X30,000).

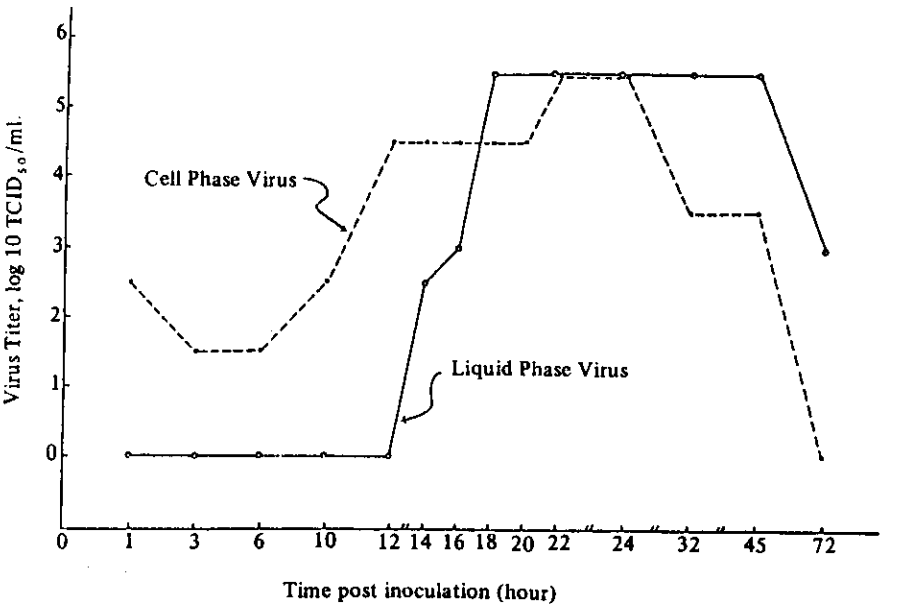

Fig. 9. The Growth of Isolated Virus in BHK 21 Cells.

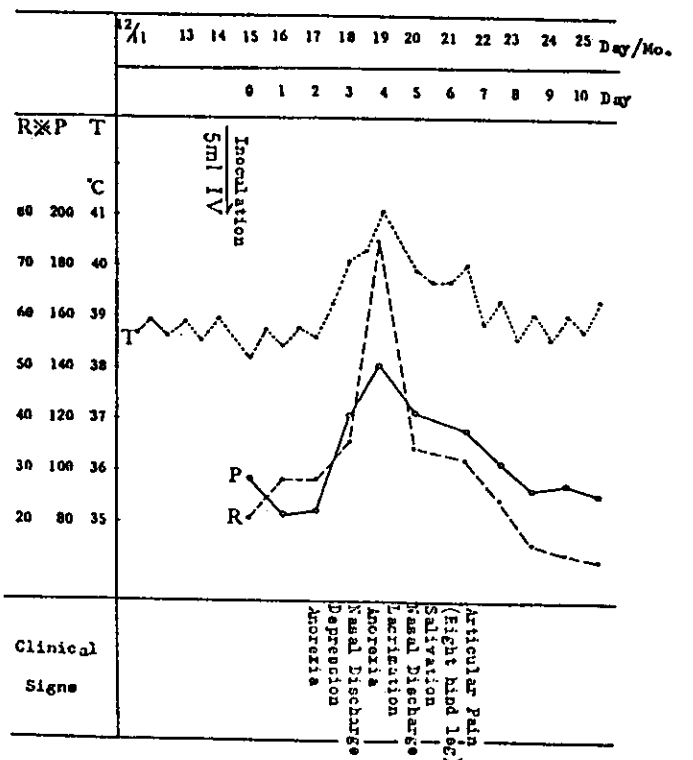

Fig. 10. The Artificial Infection Test of Isolated Virus in Cattle.
* : R: Respiration
P : Pulse
T : Temperature

PI (Fig. 9).

8. **Pathogenecity of the Isolated Virus**

An amount of 5 ml ($10^{6.0}TCID_{50}$/ml) TLRI strain cell culture fluid was inoculated intravenously to a 3 month old calf. As shown in Fig. 10, after a incubation period of 3 days, the body temperature went up to 40°C and, depression, anorexia and slight excess of salivations were observed. On the next day, the temperature further increased to 41°C and, significant increase in respiration and pulse rate, marked nasal discharge and excess salivation, lameness caused by articular pain were also observed. These clinical signs disappeared with the temperature recovered to normal 3 days later.

DISCUSSION

1. Using the method described by Van der Westhuizen et al,[19] the authors had successfully isolated 4 strains of BEFV in Taiwan in 1984. The quick adaptation of the isolated virus to new host manifested in the rapid increase of mice mortality along with the increase in passages, 60% at the first, 83% at the second and 100% at the 3rd passage.

2. The isolated virus developed CPE in BHK 21 or HmLu-1 cells at the 3rd day of inoculation.

3. Most chemicals, such as ethylether, chloroform, sodium deoxycholate and trypsin, easily inactivated the isolated virus. Low pH (pH 3.0) also inactivated the isolated virus, but at pH 8.0, the viruses showed resistance.

4. Freeze-thaw process did not affect the titer of the isolated viruses. But the presence of 1 M $MgCl_2$ did not help prevent the inactivation of the viruses by heat, i.e. at 50°C for 1 hour. The virus reduced the titer of 1.5 and 3.0 $TCID_{50}$/ml when it was treated at 56°C for 5 and 10 min. respectively, and it lost the titer completely when it was heated longer than 30 minutes. These findings were similar to those found by Heuschele,[4] Inaba,[5,6] Lecatsas[12] and Matumoto.[13]

5. The following findings identified the isolated virus with the standard BEF virus:

(1) In the serum neutralizing antibody test, the TLRI and Liu Yin strains crossed well with the standard BEF virus, YHL strain.

(2) Specific fluorescent antigen first appeared on cell membrane at 12 hours post inoculation (PI) of the isolate to BHK 21 cells and at 22 hours PI in cell plasma.

(3) Morphologically, the isolated virus under the electron microscope, appeared to be primarily a virus belonged to Rhabdoviridae, i.e. bullet shaped, concave bottomed and cross striation curved particle with a size of approximately 80 x 140 nm. In the ultra thin sections, the particles gathered around cytoplasmic vesicles and the particles budded from the cell membrane. These findings resembled to those found by Lecatsas[12] and Ito et al (1969).

(4) The success of the artificial infection of the virus to the cattle and reproduced the clinical symptoms of BEF.

6. The high virus titer ($10^{5.5}TCID_{50}$/ml) attained in the BHK 21 cell culture provided the foundation for the development of a vaccine.

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1984年在台灣分離牛流行熱病毒

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1984 年在台灣乳牛發生牛流行熱時筆者從發熱病牛血液中，以哺乳小白鼠腦內接種方法分離到牛流行熱病毒 4 株。

分離病毒容易受到各種化學藥品，如乙醚氯仿，Sodium Deoxycholate，胰蛋白酶等作用而不活化對 50 ℃ 30 分鐘熱處理亦活化，並經

(1)分離病毒與標準牛流行熱病毒 YHL 株中和抗體交叉試驗成立。

(2)在細胞膜及細胞質中證明特異螢光抗原。

(3)以電子顯微鏡觀察到Rhabdo 病毒科之病毒。

(4)分離病毒接種於小牛後產生牛流行熱症狀，即精神消沈，發高熱，食慾減退，口涎鼻漏，呼吸困難，跛腳（關節痛）等，分離病毒確認爲牛流行熱病毒。

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