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***Full Article***

# Evaluation and comparison of Hela, Hep2C and Vero cell lines sensitivity to polio vaccinal virus using micro and macro vaccine potency tests

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**ABSTRACT**

Poliomyelitis, an acute viral infectious disease caused by poliovirus, still remains a public health problem in developing countries. Despite the global effort to eradicate polio, continuing the polio immunization with a potent and safe vaccine is essential. For accurate vaccine evaluation, three types of cell lines including Hela, Hep2C and Vero were evaluated and compared using two methods of polio vaccine potency tests (micro & macro). For cells comparison, five different batches from polio vaccines were tested and to develop the test, five variables including viruses, cells, serum, media and $Co_2$ were studied. For validation, the titer of which has been well established as a working reference preparation (WRP) was applied to control the accuracy and reproducibility of the testing system. Multiple comparisons were performed by analysis of variance (ANOVA) followed by Tokey HDS and LSD. No significant differences were found between the potency of vaccine batches and between macro and micro methods. Reduction in cells sensitivity and potency of vaccines was found with increasing passage number. Significant differences were found between the sensitivity of the cell lines. The highest potency of polio vaccines was obtained using Hela cells (GMT in macro and micro test = $10^{6.35}$); Hep2C cells were afterwards (GMT in macro= $10^{6.01}$ and in micro test= $10^{5.94}$); Vero cells were lowest (GMT in macro= $10^{5.78}$ and in micro test= $10^{5.72}$). So, the sensitivity and accuracy of the potency test for evaluation of the polio vaccine in immunization program in Iran will be assured using the Hela cell line with low passage number in macro and micro methods.

***Keywords:*** Polio vaccine, Potency test, Hela, Hep2C, Vero

## INTRODUCTION

Poliomyelitis, an acute viral infectious disease caused by three types of poliovirus (1, 2, and 3), remains a serious health problem. Polio vaccine has been included in the WHO Expanded Program on Immunization (EPI). There are two types of polio vaccine: Inactivated polio vaccine (IPV), and oral polio vaccine (OPV). Inactivated polio vaccine also called salk, was developed by Jonas Salk in 1952. In 1957

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Albert Sabin developed attenuated poliovirus (Sabin *et al* 1960). Type 1, 2 and 3 monovalent oral poliovirus vaccine (MOPV) and trivalent OPV (TOPV) were licensed between 1961 and 1963 (Ronald 1984). Trivalent OPV has been largely replaced the inactivated polio vaccine (Pearce 2004). Oral polio vaccine produced by the passage of the virus through non-human cells at a sub-physiological temperature produces spontaneous mutations in the viral genome (Koike *et al* 1990).

For the success of polio vaccination program, use of potent vaccines is of utmost importance. An efficient vaccine must contain certain amount of virus particles to stimulate effectively humoral and cellular immune systems. The potency test is one of the most important procedures involved in the production of oral poliovirus vaccine. Standardization of the test procedure and selection of suitable test materials are very important in the production of vaccines. Using micro plate has been known for potency test as an exact and rapid method to estimate viral content of in process and final products (WHO TRS No. 904, 2002). The most important factor is cell culture derived from old world primates or humans which are naturally susceptible to polioviruses infection. Cell cultures from other species can be made susceptible by transformation with DNA that codes for the human or primate cellular receptor (Koike *et al* 1992, Knip *et al* 2007). So, in this study, three cell lines including Hela, Vero and Hep2C were evaluated and compared using two standardized methods of the polio vaccine potency test and most sensitive cell for potency test was introduced.

## MATERIALS AND METHODS

**Micro test method development.** One hundred samples were randomly prepared from one certified batch of polio vaccine. Vaccine samples were assayed with the micro method according to the WHO instructions (WHO/BLG/95.1 1995). Three types of cell lines including Hela (cervical cancer from Henrietta lacks, ATCC: CCL-2), Hep2C (human epithelioma, ATCC: CCL-23) and Vero (Cercopithecus aethiops kidney cell, ATCC: CCL-81) and six variables including virus (50 and 100 µl), cells number ($1 \times 10^5$, $1.5 \times 10^5$ and $2 \times 10^5$ cells per ml), cells volume (50 and 100 µl); calf serum (3 and 5 %); maintenance media (50 and 100 µl) and $Co_2$ (0 and 5%) were used in the assay. After preparation of cells (Butler 2004, Freshny 2005), serial dilutions of the samples were inoculated in the wells of a flat-bottomed Nunc microtitre plate. The cell suspension was then added to the microplate (Robins *et al* 2009). After adding the maintenance media with calf serum, the plates were incubated in a $Co_2$ incubator (Priya *et al* 2003). In parallel, macro titration was performed using Hela cell culture to evaluate the micro assay. In the macro assay, the samples were examined in tube containing monolayer of the cells which prepared two days ago (Grist *et al* 1974). Microplate and tubes containing the cells were examined microscopically every day and scored as infected or not infected based upon cytopathic effects until 7 days. Vaccine potency was calculated by Spearman-Karber method with estimation of the 50% end –point on the basis of CCID50/dose (Muhammad *et al* 2010). Geometric mean titre (GMT) was calculated using macro and micro method data in triplicate.

**Comparison of the Hela, Hep2C and Vero cell lines sensitivity.** Three hundred samples representing five different batches from the oral polio vaccine (60 samples from each batch) were tested in 6 test systems include the following: micro/macro potency test with Hela cells, micro/macro potency test with Vero cells, micro/macro potency test with Hep2C cells. The tests were performed according to the standard WHO protocol (WHO/ BLG/ 95.1 1995) based on previous results (Formulation of micro potency test using three cell cultures). The variables such as test procedures, temperature, type and volume of materials etc were fixed in all the tests.

For reliability of the results, the tests were repeated six times in each batch. Repetition of the tests, was calculated with a hypothesis that one of the cells is

most sensitive to polio vaccinal virus (d= μi - μj), if the difference of cells sensitivity be significant and is achieved in the probability error level type one ($\sigma$ = 0.05) with power 1-$\beta$ = 0.80. The elementary data of cells sensitivity (μi and μj) and standard deviation were calculated on the basis of a pilot study as 0.4 and 0.32, respectively. Therefore, repetition of the each test was calculated by, $N= [(z_1-\alpha/2+z_1-\beta) / (\mu_i-\mu_j)/\sigma]^2)$ (Shokati *et al* 2010).

**Test validation.** To determine the corrected potency of the working reference preparation, the following formula was used:
Corrected potency of the working reference preparation = The established titre of the international reference reagent as the result of an international collaborative study / The GMT of the international reference reagent × The GMT of the working reference preparation (ICH, Q2 R1, 2005).

**Statistical analysis.** Statistical studies, was performed using analysis of variance (ANOVA) followed by Tokey HDS and LSD multi comparison.

## RESULTS

**Micro test method development.** The optimum range of variables in micro method of polio vaccine potency test using three cell lines was determined (table 1).

**Comparison of the Hela, Hep2C and Vero cell lines sensitivity.** Each batch of samples was tested in 6 runs; each run with 6 test systems; in each system as triplicate. The GMT was calculated for each cell (table 2 and fig.1). Analysis of variance showed:
1. No significant difference was found between the potency of several lot numbers of polio vaccine (P> 0.8).
2. Reduction in cells sensitivity and potency of vaccines was found with increasing passage number (P= 0.033).
3. Significant differences were found between the sensitivity of the cell lines. (P=0.001). The highest potency of polio vaccines was obtained using Hela cells (GMT in macro and micro test = $10^{6.35}$); Hep2C cells were afterwards (GMT in macro= $10^{6.01}$ and in micro test= $10^{5.94}$); Vero cells were lowest (GMT in macro= $10^{5.78}$ and in micro test= $10^{5.72}$).
4. The difference between macro and micro test methods was not significant (P= 0.842).

**Test validation**. System suitability was achieved in these tests. In the lowest dilution, all of the wells or tubes had CPE and in the highest dilution, all of them, were negative. The number of discarded cells infected by non viral agents, such as bacterial contamination, was less than 20%. The difference between the WRP geometric mean titre (GMT) and the corrected potency of the working reference preparation was less than 2SD (standard deviation) (SD= 0.758; ±2SD range) (table 3-6 and figure 2). The coefficient variation (CV) was 1.4% (CV for standard WRP=1.2%).

## DISCUSSION

Oral poliomyelitis vaccine, one of the most effective human viral vaccines, has an important role in polio eradication in the world. In 1988, the world health assembly resolved to eradicate polio globally by the year 2000 (Kew *et al* 2005). By this program, the numbers of poliomyelitis cases were reduced from 350000 cases to 49 cases in 2010 and these cases were only in four countries containing, Afghanistan, Pakistan, Nigeria and India. Angola, Chad and Congo have active and persistent poliovirus transmission of more than 12 months following an importation with re-established transmission. Iran managed to eradicate the wild polio virus in 2000 by OPV and the last cases of wild polio in Iran were from Pakistan in 2000 and from 2001 until now, there was not any poliomyelitis case (WHO 2012). Use of potent and safe vaccine, is very important in expanded program on immunization. Production of safe and efficacious vaccines has depended in part on consistency of the manufacturing process and in part on laboratory tests of working seed viruses and vaccine lots derived from them (Sutter *et al* 2004). For this purpose, laboratories should have effective and validated quality control tests such as

Potency, Identity and Stability. Although recent developments in molecular detection technology make it probable that enterovirus diagnosis/surveillance will increasingly be achieved by non-culture-based methods, culture of poliovirus is the gold-standard method for virological surveillance in the worldwide initiative (WHO manual 2002). The study was aimed to examine critically the role of established laboratory tests in assuring the quality of live attenuated poliovirus vaccines. To ensure the accurate evaluation of polio vaccine potency test in the Iran immunization program, the sensitivity of three common cell lines including the Hela, Hep2C and Vero to polio vaccinal virus was assessed and compared using micro and macro culture potency test. There are a few cell culture studies about polio virus with many limitations. In 1999, Wood et al used the clinical samples such as feces for determination of cell sensitivity, but there was no evidence for presence of polio virus in these samples. Shokati et al. (2010) compared the cells used for detection and isolation of entroviruses in polio laboratory, containing RD, Hep2 and L20 to polio vaccinal

**Table 1.** The optimum range of the variable factors in micro titration potency test.

| Procedure | Variable Factors | Macro method by Hela (-Log CCID50/dose) | **Micro method** (-Log CCID50/dose) | | |
|---|---|---|---|---|---|
| | | | **Hela** | **Hep2C** | **Vero** |
| 1 | Virus= 100 µl<br>Cell No.= 2× $10^5$/ml<br>Cell Volume= 100µl<br>Serum= 3%<br>Media= 0<br>$CO_2$= 5%<br>Incubation Temperature= 36 $^0$C<br>Incubation Time= 7 days | 6.32<br>6.45<br>6.45<br>6.32<br>6.32<br>GMT: 6.37 | 6.30<br>6.30<br>5.92<br>5.92<br>6.17<br>GMT: 6.12 | 6.17<br>6.17<br>6.17<br>5.92<br>6.05<br>GMT: 6.10 | 5.92<br>5.92<br>5.80<br>6.05<br>6.05<br>GMT: 5.95 |
| 2 | Virus= 50 µl<br>Cell No.= 2× $10^5$/ml<br>Cell Volume= 100µl<br>Serum= 3%<br>Media= 50 µl<br>$CO_2$= 5%<br>Incubation Temperature= 36 $^0$C<br>Incubation Time= 7 days | 6.32<br>6.32<br>6.32<br>6.45<br>6.45<br>GMT: 6.37 | 6.30<br>6.30<br>6.30<br>6.17<br>6.30<br>GMT: 6.27 | 6.17<br>6.17<br>6.05<br>6.30<br>6.30<br>GMT: 6.20 | 6.05<br>6.05<br>5.92<br>5.92<br>5.80<br>GMT: 5.95 |

**Table 2.** The comparison GMT result of OPV potency test by different cells.

| Vaccine (Lot Number) | Hela | | Hep2C | | Vero | |
|---|---|---|---|---|---|---|
| | **Macro** | **Micro** | **Macro** | **Micro** | **Macro** | **Micro** |
| **1** | 6.34 | 6.30 | 6.01 | 5.90 | 5.74 | 5.70 |
| **2** | 6.34 | 6.31 | 6.01 | 6.01 | 5.74 | 5.79 |
| **3** | 6.36 | 6.40 | 6.05 | 6.00 | 5.92 | 5.80 |
| **4** | 6.32 | 6.32 | 6.00 | 5.90 | 5.67 | 5.52 |
| **5** | 6.38 | 6.40 | 5.96 | 5.90 | 5.82 | 5.80 |
| **Mean of GMT** (-LogCCID50/dose) | 6.35 | 6.35 | 6.01 | 5.94 | 5.78 | 5.72 |
| **Passage Number** | 71-72-73-74-75-76 | | 163-164-165-166-167-168 | | 247-248-250-251-252-253 | |

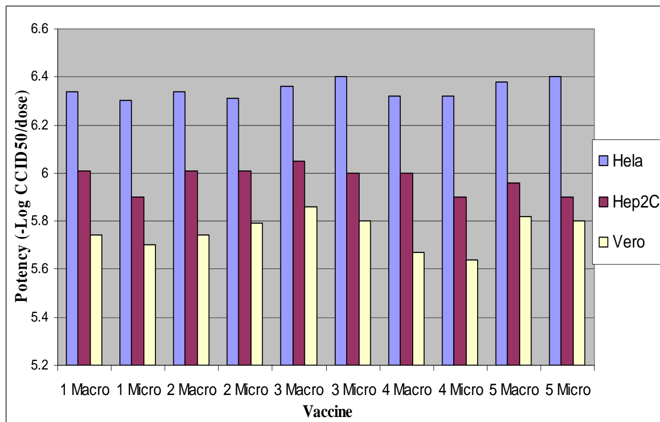


**Figure1.** The comparison GMT result of OPV potency by different cells

virus and polio standard virus. This study showed RD is most sensitive to polio vaccinal virus, L20 is most sensitive to polio standard virus and Hep2C had less sensitivity to polio vaccinal virus and polio standard virus in compare to other cell lines but Hep2C is recommended for polio potency test by WHO. The study has taken advantage of the comparison between the cells sensitivity to polio vaccinal virus and polio standard virus together and cells sensitivity to each type of poliovirus, separately. In a similar study performed by Abey et al. (2005), L20B was introduced as most specific for isolation of poliovirus. In 2007, Sarmiento et al. indicated that L20B are highly sensitive and selective cell for polioviruses replication. In spite of two mentioned study showing that L20B is most sensitive to polio virus, Wood et al. (1999) and Dowdle et al. (1998) demonstrated that RD is most sensitive to polio virus. In some studies (Abbasian *et al* 2004, Tabatabaii *et al* 2007), the sensitivity of RD, L20B and Hep2 was similar. In these studies, there were not any standard situations in sensitivity tests. Robins et al (2009) determined the effect of difference serum concentration on polio potency using RD. In a study, Hep2, RD and L20B were used for isolation of entroviruses in feces and cerebrospinal fluid of patients with acute flaccid paralysis (AFP). Ozkaya et al. (2002) showed L20B, RD and Hep2 were most sensitive respectively to poliovirus, echovirus and coxakivirus type B.

In the previous studies, there was no standard for validation of the tests and effective situation such as temperature, pH, media, serum and etc were not considered. Also, they applied only one approach in potency test and didn't compare common cell lines in vaccine potency. In the present study, micro potency test was developed using three common cell lines containing Hela, Hep2C and Vero. For micro potency test, two approaches were established using each of the cells. Three cell lines were evaluated and compared using micro and macro potency test to polio vaccinal virus. The validation assay was performed for all of the tests. Statistical analysis and multiple comparisons were performed using ANOVA and Tokey HSD and LSD. Analysis results showed that the difference of cells sensitivity was significant ($p= 0.001$). Figure 1 and table 2 showed that no significant differences were found between the potency of vaccine batches (confirmation of the consistency in polio vaccines production) and between macro and micro methods. Significant differences were found between the sensitivity of the cell lines. The highest potency of polio vaccines was obtained using Hela cells (GMT in macro and micro test = $10^{6.35}$); Hep2C cells were afterwards (GMT in macro= $10^{6.01}$ and in micro test= $10^{5.94}$) and Vero cells were lowest (GMT in macro= $10^{5.78}$ and in micro test= $10^{5.72}$).

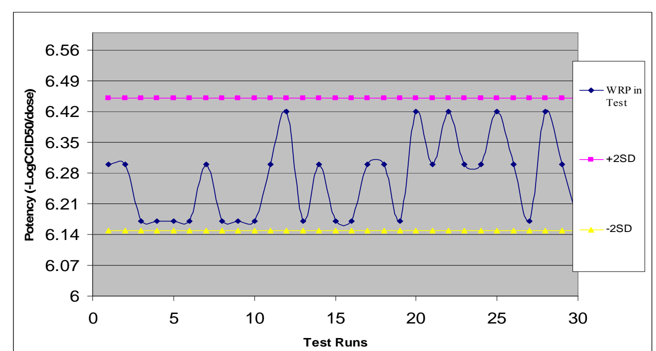


**Figure2.** Potency of Working References for determination of standard deviation

Another advantage of this study is evaluation of correlation between cells passage number and polio vaccine potency titre. Reduction in cells sensitivity and

potency of vaccines was found with increasing passage number. The difference of mean potency in different passage number was significant (p= 0.33). In all of the tests the maximum passage of each cell was 6 passages. The other advantage of this study was test validation. In all of the tests, the difference between the working references preparation (WRP) geometric mean titre (GMT) and the corrected potency of the WRP was less than 2SD (±2SD range). The CV for standard WRP was 1.2% and for WRP in this study was 1.4%. Therefore, the accuracy and the reproducibility of the tests were acceptable. So, the sensitivity and accuracy of the potency test for evaluation of the polio vaccine in immunization program in Iran, will be assured using the Hela cell line with low passage number in macro and micro methods (with special designing and certified formulation of material).

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