CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Viruses

Vaccine Strain : ERA strain of Rabies Virus (Dr. M.K. Abelseth) (2).

Challenge Virus : Street Rabies Virus from the brain of rabid dog supplied by the Virus Research Institute.

Virus used in Neutralization Test : Fixed rabies virus, CVS strain received from the World Health Organization.

Tissue Culture : Pig kidney tissue culture (in Roux bottle)

Apparatus for the preparation of pig kidney cells

- Pointed scissors
- Tooth forceps
- Abdominal tractor or Arterial forceps
- Petri dishes
- Beaker
- Measuring cylinder 50, 100, 250 ml
- Corucal bottle
- Graduated pipet 10, 2, 1 ml

- Roux bottles
- Inverted Microscope

Trypsinizing solution consists of 0.25% of trypsin in PBS(+)

Phosphate buffer saline [PBS(+)] pH 7.4

Stock solution A

Sodium Chloride (NaCl)	8.0	g
Potassium Chloride (KCl)	0.2	"
Disodium Hydrogen Phosphate (Na_2HPO_4) (Anhydrous)	0.288	"
Potassium Dihydrogen Phosphate (KH_2PO_4)	0.16	"
Double distilled water to make	800	ml

Stock solution B

Magnesium Chloride ($MgCl_2 . 6 H_2O$)	0.1	g
Double distilled water to make	100	ml

Stock solution C

Calcium Chloride ($CaCl_2 . 2 H_2O$)	0.14	g
Double distilled water to make	100	ml

Autoclaved at 15 lb for 20 minutes each stock separately.

The cooled stocks were combined after autoclaving.

Phosphate buffer saline [PBS(-)] = PBS(+) without calcium chloride and magnesium chloride.

Medium for growing pig kidney cells

(1) Lactalbumin hydrolysate in Earle's balanced salt solution (LE)

Components

Lactalbumin hydrolysate	5 g
Earle's balanced salt solution	100 ml
Double distilled water to make	1000 ml

Melt lactalbumin hydrolysate with 50 ml of double distilled water in the boiling water bath until dissolve, add Earle's balanced salt solution 100 ml, and double distilled water to make 1000 ml.

The solution is sterilized by Seitz filtration through filter sheets stored in the refrigeration.

Freshly prepared by adding about 7% solution $NaHCO_3$ to make pH 7.4

Add Kanamycin 10 mcg/ml and Fungizone 1 mg/ml

Add Calf serum or Fetal bovine serum

Earle's balanced salt solution

Solution A

$CaCl_2 \cdot 2H_2O$	0.26 g
Double distilled water to make	20 ml

Solution B

Glucose	1.00 g
Phenol red 1% Solution	1.5 ml
NaCl	7.18 g
KCl	0.4 "
$MgSo_4 \cdot 7H_2O$	0.20 "
NaH_2PO_4	0.14 "

Double distilled water to make	80	ml

Keep in refrigerator.

(2) Hank's balanced salt solution with lactalbumin hydrolysate (LH)

NaCl	8.00	g
KCl	0.40	"
$CaCl_2$	0.14	"
$MgSO_4 \cdot 7H_2O$	0.20	"
KH_2PO_4 (Potassium dihydrogen phosphate)	0.06	"
Na_2HPO_4 (Disodium hydrogen phosphate)	0.05	"
Glucose	1.00	"
Phenol red	0.02	"
Lactalbumin hydrolysate	5.00	"
Streptomycin sulfate	0.20	mg(156 IU)per ml
Double distilled water	1000.00	ml

Melt lactalbumin hydrolysate in approximately double distilled water 50 ml in the boiling water bath until dissolve. Dissolve the salts in double distilled water in the order listed. Pour the salt solution into the solution of lactalbumin dydrolysate and mixed well. The solution is then sterilized by Seitz filtration. After filtration, the solution is ready for used.

Stabilizing Medium pH 7.6

Solution 1

Dipotassium hydrogen phosphate (K_2HPO_4)	18.75 g
Cysteine hydrochloride	7.5 "
Lactose	375.0 "
Double distilled water to make	2500.0 ml

(1) Dissolve the dipotassium phosphate, cysteine hydrochloride and lactose in water. Add each constituent separately in the order listed and dissolve completely before adding the next.

(2) Sterilize the solution by positive pressure filtration.

(3) Store in completely filled, tightly stoppered bottles at approximately 4°C.

Solution 2

Gelatin	15.0 g
Double distilled water	2500.0 ml

(1) Dissolve the gelatin in water warmed to 43-45°C.

(2) Sterilize the warm solution by positive pressure filtration, using a filter and pressure vessel pre-warmed to 36-37°C. Employ a filtration pressure of about 20 N/cm^2 (2 kgf/cm^2).

(3) Store at approximately 4°C.

Preparation of working stabilizing medium

(1) Melt the gelatin (Solution 2)in a water bath maintained at 36-37°C. Cool to approximately room temperature.

(2) Mix equal volumes of Solutions 1 and 2.

(3) Measure the pH of the mixture and adjust it to 7.6 by the addition of 0.1 N sodium hydroxide solution; approximately 30 ml are required.

(4) Store in completely filled, tightly stoppered bottles at approximately 4°C.

Test Animals

Mice : Mice, 3-4 weeks old and weight about 14-15 g.

Guinea Pigs : weight about 350-450 g.

2.2 METHODS

2.2.1 Preparation of Primary Pig Kidney Cell Culture

a. A healthy pig 1-2 months of age was sacrificed and the kidneys sterilly excised and sent to a sterile room as hood.

b. A pair of kidneys weighing about 20-30 g, were transfered to a petri dish containing PBS(-) where the capsule and pelvis were removed.

c. Kidneys transfered to another petri dish containing PBS(+) where they were washed two times.

d. Kidneys were then minced using two pairs of pointed scissors.

e. Minced kidneys washed two times in PBS(+) and placed in corucal bottle with 200 ml 0.25% trypsin solution at 30°C for 30 minutes while spinning (a spinner was magnetic stirror).

f. Fluid decanted and discarded, thus would remove most of the blood and fragmented cells.

g. Carried out further trypsinizations, with 200 ml of 0.25% trypsin solution, stirred overnight at 4°C.

h. Pooled cell suspension strained through sterile cheese-cloth into centrifuge bottles.

i. Centrifuged the filtrate for approximately speed 1000 rpm/min 30 minutes at 4°C.

j. Supernate discarded and cells resuspended in 10 ml growth medium (LE medium + 5% fetal bovine serum or calf serum + Kanamycin 10 mcg/ml + Fungizone 1 mg/ml) in centrifuge tubes.

k. Centrifuged 15 minutes, speed 1000 rpm/min at room temperature.

l. Supernate and top layer of red blood cell discarded. Pellet resuspended with 10 ml growth medium and centrifuged 10 minutes speed 1000 rpm/min at room temperature. Repeated this step 2 times.

m. Suspended the packed cell with 10 ml of LE medium. Cells counting in the dilution of 1:10 or 1:100.

n. Cell culture, inoculum size 300,000 per ml of growth medium, which consists of Earle's balanced salt solution or Hank's balanced salt solution, with 0.5% lactalbumin hydrolysate and 3.5% of fetal bovine serum or calf serum. The final pH of the growth medium of cells should be 7.2 - 7.4. The mixture of cells

and medium should be prepared in a single container to ensure the uniformity of cell suspension.

o. Dispensed the suspended cells into sterile Roux bottles at the rate of 80 ml, respectively, per bottle.

p. Kept the bottles at 37°C incubator for 4-6 days studied the confluent growth of cells under inverted microscope. Any bottles in which the cells have not became confluent or did not appear healthy should be discarded. If a large number of bottles with poor cells were found, discarded the entire batch. It was not advisable to extend the incubation period in order to improve the cells since complete cell sheets may over grow and became granular, resulting in inadequate virus production, or they may fall off the glass before the production was complete.

2.2.2 Infection and Incubation of Pig Kidney Cells

a. Infected the cells with 1.0 ml of ERA strain of Rabies virus, kept the bottles at 34°C incubator one hour with two times rolling. Eighty millilitres of the medium, LE medium or LH medium, was replaced that contains 2% fetal bovine serum or calf serum.

b. Incubated the cells at 34°C and 36°C.

c. Fluid was harvested and fresh medium was added to the cells. Repeated after a further incubation of every 2,7 or 9 days according to the propagation of virus. This process may be repeated again depending on the condition of the cell sheet.

2.2.3 Vaccine Preparation

a. Preparation of seed virus : Pooled virus suspension from fluid medium harvested every 2 days at 34°C and 36°C, and at 34°C of LE medium and LH medium three successive fluid harvested 7, 7, 7 days; 9, 9, 9 days; 5, 7, 7 days at 34°C in LE medium.

b. Virus titration

The pooled fluid harvested was tested by injecting, intracerebrally, 14-15 g mice with each of the dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, of the fluid harvested vaccine and observing them for 21 days.

Procedure

a) Virus dilution, the 10 fold serial dilution of fluid was made from 10^{-1} to 10^{-6}.

b) Injected 0.03 ml intracerebrally, 14-15 g mice, using 5 mice per one dilution, starting from the highest to the lowest dilution.

c) Observed the mice for 21 days.

d) At least 80% of the mice injected for each dilution must survive longer than 4 days, or the test is considered to be "No Test" and must be repeated.

The LD_{50} must be calculated by the Reed and Muench method. Mice dying before the 4th day will be considered nonspecific deaths.

Calculation of the Reed and Muench method (26).

Example:

Vaccine Dilution	Mice		Cumulative totals		Percentage Mortality
	Death	Survived	Death	Survived	
10^{-2}	4	1	9	1	90
10^{-3}	4	1	5	2	71.43
10^{-4}	1	4	1	6	14.29
10^{-5}	0	5	0	11	

In this example the dilution factor is 10 and the starting point dilution (showing a mortality next below 50%) is 10^{-4}.

Calculate the "difference of logarithms" from the formula :

$$\frac{50\% - (\text{mortality next below } 50\%)}{(\text{mortality next above } 50\%) - (\text{mortality next below } 50\%)} \times \text{logarithm of dilution factor}$$

Hence

$$\text{"difference of logarithms"} = \frac{50 - 14.29}{71.43 - 14.29} \times 1$$

$$= \frac{35.71}{57.14}$$

$$= 0.62$$

Since, in the example, the mortality decreases with increasing dilution, the 50% endpoint dilution is lower than the starting point dilution and is calculated by subtracting the "differences of logarithms"

as follows :

log (reciprocal of 50% end point dilution) = log (reciprocal of starting point dilution) -"difference of logarithms"

$= \log 10^{-4} - 0.62$

$= 4 - 0.62$

$= 3.38$

Hence

log (50% endpoint dilution) $= -3.38$

and 50% endpoint dilution (LD_{50} titre) $= 10^{-3.38}$

c. Sterility test (29) Bacterial contamination

a) A 5 ml liquid vaccine is withdrawn and 6 drops placed on a tryptose agar plate and 6 drops placed on a blood agar plate. The remainder is placed in a tube of Nutrient Broth. These are incubated at 37°C for 7 days.

Also the sample shall be tested for the presence of Salmonella as follows.

b) One ml of bulk is inoculated into 20 ml of tetrathionate broth. This is incubated at 37°C for 24 hours. At the end of this time two loopsful are both deposited in the same place and then streaked on SS Agar. At the end of 24 hours plated are read.

c) One ml of bulk vaccine is inoculated into 20 ml of tryptose broth and incubate and incubated at 37°C, for

24 hours. At the end of this time 2 loopsful are placed in the same place on a Mac Conkey plate and 2 loopsful are placed in the same place on an SS Agar plate.

At the end of 24 hours the plates are examined. If negative reincubate for 24 hours and examine.

Transfer after 24 hours

From Medium	To Medium	Quantity	Temperature
Mac Conkey	Triple Sugar Iron Slants	1 Colony	37°C
SS Agar plates	" " " "	1 Colony	37°C

Standard of sterility : The fluid vaccine must be free of contamination with Salmonella.

2.2.4 Final preparation of vaccine

a. Mixed equal volumes of the fluid harvested and the stabilizing medium.

b. Dispensed into single dose (4.0 ml).

c. Cooled to approximately -50°C (minus 50°C)

d. Transferred to the lyophilizer. The vials were closed with sterile rubber stoppers under vacuum and then capped with a single aluminium seal.

e. Test for lyophilized product. (see below)

f. The finished vials were checked for imperfections and packed

in boxes labelled for identification of product, serial number, quantity, and filling date.

2.2.5 Storage of finished product

Thirty vials were submitted for quality control testing, the remainder of the lot is stored at 4°C pending completion of the tests required for release. The label on each vial should indicate the name of the vaccine, the animals for which it is recommended, the minimum age of animal to be vaccinated, dosage, route of administration, expiry date, and lot number.

A leaflet should be inserted into each package with any further recommendations regarding proper handling, reconstitution, and revaccination.

A diluent consists of pyrogen free distilled water dispensed into vials, which are closed with rubber stoppers and sealed with an aluminium seal. The filled, stoppered, and capped vials are sterilized by autoclaving. The diluent must be supplied in each package.

2.2.6 Test for lyophilized Product (29)

a. Sterility test

a) Bacterial Contamination

Collect at random 10 vials of the final filled container. Reconstitute the contents of each vial with diluent (pyrogen-free distilled water). The reconstituted vaccine from each of the 10 vials, is added to the following media in the quantities noted, unless the

contents of the vial is 2 ml or less, in which case the contents are divided equally between the Aerobic and Anaerobic media. These are then incubated for one week at the temperature indicated. Observe daily for seven days.

Medium	Reconstituted	Temperature
Aerobic Medium		
Nutrient broth tube (20 ml)	1 ml	37°C
Tryptose Agar Plate	6 drops	37°C
Blood Agar plate	6 drops	37°C
Anaerobic Medium		
Thioglycollate Broth tube (20 ml)	1 ml	37°C
Thioglycollate Broth tube (20 ml)	1 ml	Room temperature
Transfer after seven days		

From Medium	To Medium	Quantity	Temp.
Nutrient Broth	Blood Agar plate	6 drops	37°C
Thioglycollate 37°C	Thioglycollate Broth tube (20 ml)	1 ml	37°C
Thioglycollate R.T*	Thioglycollate Broth tube (20 ml)	1 ml	R.T.

Distributed over 6 different areas.

b) Mold contamination : The reconstituted vaccine from each of the ten vials are tested by inoculating Saboraud's agar plates with 6 drops. The plates are incubated at 37°C for two days and then at room temperature for five days.

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* R.T. = Room temperature

Standard of Sterility : The final product must be free of contamination (29).

b. Physical appearance of Vaccine : The ten vials reconstituted as under "Sterility" above are used for the examination for physical appearance.

a) Examination of dried product : The dried product should be a wellformed "button" in the bottom of the vial. Breaks away easily from the vial.

b) Reconstitution, the dried product should reconstitute easily and completely on addition of the diluent followed by gentle rotation.

c) After Reconstitution, the product should reconstitute to a uniform suspension (on shaking).

c. Virus titration

The virus titration of the vaccine is tested by injecting, intracerebrally, 14-15 g mice with dilution of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, of the vaccine and observing them for 21 days.

a) Each of 2 vials from each lot is reconstituted with sterile distilled water containing 2% normal calf or fetal bovine serum (using 3 ml per dog dose) withdraw 1.5 ml from each vial and add to 7 ml of distilled water containing 2% normal calf or fetal bovine serum, 500 units of penicillin per ml and 1 mg of streptomycin per ml in a test tube. This 10 ml represents the 10^{-1} dilution.

b) Prepared dilutions of the reconstituted vaccine corresponding to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, using the above diluent.

c) Injected, intracerebrally, 14-15 g mice, each with 0.03 ml of each of the dilutions of the vaccine, progressing from the highest to the lowest dilution.

d) Observe the mice for 21 days.

e) At least 80% of the mice injected for each dilution must survive longer than 4 days or the test is considered "No Test" and must be repeated.

Standard of Potency : The LD_{50} must be calculated using the Reed and Muench method. A satisfactory vaccine shall have a titer of not less than $10^{-2.5}$ LD_{50} per 0.03 ml (29).

Mice died before the 4th day will be considered nonspecific deaths.

d. Antigenicity test

a) Vaccination of Guinea Pigs

Each of 2 vials of vaccine representing each serial are reconstituted with sterile distilled water containing 2% normal calf or fetal bovine serum (using 3 ml of diluent per dog dose vials and add to 17 ml of distilled water containing 2% normal calf or fetal bovine serum. Each of at least 10 healthy guinea pigs are injected intramuscularly on the inside of the leg as near to the nerve as possible with 0.25 ml of the diluted vaccine. At the same time at least 5 healthy guinea pigs are not a side to act as unvaccinated

controls. At the start of the test the guinea pigs should weigh at least 350 g.

b) Challenge of Guinea Pigs

Three weeks after vaccination, 10 guinea pigs are challenged by intramuscular injection of street virus. The challenged dose is 0.25 ml of 8000 LD_{50} of the virus suspension injected intramuscularly into the leg opposite to that used for vaccination. One LD_{50} of Challenge virus is 5.4/0.03 ml.

c) Interpretation of Results

A test is considered satisfactory when at least 70%, but not less than seven of the challenged vaccinated animals survive the challenge without signs of paralysis for a period of 21 days, providing that at least 80% of the controls die or show symptoms of rabies within the 21days observation period. In the event of non-specific deaths in challenged vaccinates, on or before the second day post-challenge, the deaths may be disregarded, all guinea pigs must be accounted for on the test protocol.

e. Safety test in Animals (free from pathogenic organisms)

Safety of the vaccine is measured in mice.

Mice: Each of at least eight mice are injected with 0.5 ml of the restored vaccine intraperitoneally.

Standard of Safety:

Mice: At least 7 out of 8 of the mice must remain alive and healthy for an observation period of seven days.