

## **Evaluation of Potency of Measles Vaccine used in Iran: Comparison of WHO and NIBSC Method in Cell Culture**

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### **Abstract**

Measles has been a major cause of illness and death in children and vaccination against the disease is part of the WHO global immunization program. A suitable vaccine should create maximum immune response against the pathogen and must be safe for the user. Thus, after production, vaccines must be analyzed and controlled by the producer and confirm by relevant governmental organizations. The Food and Drug Control Lab (FDCL), Ministry of Health, is the secondary control center on potency of vaccines in Iran. In this study, we have set up the WHO and NIBSC methods in FDCL and compare these methods on determining the potency of measles vaccine.

Measles vaccines were obtained from Razi Institute Iran. Nine dilutions of vaccine ( $10^{-1}$  to  $10^{-5}$ ) in 0.5 log interval were mixed with Vero cell suspension and seeded. In WHO method, the cells were incubated at 36°C for 10 days, during which the cells were checked for cytopathic changes everyday. To set up the assay, we tested the vaccine dilution with four different cell suspensions ( $2 \times 10^5$ - $5 \times 10^4$ /well) and four different concentration of serum (2.5-10%). Based on our results, in the assays, 5% serum and  $1 \times 10^5$  cells were used. The potency assay was performed with six different vaccines produced in one batch and the mean potency for Measles was  $10^{4.32 \pm 0.24}$  CCID<sub>50</sub>/vial for a ten-dose vial. In NIBSC method following seeding of Vero cells, the medium was removed after 3 hours and overlay was added. Then the plates were incubated at 35°C for 10 days. After incubation period, the overlay was removed, the plaques were stained with methyl violet and counted. This assay was repeated three times and the mean of the results was  $5.83 \pm 0.03$  log<sub>10</sub> PFU/dose.

In this study, we have set up the WHO and NIBSC methods and results indicated that the potency of the vaccine is in acceptable range in either method. Furthermore, the WHO method is simple and less time consuming compared to NIBSC which is complicated and requires more effort to produce reproducible results.

**Keywords:** Measles; potency; cytopathic effect; Vero cell.

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### **Introduction**

Vaccines are essential tools in the prevention of

diseases. They protect the vaccinated individual from developing a potentially serious disease and help to protect the community by reducing the spread of infectious agents. The success of immunization initiatives is evident from the impact they have had on the occurrence of

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diseases in various parts of the world. Smallpox has been eradicated worldwide, poliomyelitis is on the verge of eradication, and there have been dramatic reductions in the incidence of measles and neonatal tetanus in many areas (1, 2).

Measles, previously an almost invariable clinical experience of childhood, in some countries is a major cause of illness and death in children. Immunization against measles has been of interest to (WHO) for many years and more especially, since the Expanded Program on Immunization was launched with measles as one of the principal diseases against which it is directed (1). In 1982, with the aim of providing guidance for the standardization of tests needed to ensure the safety and efficacy of vaccines, WHO issued a document entitled *Manual of details of tests required on final vaccines used in the WHO Expanded Programme on Immunization* (1, 2). Live attenuated virus can prevent measles effectively (2, 3). A valuable vaccine must create high immune response with minimum harm. The prime responsibility for the safety and efficacy of a biological product rests with the manufacturer. However, it is the responsibility of the national control authority to establish proper procedures, assuring the adequate safety and efficacy of the biological products intended for use in the country. The activities required to meet these responsibilities are incorporated in the concept of assurance of quality (1, 4, 5). The relevant authorizations from control agencies such as WHO or European health organization along with the report from manufacturer has been used as authorized report. As a health issue, in Food and Drug Control Lab (FDCL) of the Ministry of Health of Iran, we decided to set up the vaccine potency evaluation using WHO and NIBSC method as two valid and internationally accepted methods. In this experiment, we have also compared the result obtained from these two methods and the assay procedures, in order to select the proper method for this propose.

## Experimental

### Material

Vaccines were obtained from Razi Institute, Tehran, Iran. All the mediums and fetal bovine serum were purchased from Gibco, UK. The

cell lines were obtained from Pasture Institute of Iran, Tehran, Iran.

### WHO Method

In conformity with WHO method (1, 2, 6), the serial dilution of vaccines were prepared in  $0.5 \log_{10}$  steps from  $10^{-1}$ - $10^{-5}$  (nine dilution) in medium 199 supplemented with 2% fetal bovine serum. The Vero cells (passage number 12) was mixed with vaccine dilution and seeded in eight replica (eight well per dilution) in 24 well plates. In primary set up, we used four different serum concentrations (2.5, 5, 7.5 and 10%) in combination with four different cell concentrations ( $2 \times 10^5$ ,  $1 \times 10^5$ ,  $7.5 \times 10^4$  and  $5 \times 10^4$  cells per well). The plates were incubated in 5%  $\text{CO}_2$  at  $36^\circ\text{C}$  for 10 days. After the primary set up, the assays were performed at 5% serum and  $1 \times 10^5$  cell per well. During incubation period, the cells were checked for cytopathic changes and positive wells recorded. The titer was calculated in  $\text{CCID}_{50}$  per vial on the basis of the final reading using the Kärber formula:  $-\log \text{CCID}_{50} = L - [d(S - 0.5)]$  where  $L = \log$  starting dilution,  $D = \log$  dilution step,  $S = \text{Sum of the proportion of positive replicate}$ .

The Cytopathic effect (CPE) in each assay was checked under microscope by two different individual and recorded. The assay was repeated six times and the potency was calculated.

### NIBSC Method

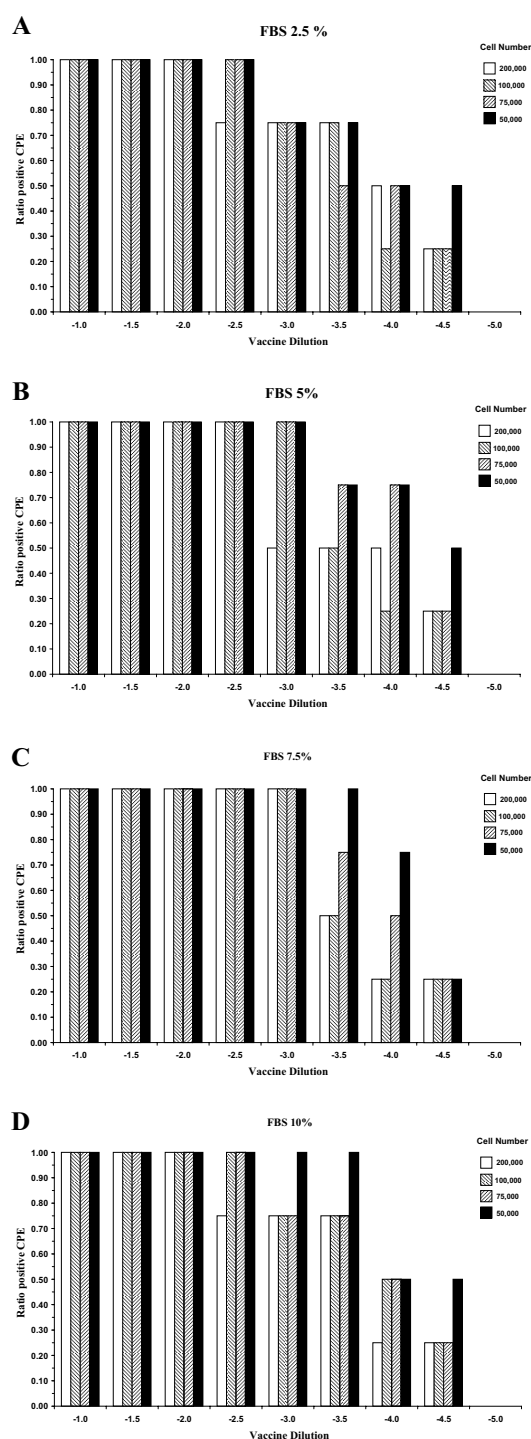
According to NIBSC method (7, 8), in the first stage, the medium composed of DMEM 91%, Fetal calf serum (FCS) 4%, sodium bicarbonate 4.4%, Penicillin-Streptomycin 1% and the overlay medium contain of DMEM 67%, FCS 4%, sodium bicarbonate 4.4%, penicillin-streptomycin 1%, carboxy methyl cellulose 25% were prepared. The vaccine dilutions were prepared similar to WHO method. The cells were mixed with vaccine and plated in 24 well plates (six of each). After 3 h, the medium was removed and the overlay was added to each well. The plates were incubated at  $35^\circ\text{C}$  with 5%  $\text{CO}_2$  for 10 days. After incubation period, the tissue culture fluid was discarded into 10% chlorox and the cells gently washed three times with PBS. Then, plates were stained with 1ml methyl violet (5% in IMS (ethanol: methanol 19:1v/v

mixture) for 20 min. The dye was then removed and wells were washed carefully with PBS and were allowed to dry. The plaques were counted and based on NIBSC protocol, counts above 80 and below 10 were regarded as inaccurate or statistically unreliable and were not included in the calculation. To calculate log of plaque forming unit per dose (Log PFU/dose), NIBSC protocol was followed. Briefly, the valid counts were multiplied by the dilution factor to obtain the number of plaques per well. The mean of this figure over all dilutions were taken and the  $\log_{10}$  of the mean gave the potency per volume used to inoculate each well. Since a 10-dose vaccine reconstituted in 1 ml was used, for assays using 100 $\mu$ l, the log PFU/dose has been obtained from the assay. In assays using 50 $\mu$ l (half dose), the log PFU/dose was calculated by adding 0.3 ( $\log_{10}2=0.3$ ) to the log PFU obtained from the assay.

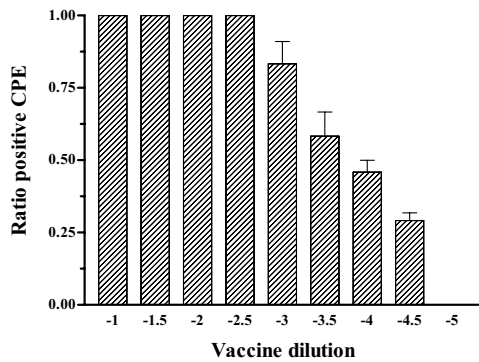
## Results and Discussion

### The effect of cell and serum concentration in WHO method

To observe the cytopathic effect (CPE) of vaccine on cells, the cells must be in a confluent monolayer. Therefore, the cell seeding and serum content of the medium are important in the sensitivity of the assay to observe CPE of the vaccine in WHO method. Therefore, we tested different concentration of serum and different cell number to evaluate their effect. Fig. 1 indicates the effect of serum concentration and cell number on positive CPE observed in different vaccine dilutions. As figure shows low number of cells and low FBS in medium could affect the positive CPE in the assay (Fig. 1 A,B,C,D). The  $CCID_{50}$  was calculated for these conditions and presented in Table 1. These results indicate that high cell number ( $2 \times 10^5$ ) lowers the sensitivity of the assay. On the other hand, although the serum concentration has little effect on the  $CCID_{50}$  calculated in one cell seeding (Table 1), high concentrations of serum did affected the sensitivity of the test, since higher concentration of vaccine showed CPE (Fig.1). Furthermore, in the absence of vaccine, it is found that low cell seeding doesn't generate confluent monolayer and high cell number plates

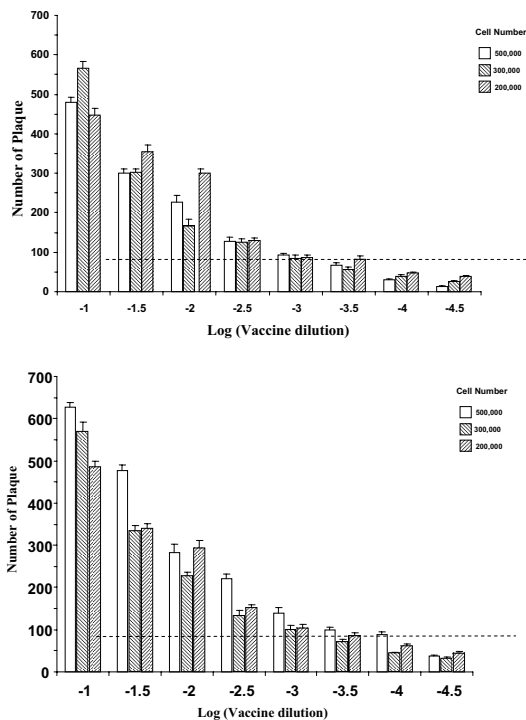


**Figure 1.** The effect of cell number and serum content of the assay medium on cytopathic effect of Measles vaccine in Vero cells. The graph indicates the effect of 2.5 (A), 5.0 (B), 7.5 (C) and 10 (D) percent serum on cytopathic effect of various cell seeding. The assay was performed as indicated in the Experimental. The graphs show the ratio of positive CPE for each dilution. The Ratio of positive CPE were calculated as ratio=total positive CPE/total replica in dilution. The results recorded on day 10. All dilutions were plated in quadruplicate (n=4).



**Figure 2.** The cytopathic effect of Measles vaccine on Vero cells. The cells were mixed at density of  $1 \times 10^5$ /well with vaccine dilution and plated. On the day 10, the positive CPE in replica of each dilution was recorded and calculated as Ratio=total positive CPE/total replica in each dilution. The results were plotted as mean  $\pm$  SEM (n=8).

have many dead cells. Both of these conditions could create false positive results. In the course of our study, it was found that  $1 \times 10^5$  cells/ml seeding gave a confluent monolayer within three days and by the end of day 10, there were only



**Figure 3.** The effect of cell number and vaccine volume on the plaque formation of Measles vaccine in Vero cells. The assay was performed as indicated in the material and methods. The graph shows the plaque number obtained for each dilution and the effect of 50  $\mu$ l (A) and 100  $\mu$ l vaccine volume added to various cell numbers in the assay. The results recorded on day 10 and Mean  $\pm$  SE of all dilutions is plotted (n=3). The dotted line indicates plaque counts of 80, the count limit of NIBSC method.

few dead cells. Therefore, based on these results it was preferred to use 5% FBS and  $1 \times 10^5$  cells for the rest of the assays.

### Measles Vaccine potency evaluated by WHO method

In order to determine the potency of the Measles vaccine, Vero cells were plated at  $1 \times 10^5$ /ml mixed with  $10^{-1}$ – $10^{-5}$  dilution of vaccine. Each dilution was plated in eight replica (eight well /dilution). The CPE was observable on the third day in high concentrations of vaccine ( $10^{-1}$  to  $10^{-2}$ ). However, as indicated in WHO method, the CPE reading was recorded on the tenth day by two independent observers. Fig.2 indicates that CPE was observed in all dilutions except  $10^{-5}$  dilution. This assay was repeated independently on six different vials of vaccine. The mean potency calculated for Measles vaccine was  $10^{4.32 \pm 0.24}$  CCID<sub>50</sub>/vial for each ten-dose vial (n=6). Since in WHO method the potency of one dose should be at least  $3 \log_{10}$ CCID<sub>50</sub>, then a ten-dose vial should be at least  $4 \log_{10}$ CCID<sub>50</sub>/vial and therefore, the potency of vaccine under test was in the acceptable range.

### The effect of cell number on potency evaluation in NIBSC method

In NIBSC method, the measurement of the potency of the vaccine is based on measuring plaques formed by vaccine in Vero cells. Based on this method, plaque counts above 80 and below 10 are regarded as inaccurate or statistically unreliable and will not be included in calculation of potency. Therefore, to determine the proper conditions for this assay, we tested different cell seeding in presence of various vaccine dilutions. We also tested two different vaccine volumes added onto the cells (Fig. 3 A, B). As shown in Fig. 3. high concentrations of vaccine ( $10^{-1}$  to  $10^{-2.5}$ ) produce plaque counts over the method's acceptable range (80 counts per dilution) and dilutions lower than  $10^{-4.5}$  had plaque counts below NIBSC range (less than 10 counts per dilution) in all conditions. Thus, the measles vaccine potency was calculated using the concentration range of  $10^{-3}$  to  $10^{-4.5}$  (Table 2.). As indicated in Table 2 in assays containing  $3 \times 10^5$  cell per ml with 50 and 100  $\mu$ l of vaccine volume and  $5 \times 10^5$  cell per well with 50  $\mu$ l of

vaccine, the PFU has been calculated as mean of three different dilutions which this will lower the error of PFU calculation among dilutions (Table .2). Moreover, we have observed that in the assay with  $5 \times 10^5$  cell per ml with 50  $\mu$ l of vaccine, the plaques are more clear, and easier to read with less empty spots in the well (data not shown). Therefore,  $5 \times 10^5$  cell per ml with 50  $\mu$ l of vaccine volume were used to assess the potency of Measles vaccine.

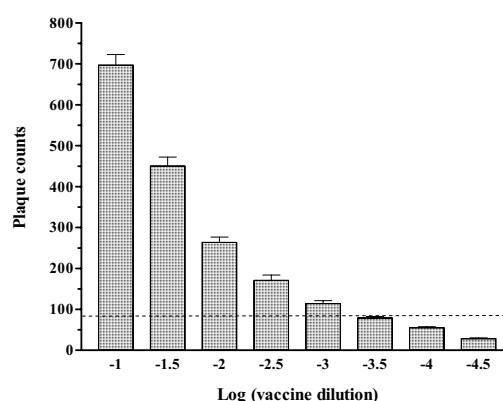
### Measles Vaccine potency evaluated by NIBSC method

In order to determine potency of the Measles vaccine, we plated Vero cells at  $5 \times 10^5$ /ml mixed with 50 $\mu$ l of  $10^{-1}$ – $10^{-5}$  dilution of vaccine. Each dilution was plated in six replica (six well /dilution). Fig 4. indicates the result of potency evaluation in this assay. The assay procedure was repeated independently on three different vials of Vaccine and the log PFU/dose of each assay was calculated. The mean log PFU/dose of the vaccine was  $5.83 \pm 0.03$  (n=3).

In this study we have setup WHO and NIBSC methods in FDCL to determine measles vaccine potency and to compare these methods. In WHO method, the assay will measure the potency of Vaccine based on the observation of CPE. Therefore, this method can measure the concentration of vaccine that produces CPE in 50% of cultures. In order to have a clear CPE, as the end point of the assay, and avoid any error in observation, we have tested different cell number plated for the assay and various serum concentrations. Our result showed that in, high concentrations of the Measles vaccine, independently of cell number or serum concentration, CPE was observed. In lower concentration of vaccine, the CPE is dependent on cell number and serum content. This effect is

**Table 1.** The CCID50 of Measles vaccine was calculated as described in Experimental The results are tabulated for assays with different cell number and serum concentrations.

Serum (%)	Cell number x 1000			
	50	75	100	200
2.5	4.6	4.4	4.4	4.1
5	4.7	4.7	4.3	4.1
7.5	4.7	4.5	4.3	4.1
10	4.7	4.4	4.4	4.1



**Figure 4.** The effect of plaque formation induced by Measles vaccine in Vero cells.

The cells were mixed at density of  $5 \times 10^5$ /well with 50  $\mu$ l of vaccine dilution and plated as described in methods. On the day 10, the plaques in replica of each dilution was counted and Mean  $\pm$  SE of all dilutions is plotted (n=6). The dotted line indicates plaque counts of 80, the count limit of NIBSC

oppositely correlated with serum concentration and cell number, where in 10% serum and low cell number (50000), the CPE is 100% even in  $10^{-3.5}$  dilution of vaccine (Fig.1). When we lower the serum content or increase the cell number, the CPE is detectable in higher vaccine concentrations (Fig.1.). On the other hand the high cell number has lowered the sensitivity of the assay (Table.1). Besides, we have found that the CPE is very clear in 5% serum and  $1 \times 10^5$  cell/ml. Therefore, we have used this condition to calculate the CCID50 of the measles vaccine. We tested six different vials of the measles vaccine and, the mean potency calculated for was  $10^{4.32 \pm 0.24}$  CCID<sub>50</sub>/vial for each ten-dose vial (n=6) (Fig.2). In WHO method the acceptable potency for one dose is at least 3 log<sub>10</sub>CCID<sub>50</sub> and for a ten-dose vial should be at least 4 log<sub>10</sub>CCID<sub>50</sub>/

**Table.2.** The Log PFU/dose of Measles Vaccine calculated for different cell number and vaccine volume. The results are tabulated for different cell number and vaccine volume used in the assay. The mean of valid log PFU/dose were presented with SE. The number of valid dilution included in calculation is indicated as n.

Cell number	Vaccine Volume	
	50 $\mu$ l	100 $\mu$ l
$2 \times 10^5$	$6.14 \pm 0.24$ n=2	$5.90 \pm 0.20$ n=2
$3 \times 10^5$	$5.80 \pm 0.17$ n=3	$5.64 \pm 0.21$ n=3
$5 \times 10^5$	$5.70 \pm 0.12$ n=3	6.07 n=1

vial. Our result indicates that the potency of vaccine under test is in acceptable range.

In NIBSC method, the potency is measured by the number of plaques formed in the assay. And the plaque forming unit is calculated using the doses of the vaccine which has produced plaques in acceptable range i.e. 10-80/well (based on the protocol) as an index for the potency of the vaccine. This assay will measure the maximum number of plaques produced by the vaccine. Based on the NIBSC protocol, the number of the cells and the volume of the vaccine added to the assay could affect the sensitivity of the test. Therefore, we tested these two conditions. Our results indicate that in high concentration of vaccine (higher than  $10^{-3}$ ) the plaques counts were above the acceptable range; therefore, those concentrations couldn't be used to calculate the PFU (Fig.3). However, lower dilutions produced plaques in acceptable range and calculating PFU using different dilutions of the vaccine will lower the error in determining vaccine potency (Fig.3., Table.2). Furthermore, we found out that in conditions with  $5 \times 10^5$  cell per ml with 50  $\mu$ l of vaccine, the plaques are clearer, and easier to read with less empty spots in the well (data not shown). Therefore, we used  $5 \times 10^5$  cell per ml with 50  $\mu$ l of vaccine volume to determine the potency of Measles vaccine in this method. We have tested different vials of the measles vaccine and the mean log PFU/dose was  $5.83 \pm 0.03$  (n=3).

Although the NIBSC method is more quantitative than WHO method, the procedure requires very restrict controls and is very time consuming. Besides, the cell number and the amount of vaccine added, the concentration

and preparation of the overlay, the fixation and staining procedure and counting clear plaques, make this assay hard to set up in a lab and could affect the validity of the test from lab to lab. However, the WHO is easier to set up and experience of observation of CPE is adequate for potency determination. Moreover, the potency obtained from WHO, indicates the minimum vaccine concentration produced CPE in 50% and therefore immunogenic response.

Taken together, our results indicate that the potency of measles vaccine based on the results obtained from these two methods is in acceptable range. Furthermore, the WHO method is the preferred method to evaluate the potency.

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