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Comparative Study of Japanese Encephalitis, Live Attenuated Vaccine Potency by $CCID_{50}$ and PFU Method

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Abstract: ***Japanese Encephalitis, live attenuated vaccine is a cell culture derived (Primary Hamster Kidney), live attenuated vaccine based on the SA 14-14-2 strain manufactured by China and is imported to India .The potency of a live vaccine is typically expressed in terms of the number of infectious unit of viruses contained in human doses using a specified tissue culture substrate. Both BHK-21 cell line as well as vero cell line shows good susceptibility for JE Virus. WHO recommends the use of BHK-21 cell line or any other cell line acceptable to the National Control Authority. In the present study the potency of the JE vaccine was performed by $CCID_{50}$ & PFU method using Vero cell line.***

Keywords: $CCID_{50}$, Vero cell line, BHK-21 cell line, Cytopathic effect, PFU, Vaccines

1. Introduction

Japanese Encephalitis is an acute Central Nervous system (CNS) inflammatory disease caused by infection with Japanese encephalitis virus (JEV); a small, enveloped, plus strand RNA virus belonging to the *Flaviviridae* family [1].Acute Inflammation of the brain is hallmark of encephalitis. Many viruses causing encephalitis are documented in medical literature, among which Japanese encephalitis is important [2]-[3]-[4]. Japanese Encephalitis (JE), a mosquito borne arboviral infection, is the leading cause of viral encephalitis in Asia [5].The term arbovirus or arthropod borne viruses is a descriptive ecologic term without taxonomic significance, meaning that the virus is transmitted through arthropods [6]. JEV is transmitted primarily by Culex mosquitoes, and circulates in an enzootic cycle in pigs and wading birds which serves as amplifying hosts. *Culex tritaeniorhynchus*, the most important vector species ,breeds in water pools and flooded rice fields and bites mainly during the night .Due to animal reservoir JE cannot be eliminated but the diseases can be prevented by universal vaccination in endemic areas. Human are considered dead end hosts, with viremia too low to allow further transmission [7].

There have been many attempts to prepare vaccine against JE since the time when causative agent was first isolated in human being in Japan. After the diagnosis of JE in the vaccinated individuals vaccinated with Crude mouse brain and chick embryo vaccine, new cases of allergy and encephalitis were also reported after such vaccination [8]. This led to intense research to prepare safer and potent vaccine. There are four different types of JE vaccines available for humans: Mouse brain derived killed vaccine, cell culture derived live –attenuated ,cell culture derived killed Inactivated and genetically engineered live-attenuated chimeric vaccines [9]-[10]-[11].Summary of the currently available JE Vaccine .[12]**Table.1**

Table 1: Summary of currently available JE vaccines

Vaccine Features	Live attenuated	JE-MB	JE-VAC		Live attenuated Chimeric
			With aluminum Adjuvant	Without adjuvant	
Strain	SA 14-14-2	Nakayama-NH Beijing-1	SA14-14-2	Beijing-1	SA14-14-2
Trade Name	Japanese encephalitis vaccineLive(RS. JEV)	JE-VAX	IXIARO/JESPECT	JEBIK-V[a] ENCEVAC[b] TC-JEV[c]	
Manufacturers	Chengdu Institute	BIKEN	Intercell Biomedical	BIKEN	Sanofi-Pastuer
Licensed	1988	1954-Japan 1993-US	2009[d]	JEBIK-V(2009[j] ENCEVAC(2011) TC-JEV(2013)	2010
No. of doses from primary series	1dose	2doses	2doses	3doses	1dose
Schedule age first shot	8 months	15-27 months	2months	36 months	≥ 12 months
Booster	At 2years And 6-7 years	12 months after primary series, aged >5years	Aged≥ 17 years,12months after primary series	12-24 months and thereafter every 3 years	Not yet
Total doses	3	4	4	5	2
Route	SC	SC	IM	SC	SC

IM-intramuscular; JE-MB-inactivated mouse brain-derived JE vaccine; JE-VC-inactivated vero cell culture-derived JE vaccine; SC-subcutaneous; a Japan, BIKEN/TAKEDA .b Japan, Kaketsuken. C Korea, Boryung/Star-Bio.d Licensed

in 2009 for aged-17 years and in 2013 for aged 2 months through 16 years. [12]

Live attenuated cell culture vaccine was developed in china based on the SA14-14-2 strain . The vaccine was first Licensed in china in 1988and is currently being produced in Primary Hamster Kidney cells, Since the Licensure, greater than 300 million doses of SA 14-14-2 have been produced for administration to Chinese children, with an excellent record of safety and efficacy [13].Over the past decades, SA14-14-2 has been progressively licensed in other Asian countries, including South Korea, Nepal, India, Sri Lanka, Cambodia, Laos, Myanmar and Thailand. Today SA14-14-2 is the most widely used vaccine in the JE –endemic area [14].

JE is one of the commonest causes of acute encephalitis syndrome in many states of India. In India JEV shows epidemicity annually during monsoon and post monsoon seasons in Chennai, Karnataka, Andhra Pradesh, Maharashtra, Goa, Kerala, Uttar Pradesh, Bihar, West Bengal ,Assam and Meghalaya. In Assam JE has appeared in sporadic outbreak since 1976[15].

Incidence of JE has been increasing in Assam National Vector Borne Diseases Control Programme (NVBDCP), Assam ,data in 2008,no.of patients diagnosed with JE was 157 with 33 deaths affecting 27 districts in 2013, no. of patients increased up to 502 with 139 deaths affecting 27 districts. In 2014 there was epidemic of JE in Assam during monsoon season from June to August [16].According to directorate of NVBDCP, Delhi,1661 cases of JE were reported in the year 2014 from 15 states and union territories, out of which 293 (17.6%) died, Assam, West Bengal, Uttar Pradesh(UP) and Jharkhand reported maximum number of cases [17].

The Immunization strategy of India recognizes that JE is not a uniformly distributed disease, and therefore targeted immunization will be most cost effective. Human vaccination is the most feasible, reliable and cost effective tool for JE Control. JE vaccination in India started in 2006 following large outbreak of JE in some districts of Eastern UP and Bihar. Large vaccination campaigns were carried out in 11 of the highest risk districts of the country in 2006,27 districts in 2007,22 districts of 2008,and 30 districts in 2009.Chidren between the age of 1 to 15 years were vaccinated with a single dose of Chinese live attenuated SA-14-14-2JE vaccine. In 2011, the same SA-14-14-2 JE vaccine was introduced in the routine immunization under Universal Immunization Program (UIP) in the 181 endemic districts as a single dose of SA-14-14-2 vaccine was added at 9 month of ages along with measles vaccine. So far, 155 out of 181 identified JE campaign and overall 10.8 crore children have been immunized with JE vaccine through campaign.[18].
It is important to establish quality control of the vaccines before they are released. The various Quality control tests are conducted as per IP 2016 [19]. Potency is one of the most important parameters for the quality control of the vaccines. The potency of live vaccine is typically expressed in terms of the number of infectious units of virus contained in a human dose using a specified tissue culture substrate. In case of Live attenuated JE Vaccines, potency will have to be assessed in terms of Virus infectivity titres. The two main methods to check the Titres of JE Live vaccine are Plaque Forming Unit (PFU) assay and Median Cell Culture Infective Dose method [20].

Presently the method used to estimate the potency of JE vaccine at Central Drugs laboratory Kasauli is Median Cell Culture Infective dose ($CCID_{50}$) in BHK-21 cell Line .Both BHK-21[21] as well as Vero cell line [22] shows good susceptibility for JE Virus and are used as testing cell lines for JE vaccines. According to WHO recommendations the use of BHK-2 cell line or any other alternative cell line acceptable to National control Authority can be used [23] . JE vaccine has been titrated by Plaque assays on Vero cell Line [24]. $CCID_{50}$ method is standardized in Central Drugs Laboratory in BHK-21 cell line. For this study an alternative cell line i.e Vero was used and a comparative study was conducted for the feasibility of use of vero cell line as an alternative cell line [20-21-22].

2. Materials and Methods

2.1 Vaccine Samples, Reagents and Cell Lines.

The samples received for Lot Release of Japanese Encephalitis vaccine (Live attenuated); strain SA14-14-2, vaccine diluents (PBS) at Central Drugs Laboratory Kasauli were taken for the study. Cell line used were BHK-21 Cell Line at Passage-37and vero cell line at Passage-124 were taken .Vero (Vervet Renal origin) African Green Monkey Kidney cell line is a continuous cell line derived from the kidney of a normal African Green Monkey, *Ceropithecus aethiops.*It was established by Y.Yamasura and Y.Kawakita at the Chiba university in Japan, in March 1962.Vero cell is one of the most common mammalian cell lines used in research .This anchorage –dependent cell line has been extensively used in virology studies. [25] The BHK-21 cell line was derived from baby Syrian hamster (Mesocricetus auratus) kidney [26].

2.2 Cell preservation, revival and propagation

Vero cell and BHK cells procured from ATCC were taken and were tested for freedom from contamination with bacteria, fungi and its ability to grow as a monolayer. They were stored at -196 ^{0} C (Liquid Nitrogen) in cryopreservation system.

2.3 Revival of the Vero cell Line.

Ampoule of the Cell lines were taken from the cryocan and thawed in the water Bath.[27] The ampoules were opened in the Bio Safety Cabinet under Class100[28].The entire content of the ampoule were mixed thoroughly and put into the tissue culture flask containing 10 ml MEM ,supplemented with10% v/v inactivated fetal calf serum [25].The Media is changed after 4 hrs or overnight for the removal of DMSO. The cells are allowed to grow at 36 ^{0}C $\pm$ 1 ^{0}C until a complete monolayer is formed.

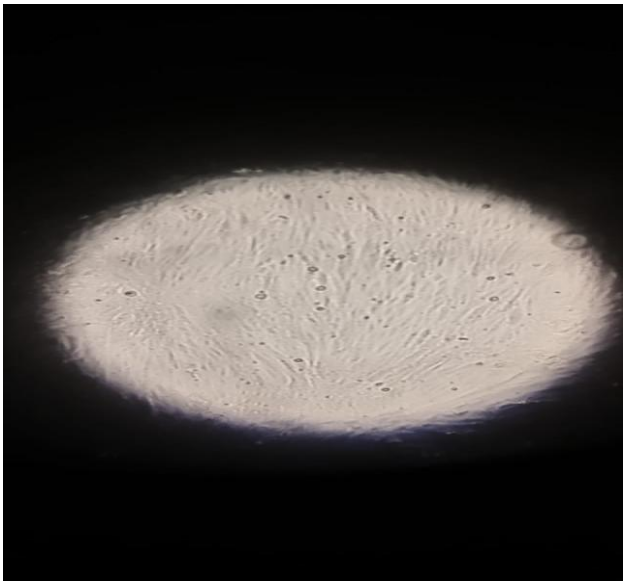

Figure 1: Vero cell Line after formation of complete monolayer

2.3 Method

The two main methods to check the Titers of JE Live vaccine are Plaque Forming Unit (PFU) assay and Median Cell Culture Infective Dose method [20].

2.3.1 Median Cell Culture Infective Dose Assay

Alternative assays such as that to determine the cell-culture infective dose 50% (CCID50) may be used with the approval of the national control authority, provided they have been calibrated against the PFU assay [20-23]. The virus content should be determined after reconstitution of the freeze–dried product. Limits for accuracy and precision of the virus titration should be agreed with the national control authority. Since no international reference materials have been established for live JE vaccine, no recommendations for potency based on such materials can be formulated. The national control authority should provide or approve a reference preparation of live JE vaccine for use in tests to determine virus concentration. The national control authority should specify the minimum amount of vaccine virus that one human dose should contain. The minimum amount of vaccine virus in one human dose is 5.4 log PFU per 0.5 ml at the time of release in one country [23].

Ten samples of different batch No's of J.E vaccine live attenuated vaccine were taken for the study. Ten fold dilution of the vaccine were made in Minimum Essential Medium with 2% FBS .Range of Dilution 10^{-1} to 10^{-7} were taken . 100 µl dilution of the vaccine was inoculated in eight wells of the micro-titre plate. Confluent layer of the cell lines were trypsinized to prepare a cell suspension having cell concentration adjusted to 3-4 x 10^{5} cells per ml, 11 and 12 row was inoculated with 100 µl MEM with 2% FBS. 50 µl of the cell suspension prepared was added into the microtitre plate. development. Titer was calculated by Spearman- Karber Method. [29].

2.3.2 Plaque Forming Unit (PFU) Assay

The infectivity of each final lot should be established in an assay approved by the national control authority, such as the PFU method in the baby hamster kidney (BHK)-21 cell line. Alternative cell lines may also be acceptable to the national control authority. Manufacturers should determine the appropriate titre to reliably produce vaccine. In the PFU method in BHK-21 cells used in some laboratories, samples from the virus suspension are diluted 10-fold serially, and the titre is estimated after plaques have developed. The titration should be made in parallel with a reference vaccine that is approved by the national control authority. [23]

Ten samples of same batch No's of J.E vaccine live attenuated vaccine used in CCID50 method were tested by PFU method. 75 cm^2 tissue culture bottle containing vero cell monolayer was trypsinized and cell culture was adjusted to contain 3-4 x 10^5 cells per ml . 2 ml of the cell suspension were added to each well of the Tissue culture plates. The plates were incubated at 36 ^{0}C ± 1^{0}C under 90% humidity and 5% CO_2 overnight .Ten fold dilution of the vaccine were made in Minimum Essential Medium with 2% FBS .Range of Dilution 10^{-1} to 10^{-7} as used in $CCID_{50}$ method were taken. [30] vaccine dilution 10^{-3} and 10^{-4} dilution were used. Infection of the virus and adsorption step was followed, the medium were aspirated from the 6 wells Tissue culture plates with confluent Vero cell mono layer.0.2ml of 10^{-3} and 10^{-4} dilution. Were added to first two wells of each row of the other plate .2wells are kept as cell control to which 0.2ml of growth medium was added **(Fig 1, Fig 2).**Incubate the plate for 90 minutes at 36 ^{0}C ± 1^{0}C under 5%CO_2 and 90%humidity for virus adsorption. The plates were gently shaken after every 30 minutes for uniform distribution of virus. After adsorption of the virus the cell monolayer was covered with the overlay medium ,3ml per well. Incubate the plate for 7 days at 36 ^{0}C ± 1^{0}C under 5%CO_2 and 90%humidity for development of plaques. Further plaque fixation and staining, on 7 day ,the plates were taken out of the incubator and the overlay media was aspirated., the cell monolayer was washed with PBS (-) ,2 ml per well. Fixed the cell monolayer with 2ml per well with 10% formal saline solution for 30 minutes at room temperature.After fixation aspirated the fixative and washed the monolayer with PBS(-).Flooded the well with 0.5% Crystal violent solution for 20-30 minutes for staining the monolayer. Washed the excess stain with PBS(-) .Let the monolayer dry and counted the plaques. The virus titer was expressed in terms of Plaque forming unit/0.5ml (PFU/0.5ml) using the formula, PFU=P x D x 2.5 .Taking log PFU = log P + log D +Log 2.5 .Where, P = the average number of plaques in a well, D = the reciprocal of the virus dilution used.

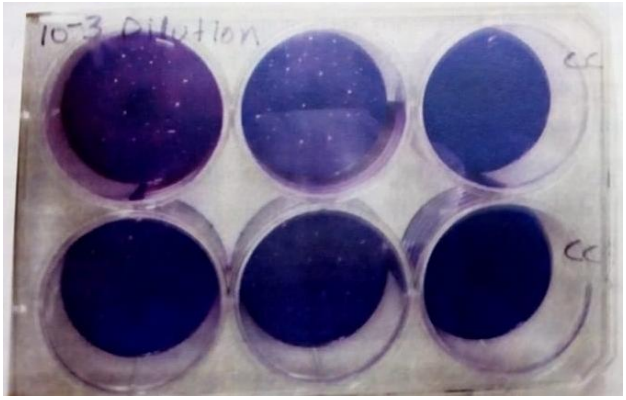

Figure 2: 6 well plate tissue culture plate showing Plaques on 10^{-3} dilution

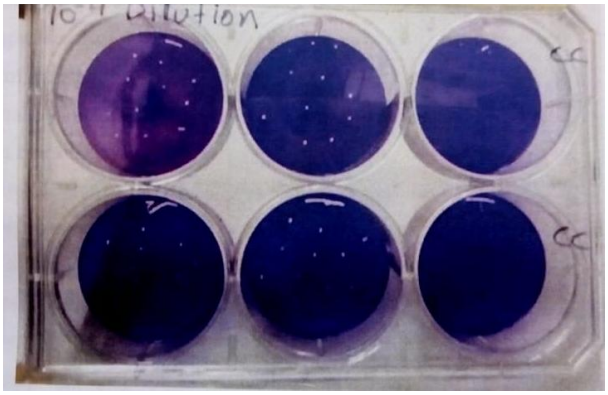

Figure 3: 6 well Tissue culture Plate showing Plaques on 10^{-4} dilution

3. Results

The two main methods to check the Titers of JE Live vaccine are Plaque Forming Unit (PFU) assay and Median Cell Culture Infective Dose method [20].

Table 2: Titer value obtained by $CCID_{50}$ method with Vero cell line

Test No.	No. of wells positive per vaccine dilution					Total no. of wells	CCID50 titre/0.5ml
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}		
1	8	8	4	3	0	23	6.073
2	8	8	3	2	1	22	5.948
3	8	7	3	2	0	20	5.698
4	8	8	4	1	0	21	5.824
5	8	8	4	2	0	22	5.948
6	8	7	5	3	1	24	6.198
7	8	7	4	2	0	21	5.824
8	8	7	5	4	0	24	6.198
9	8	8	4	2	0	22	5.948
10	8	7	5	3	0	23	6.073
Mean							5.973
Standard Deviation							0.164

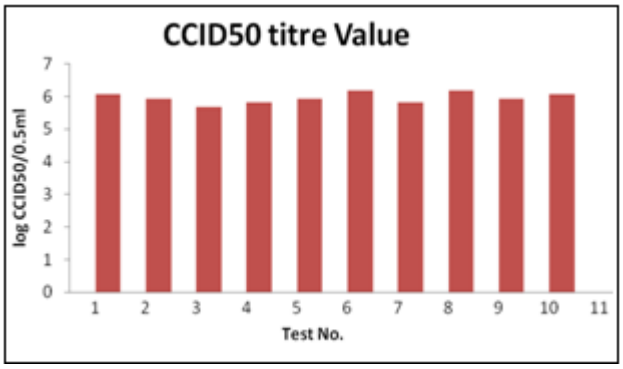

Figure 4: $CCID_{50}$ titer values obtained in 10 tests

CCID $_{50}$ method test was carried out simultaneously with the Plaque assay using the same vaccine. The lowest and highest titres obtained were 5.69 log $CCID_{50}$ per 0.5ml and 6.198 log $CCID_{50}$ per 0.5ml, respectively, with mean of 5.973 and standard deviation 0.164.

Table 3: Details of Plaque formation and corresponding titres obtained at two dilutions (10^{-3} and 10^{-4})

Test No.	No. of plaques on 10^{-3} dilution				Mean Titer (Log PFU/0.5ml)	Test No.	No. of plaques on 10^{-4} dilution				Mean Titer (Log PFU/0.5ml)
1.	136	134	138	136	5.531	1.	14	15	13	14	5.543
2.	131	130	135	128	5.515	2.	11	13	16	12	5.057
3.	105	111	114	106	5.435	3.	10	11	12	10	5.428
4.	110	111	115	104	5.439	4.	10	12	12	11	5.548
5.	125	123	130	127	5.494	5.	12	11	13	14	5.493
6.	148	145	142	145	5.559	6.	13	16	15	14	5.558
7.	115	121	127	125	5.484	7.	12	14	11	12	5.484
8.	143	142	144	143	5.553	8.	13	15	14	15	5.551
9	116	123	126	119	5.480	9	11	13	10	14	5.473
10.	139	141	139	141	5.541	10.	13	15	12	16	5.541
Mean					5.503	Mean					5.513
SD					0.044	SD					0.042
CV%						CV %					0.13%

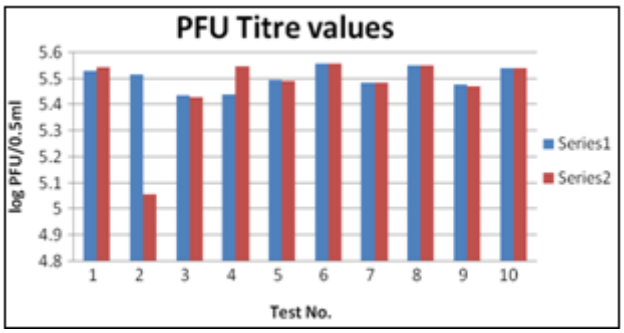

Figure 5: PFU titers obtained in two different dilutions

10 different vials of the same batch Numbers of the JE live vaccine were tested by PFU method for its potency. The results shown in the above table were obtained .It is clear from the above table that countable plaques were obtained on dilutions, i.e. 10^{-3} and 10^{-4} .The titers obtained from both the dilutions are similar. The lowest and the highest titers on 10^{-3} dilution were 5.435 log PFU/0.5ml and 5.559 log PFU/0.5ml,respectively, with mean of 5.503 and Standard deviation of 0.044. The lowest and highest titers on 10^{-4} dilutions were 5.428 log PFU per 0.5ml and 5.551 log PFU per 0.5ml,respectively, with mean of 5.513 and standard deviation of 0.042. The titers obtained were all above the lower limit of 5.4 log PFU per 0.5 ml. The titers obtained from both the dilution showed a CV% of 0.13% only. Therefore, any dilution can be chosen for the study. 10^{-3} dilution was chosen for the present study.

Table 4: Correlation between titers obtained by the two methods

S.No.	Log CCID50 titre/0.5ml	Log PFU/0.5ml
1.	6.073	5.531
2.	5.948	5.515
3.	5.698	5.435P
4.	5.824	5.439
5.	5.948	5.494
6.	6.198	5.559
7.	5.824	5.484
8.	6.198	5.553
9.	5.948	5.480
10.	6.073	5.541
	Correlation	0.945

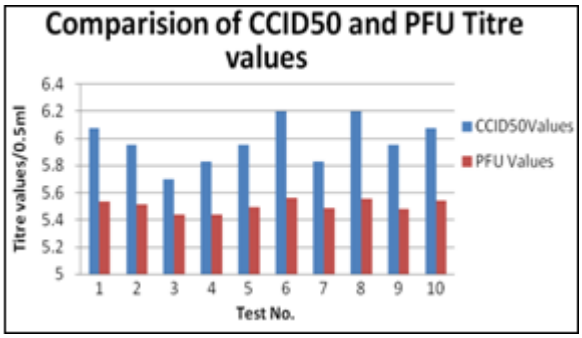

Figure 6: Chart of Comparisons of test results of two methods

It is clear from the above table that the titers obtained by the two methods for the same vaccine are different. The titres obtained by $CCID_{50}$ method are higher than the Plaque assay, but the two methods show a very high correlation of 0.945.

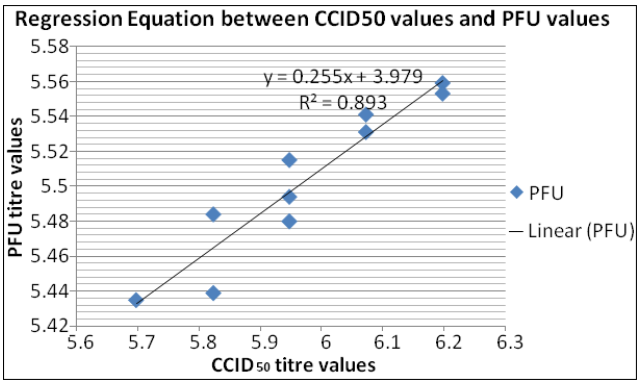

Figure 7: Chart of regression between CCID50values and PFU values.

Regression equation was plotted between the values of $CCID_{50}$ and PFU methods. The obtained equation was y=0.2551x+3.9796 and the value of R^2 = 0.893.

Table 5: PFU titer value calculated by regression equation: y=0.2551x + 3.979

S.No.	Log PFU/0.5ml
1.	5.529
2.	5.497
3.	5.433
4.	5.465
5.	5.497
6.	5.561
7.	5.465
8.	5.561
9.	5.497
10.	5.529
Mean	**5.503**
SD	**0.042**

From the above table it is clear that the $CCID_{50}$ titers converted into PFU titers through regression equation all fall above the lower limits of 5.4 log PFU per 0.5 ml; the minimum and maximum values being 5.433 and 5.561, respectively, mean of 5.503 ± 0.04.2 SD

Table 6: Comparison of the PFU values obtained by three different methods (i.e tests, regression equation and conversion factor)

S.No.	PFU/ 0.5ml	PFU/0.5ml (regression equation)	PFU/0.5ml($CCID_{50}$/0.5mlx0.7 Conversion factor)
1.	5.531	5.529	5.912
2.	5.515	5.497	5.787
3.	5.435	5.433	5.537
4.	5.439	5.465	5.663
5.	5.494	5.497	5.787
6.	5.559	5.561	6.037
7.	5.484	5.465	5.663
8.	5.553	5.561	6.037
9.	5.480	5.497	5.787
10.	5.541	5.529	5.912
Mean	5.503	5.503	5.812
SD	0.044	0.042	0.164

A more precise estimate is obtained by applying the Poisson distribution. Where P(o) is the proportion of negative tubes and m is the mean number of infectious units per volume (PFU/ml), P(o) = e(-m). For any titer expressed as a TCID[50], P(o) = 0.5. Thus e(-m) = 0.5 and m = -ln 0.5 which is ~ 0.7. Therefore, one could multiply the TCID[50] titer (per ml) by 0.7 to predict the mean number of PFU/ml. When actually applying such calculations, remember the calculated mean will only be valid if the changes in protocol required to visualize plaques do not alter the expression of infectious virus as compared with expression under conditions employed for TCID[50]. Thus as a working estimate, one can assume material with a TCID[50] of 1x 10(5) TCID[50]/ml will produce 0.7 x 10(5) PFUs/ml.[31] .The PFU titres obtained by converting the CCID50 titre were all above the Lower limit 5.4 PFU/0.5ml, with lowest and highest titres of 5.5.3 and 6.037, respectively; mean titre of 5.812± 0.164 SD.

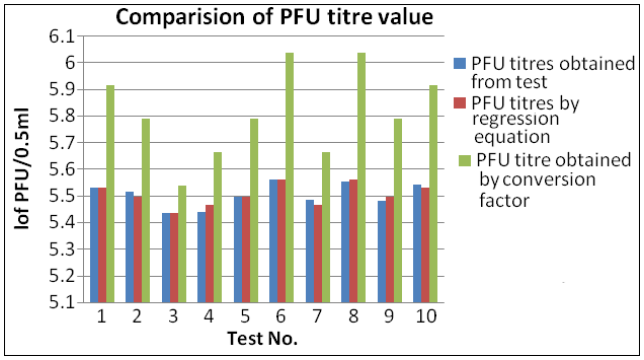

Figure 8: PFU titres obtained through different methods

4. Discussion

Japanese encephalitis is the most common vaccine preventable cause of encephalitis in Asia. It is responsible for an estimated 67, 9000 JE cases annually, has a 20-30% fatality rate and leaves neurologic or psychiatric sequelae in 30-50% of survivors [32].Since JE vaccines has been incorporated into immunization programs in all areas where JE is a Public health problem. The four available JE vaccines are registered worldwide and used in national immunization programs for different age groups. Each of the vaccines incorporates a different vaccination schedule and booster dose requirements. [12]

The present study was carried out on the JE Live Vaccines based on the SA14-14-2 strain. The Japanese Encephalitis Vaccine, Live Attenuated, is a preparation of Live attenuated Japanese Encephalitis virus (Strain SA 14-14-2) grown on monolayer of Specific pathogen free (SPF) hamster Kidney Cell (PHKC).After cultivation and harvest appropriate stabilizers are added into the virus suspension , which is then lyophilized .The major component of the Final vaccine are: Live attenuated virus (strain SA 14-14-2), human serum albumin ,gelatin, sucrose, lactose and carbamide. After lyophilization , the live Japanese encephalitis vaccine is a powder that looks like a light yellow crisp cake. It is stored and transported at 2-8^{0}C and protected from light. [33]

It is important to establish quality control of the vaccines before they are released. The various Quality control tests as per IP (1996) and WHO (1988) are potency test, sterility test, abnormal toxicity, identity tests, physical aspects and different biochemical tests. One of the most important test is the potency test, it checks the vaccine titre.

The potency of a live vaccine is typically expressed in terms of the number of Infectious units of virus contained in a human dose using a specified tissue culture substrate. In case of live –attenuated JE vaccines, potency is to be assessed in terms of virus infectivity titers. The two main method to check the titre of the JE Live vaccine are Plaque Forming Unit (PFU) assay and Median Cell Culture infectivity Dose ($CCID_{50}$) method [34].

Since the JE live vaccine is manufactured as per the guideline of Chinese Pharmacopeia, the titration method mentioned in the Chinese Pharmacopeia i.e. Plaque assay, should be preferred over other methods to estimate the potency of the vaccine. Therefore, an attempt has been made to carry out the Plaque assay for JE live vaccine titration. Both BHK-21 [36] as well as Vero cell line[37] show good suspectibility for JE virus and are used as testing cell lines for JE vaccines. WHO TRS 910, 2002 recommends the use of BHK-21 cell line or any other alternative cell line acceptable to the National Control Laboratory.JEV has been titrated by plaque assay on Vero cells by Royal *et al* .In the current study vero cell line has been used for both the assays.

Both the test methods were performed on ten different vial of the same vaccine of the same batch. The results from the table shows that the vaccine titres obtained by PFU method was in the range 5.435 to 5.559 log PFU per 0.5ml with mean of 5.503 ± 0.044SD. Whereas , the titres obtained by the CCID $_{50}$ method fall in the range 5.698 to 6.198 log $CCID_{50}$ per 0.5 ml with mean of 5.973± 0.164 SD.The results indicate tat there was some difference in titer of JE live vaccine tested by the two methods, which is in accordance with the work done by Lili, Shuqio, Guangzhi,et al [38]. The $CCID_{50}$ titres were found to be higher than the PFU, mean difference in the titres is 0.470 ± 0.123 SD; CV% is 0.620% and correlation 0.945.

The $CCID_{50}$ can be converted to Plaque forming unit (PFU) using Poisson distribution. This conversion is an estimate based on the rationale that the limiting dilution, which would infect 50% of the cell layer challenged, would be expected to produce a single plaque in a cell monolayer. However ATCC recommends that the actual number of PFUs be determined empirically.$CCID_{50}$ titer (per ml) multiplied by 0.7 predicts the mean number of PFU/ml. For example, one can assume the material with a $CCID_{50}$ of $1x10^5$ $TCID_{50}$/ml will produce approximately 0.7 x 10^5 PFU/ml. When applying this calculation, it should be remembered that the estimated mean will only be valid if the changes in the protocol required to visualize plaques do not alter viral expression as compared to conditions used to determine CCID $_{50}$[32].Thetitre btained in the study were converted using the above factor, it was found that the PFU titres obtained were all not less than 5.4 log PFU/0.5ml. The

minimum and maximum values were 5.537 and 6.037, respectively with a mean titre of 5.812±0.164SD.

From the above study it was inferred that both the methods shows good correlation =0.945, CV%=0.620%.The titres obtained were in accordance with the Chinese Pharmacopiea for Plaque assay and were more sensitive , consistent and reproducible, as compared to the $CCID_{50}$ method with a mean titre of 5.503± 0.044 SD.

5. Conflict of Interest

It is declared that this study raises no conflict of interest to any other person involved to this study and in the department.

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