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# Spectrochemical characterization of Vero cell line against PPR virus infection

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**ABSTRACT:** An in vitro virus infectivity assay based on the newly introduced spectrochemical method was performed using *Peste des Petits* Ruminants (PPR) virus infected Vero cell line as a model infection. Herein, the mitochondrial reductase enzyme activity was monitored using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent as a substrate which was reduced to a insoluble formazan products. The purple to darker color was achieved upon hydrolysis of the insoluble formazan with DMSO solution. The absorbance (OD values) of colored solution was measured by spectrophotometer at a wave length of 570nm. The infectivity/viability data were achieved from the OD values of different doses of virus infected or non-infected cells using the standard formulae. The OD values obtained from spectrochemical assay were compared with traditional Plaque assay and validated with Trypan blue assay. The data obtained from spectrochemical analysis showed similar trend as was achieved with traditional methods with a little variation in the sensitivity. The sensitivity variations are obvious due to the marked differences in the measurement unit and detection methods. However the newly introduced spectrochemical method showed superiority over the traditional methods because of its simple, label free, less time consuming measurement method and its suitability in the monitoring of large number samples.

**KEYWORDS:** Spectrochemical-Assay, PPR virus, MTT assay, Plaque assay, Trypan blue assay.

## INTRODUCTION

In vitro cell culture system is a useful alternative to animal based research including drug effect study or pathogenesis of intracellular pathogen particularly virus, chlamydia and rickettsia. Present study focuses on analysis of cellular responses against viral exposure using spectrochemical analysis based on mitochondrial reductase enzyme activity assay. The current *in vitro* virus infectivity analysis is based on trypan blue exclusion assays, optical microscopic analysis of cytopathic effect (CPE) or plaque assay. These traditional systems lack accuracy and sensitivity in studying cellular changes resulting from infection of an intracellular pathogen. Hence, accurate and sensitive detection system is required for the investigation cellular response to intracellular organism's infection or other influences. Very recently electrochemical analysis of mammalian cell appears as a very fast, sensitive and effective tool for the analysis of in vitro cytotoxicity [1;2;3;4]. However, this emerging diagnostic tool requires sophisticated transducer and recorder system, where physiologic responses of cell acquired and transduced as electrical signal [5;6]. The intensities of electrical signals represent physiologic state of the corresponding cell population [2;7;8]. In spite of the accuracy and high sensitivity and strict cell line

specificity [9], the electrochemical detection system is not suitable for Bangladesh due to the unavailability of detector device and transducer system that requires huge cost involvement [2;10;11]. Therefore, a convenient detection system for the viability of cell against infectious agents, toxicants, and pollutants is required for Bangladesh perspective. Particularly, the onsite monitoring of *in vitro* cellular response for the monitoring of viral infectivity is our current demand.

Considering the severity of illness and huge economic impacts to the small ruminant keepers and marginal farmers, *Peste des Petits* Ruminants (PPR) was considered as a model virus in this research [12]. The PPR literally named as “Plague of small ruminants" is an economically significant viral disease of sheep and goats because of its huge morbidity and severe mortality [13]. Therefore, a rapid, accurate and onsite monitoring system for this deadly viral disease is required to suggest earliest strategy to combat and save the sheep and goat population of Bangladesh. There are various traditional and conventional diagnostic methods for PPR virus isolation and identification confirmed by morphological characterization, clinical findings, postmortem lesions, cell cultures isolation, plaque assay, serological study, and followed by molecular detection (i.e. PCR, RT-PCR). Although the traditional and conventional methods are accurate and sensitive for the diagnosis of virus but all of them are time consuming, laborious and lacks economic feasibility. Therefore, herein this study, spectrochemical monitoring of PPR virus infection was performed as an alternative to traditional methods. This newly developed method was based on the mitochondrial reductase enzyme activity on 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[7]. The MTT reagent based cytotoxicity determination is a well-established spectroscopic detection method for the environmental toxicity analysis and drug effect study [14;15;16]. Where, MTT reagent is reduced by mitochondrial reductase and produces purple insoluble formazan in living cells [16]. Colored solution was achieved when dimethyl sulfoxide was employed [7;14]. Finally, the absorbance of the colored solution measured by spectrophotometer at a defined wavelength (570nm) indicates the mitochondrial activity of cells [15]. Based on this principle cytotoxicity of Rat Pheochromocytoma (PC-12), Neuroblastoma (SHS-Y5Y), HEK293, HeLa, HepG2 cell were investigated successfully against Pentachlorobenzine (PCB), Bisphenyl A, dichloro-dimethyl-trichloroethane (DDT) and other potential toxicant [1;2;4;8]. Very recently nanoparticle toxicity has also been investigated using MTT based mitochondrial reductase enzyme activity assays [17]. But no study has earlier been carried out on the MTT based spectrochemical characterization of cell against infectivity of intracellular pathogen. Therefore, the present research is aimed to establish spectrochemical characterization of PPR virus infection on vero cell line that can be used as an alternative to traditional, time consuming optical based CPE or plaque assay method for viral infectivity assay.

In the present research, MTT reagent based mitochondrial reductase enzyme activity assay [7;14] was employed with the aim of monitoring *in vitro* cellular response against viral infections. Herein PPR virus sample was considered as a candidate for this *in vitro* viral infectivity assay based on spectrochemical method. The detail experimental processes are schematically illustrated in the Figure 1.

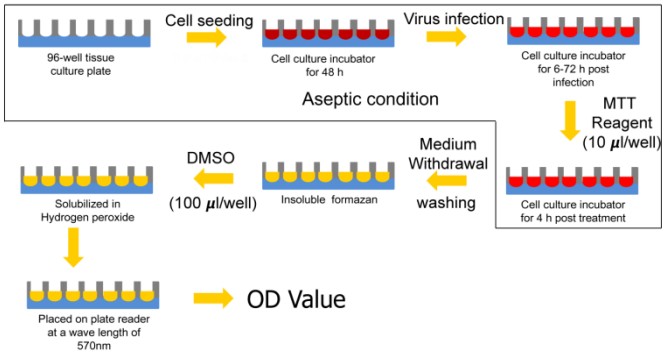


**Figure 1.** Schematic illustrations of spectrochemical analysis of PPR infected vero cell.

## MATERIALS AND METHODS

### Materials and reagents

Vero cell passage-17 and PPR virus passage-30 were obtain from SAARC RLDL-PPR; Dimethyl Sulfoxide (DMSO) purchased from Sigma-Aldrich, USA; 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent purchased from Sigma-Aldrich, USA; Cell culture plate purchased from NUNCLON, Denmark; Trypsin-EDTA (0.25%,100mL) and FBS (500 ml) were purchased from Gibco, South America; Agarose (500g) was purchased from Agarose-Seakem (R) GTG (R), Rockland, USA; Crystal violet (100g) and Trypan blue(25g) were purchased from BDH VWR International Ltd, Poole, BH15, Ltd, England; Hemocytometer purchased from HBG Germany; Spectrophotometer was purchased from Erba(R) Erba Lisa Scan II tm, Erba, Mannheim, Germany.

### Formulae used for deriving Infectivity and Viability

$$\% \text{ Viability} = \frac{OD \text{ of infected} - OD \text{ of Blank}}{OD \text{ of control} - OD \text{ of Blank}} \times 100 \quad \ldots\ldots 1$$

$$\% \text{ Infectivity} = 100 - \% \text{ Viability} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots 2$$

### Cell line and viral sample selection

Considering the susceptibility of *Peste des Petits* Ruminants (PPR) virus, vero cell line was used as a candidate for the present spectrochemical investigation. The infectivity of PPR on vero cell was spectrochemically investigated in parallel maintaining vero cell in virus free medium as a negative control.

### Inoculum preparation

PPR virus infected vero cells (passage-30) obtained from SAARC RLDL-PPR were scrapped with the sterilized cell scraper and collected in a centrifuged tube. The tubes were spinned at 5000 rpm for 10 min. The supernatant was collected and remaining cell suspension was vortexed and centrifuged again for the collection of supernatant. The collected fluid was filterred in using syringe filter (0.22$\mu$m) and treated with 1% penicillin and streptromycin solution. Thus inoculums were prepared using 10-fold dilution for experimental use.

### Maintenance of cell culture and virus inoculation

Vero cells were maintained in Minimal Essential medium (MEM) supplemented and were cultured at $37^0$C in MEM supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% antibiotics (streptomycin and penicillin), 2% L-glutamine, 2% Sodium bicarbonate. Similarly, maintenance media contained 2% FBS in place of 10%. Cells were maintained under standard cell culture conditions at $37^0$C in an atmosphere of 5% $CO_2$. The medium was changed twice in a week. At 80-90% confluence the cells were sub-cultured at a density of $2\times10^6$ cells/ml on culture plates, and then incubated for 4–5days. When the cells reached sub-confluence, they were harvested with trypsin and sub-cultured. The cells from passages were used in the experiments. The cell cultured plate with confluent layer was subjected to virus inoculation. For that cell cultred plates were washed with PBS and virus inoculum was added spread throughout the plate by swirling movement left at $37^0$C for 30min. Then maintenance medium was added and kept in incubator for experimental investigations.

### Spectrochemical investigation

For spectrochemical assay, the medium was removed and replaced with 100µL of fresh culture medium. Then 10µL of 12mM MTT stock solution was added to each well and allowed for incubation at $37^0$C for 4 hours. Afterwards 100µL of the SDS-HCl solution was added to each well and mix thoroughly using the pipette to stop reaction. Then medium was removed gently and DMSO was added as a solubilizing agent to dissolve the insoluble formazan product. Finally the solution was gently pipetted and absorbance (OD values) was determined spectroscopically at 570 nm. The percent infectivity of the cell was determined by analyzing and quantifying the OD values obtained using the spectrophotometer.

## RESULTS

### Titration of virus ($TCID_{50}$)

For the titration of PPR virus $TCID_{50}$ was determination using vero cell seeded 96-well plates at a density of $0.4\times10^{-6}$ cells/well and maintained in cell culture incubator. The cell cultured wells were infected with six different dilutions ranging from $10^{-1}$ to $10^{-6}$ of a virus sample and observed twice daily for 7 days. Four wells of the cellcultured plates were infected with each dilution. All the infected wells of dilutions $10^{-1}$, $10^{-2}$, and $10^{-3}$ showed CPE (100%) and three wells of dilution $10^{-4}$ (75%) and one well of dilution $10^{-5}$ (25%) showed CPE whereas none of the well was infected with dilution $10^{-6}$ showed CPE (0%) as illustrated in the Figure 2.

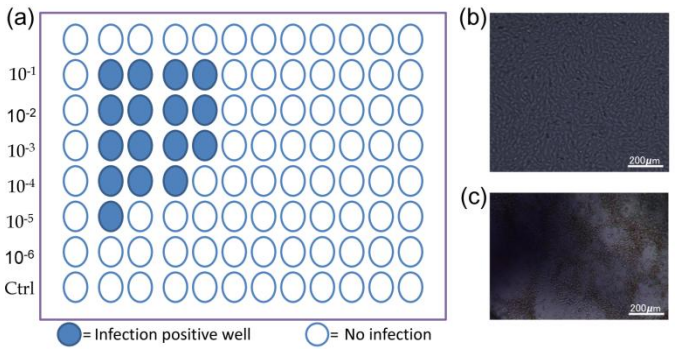


**Figure 2.** (a) Illustrations of the results of results of TCID50, (b) Optical microscopic image (5x) of non-infected vero cells and (c) PPR virus infected vero cells.

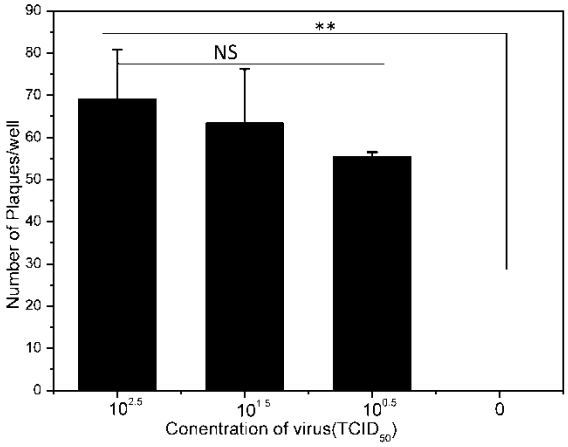


**Figure 3.** TCID50 of the inoculum obtained from PPR virus infected vero cell culture fluid.

### Plaque assay for virus infectivity analysis

For plaque assay, three 96-well plates were seeded with $0.4\times10^{-6}$ cells/well and maintained in the cell culture incubator. The cell seeded wells were infected with four different concentrations of a PPR virus sample and observed twice daily for 7 days. For each dilution, three wells of tissue culture plates with 80-90% confluence were used with control non-infected wells of similar confluence. The original virus concentration $10^{3.5}$ $TCID_{50}$ showed numerous plaques which was difficult to count them individually because one plaque coalesced with another forming a large plaque. Hence, the figure 3 showed the number of plaques obtained from concentrations ranging from $10^{2.5}$to 0 $TCID_{50}$. The plaque assay showed significant variation (**p= 0.000145) between infected and noninfected cells. However for various doses of virus infections the differences in infectivity was non-significant (NS) indicating that the traditional plaque assays are not sensitive for virus infectivity analysis.

### MTT based spectrochemical analysis of virus infectivity

For spectrochemical analysis of virus infectivity, three 96-well plates seeded with $0.4\times10^{-6}$ cells/well were maintained in cell culture incubator. The cell seeded wells were infected with four different concentrations of a PPR virus and observed twice daily for 7 days. For each dilution, three wells of tissue culture plates with 80-90% confluence were used keeping non infected control groups. At day-7 of post-infection, MTT reagents were treated at a dose of 10µl/well and allowed for 4 hours incubation for mitochondrial reductase enzyme activity. Mitochondrial reductase enzyme reduces MTT reagent to a non-soluble formazan product. After 4 hours media was pipetted out and DMSO was added in each well and allowed for 15 minutes to hydrolyze the insoluble formazan product. The Figure 4a showed formazan hydrolyzed product where a gradual increase in color intensity with the increasing concentrations of virus suspensions and the highest color intensity in the control non infected groups which was clearly visible even with the naked eyes. Whereas in the Figure 4b, the cell culture medium was taken out prior to the DMSO treatment which showed no changes in color intensity irrespective of virus concentrations but the wells of control group showed variations in color intensities due to trace amount of formazan that came out during the media removal because of the huge amount of formazan accumulation in the control groups. Optical Density (OD) value obtained from the hydrolyzed formazan product of the representative concentrations of virus infected groups and non-infected control groups with the corresponding blank were used for determining the percent viability and infectivity using the equation-1 and equation-2 (shown in methodology section), respectively. Figure 5 represents percent infectivity and viability with respect to the corresponding doses of virus infections ranging from $10^{3.5}$ to 0 $TCID_{50}$. The spectrochemical assays showed highly significant (**p=0.00317) differences in infectivity was observed between the PPR virus infected and non-infected cells. The infectivity decreases significantly (*p= 0.049) with the decreasing concentrations of viruses. However, the viability increases with decreased concentrations of viruses which is obvious phenomenon in cell virus interactions.

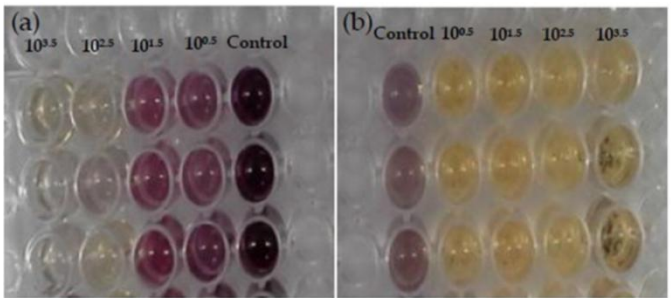


**Figure 4.** (a) The image represents hydrolyzed formazan products with DMSO showing color intensity with decreasing concentrations (TCID50) of virus suspension, (b) represents MTT treated cell culture fluid taken out prior to the solubilization of formazan

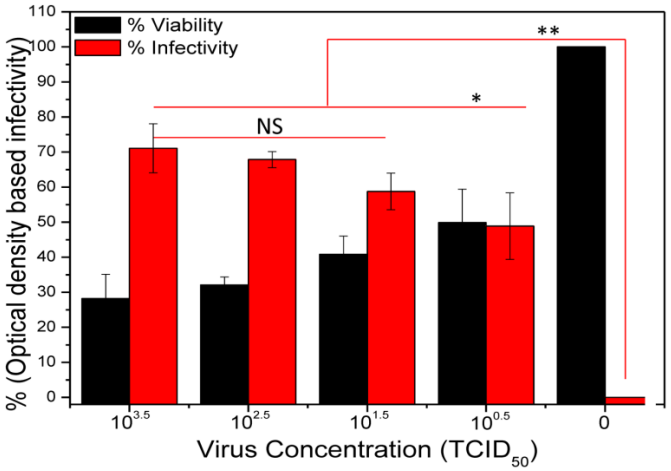


**Figure 5.** Comparison between percent viability and percent infectivity obtained from Optical Density (OD) based spectrochemical analysis where X-axis represents virus concentrations (TCID50) and Y-axis represent percent viability/percent infectivity.

### Trypan blue exclusion assay for cell viability

For the confirmation of spectrochemical analysis of virus infectivity, similar sets of cells were seeded and infectivity/viability was determined using trypan blue based traditional cell counting method. Percent viability

of the infected tissue culture plate with various concentrations of virus maintaining non infected control groups are presented in the figure 6. The trypan blue exclusion assay showed similar trend of enhancement of infectivity with the treated virus infections where the infected and non-infected cell showed significant differences (*p=0.013). However, infectivity levels among the virus concentrations do not show any significance differences indicating the less sensitivity of this traditional assay. The corresponding percent viability also showed similar observations with respective concentrations of virus infections.

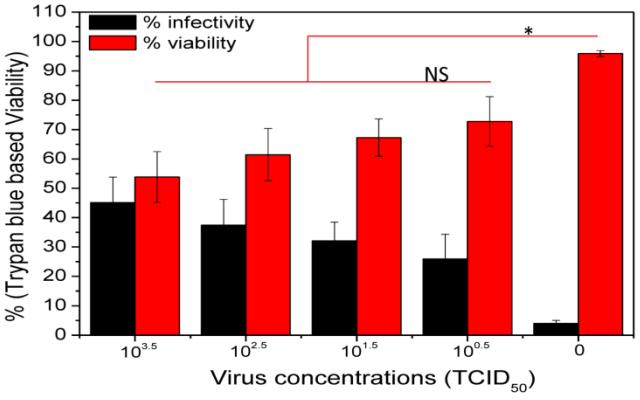


**Figure 6.** Comparison between percent viability and percent infectivity obtained from Trypan blue exclusion assay where X-axis represents virus concentrations (TCID50) and Y-axis represent percent viability/percent infectivity.

### Validation of spectrochemical assay with conventional trypan blue assay

For the validation of optical density based spectrochemical assay with conventional trypan blue based optical cell counting method, two sets of experiments were carried out with similar concentration of cells and treated with the similar concentration of virus suspensions and maintained in an identical conditions.

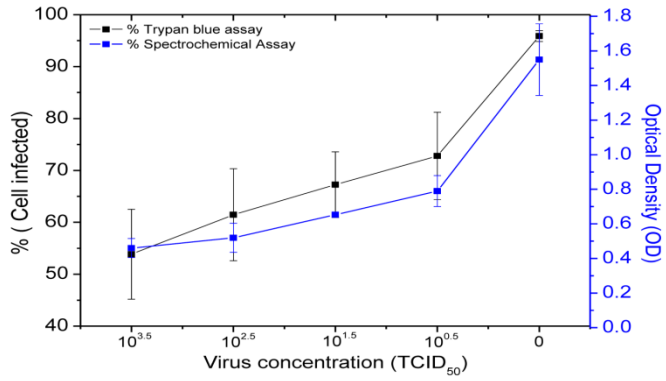


**Figure 7.** Validation of spectrochemical assay with conventional trypan blue assay.

Data obtained from both the experiments were presented in the Figure 7 where two linearly fitted trend lines were shown to validate the newly developed method. The trend lines obtained from both methods showed similar trend with little variations in sensitivity. The sensitivity variations are obvious due to the marked differences in the measurement unit and detection methods.

## DISCUSSION

In the present study, it was focused a spectrochemical based viral infectivity assay which was based on the mitochondrial reductase enzyme activity of live non infected healthy cells. MTT reagent was used as the substrate of reductase enzyme. The dissolved MTT reagent when employed to a live cell, the mitochondrial reductase enzyme reduced the MTT forming insoluble formazan products as the precipitated [16]. Afterwards the media with unused MTT reagent was decanted out leaving the insoluble formazan products at the bottom of the tissue culture plate. The purple to darker color (Figure 4a) was achieved upon hydrolysis of the insoluble formazan with DMSO solution [7;14]. The intensity of the color varies from purple to dark due to the number of viable cells. However the MTT treated cell culture medium do not show such color intensity variations with exception to the control wells (Figure 4b). The control well might have some formazan products because of their huge accumulation in the control wells. As the concentration of viable cells increased the color was darker because of the activity of mitochondrial reductase enzyme was increased. Therefore, the color intensity directly correlates with the viability of cells.

The measurement of optical density (OD) value has long been used as a sensitive method to quantify the density of a suspension [15]. Keeping this in mind, herein this experiment, OD values were measured at a wavelength of 570nm (As per manufacturer's instruction) which reflects the concentration of viable cells in a tissue culture plate. The optical density measurement was performed conveniently using a spectrophotometer where all the wells of a 96-well plate were measured at a time within few seconds. Thereby the number of samples can be measured within short time which most important for analyzing and quantifying the viral infectivity of a population sample. Whereas the traditional methods employ microscopic quantification of Plaques or Trypan blue based exclusion assay which is time consuming, laborious and difficult to analyze accurately [18]. Therefore, the newly developed method can be suitable for monitoring viral infectivity of a population to suggest appropriate measures to combat with the outbreak and save the population.

The developed spectrochemical method was compared with the traditional plaque assay [19]. In the plaque assay, quantification of highly concentrated virus suspension ($10^{3.5}$ $TCID_{50}$) was not possible because the numerous plaques formed and coalesced with other forming a large empty area (Figure 3). However, the diluted suspensions were quantified by counting plaques whereas a lot of plaques count might be missed or duplicated due to the optical limitations, where the developed spectrochemical method does not possess such limitations because the quantification of optical density performed using a spectrophotometer which is accurate and sensitive at a definite wavelength (570 nm).

The newly developed method was further verified and validated with the traditional trypan blue based cell counting method where the trend line developed from both methods were fitted completely with a little variations in their sensitivity. The variation in sensitivity is obvious due to the marked differences in their measurement unit and detection methods. However, the developed method showed complete fitting with the optical based cell counting method proving the accuracy of the newly developed spectrochemical detection method. Considering the suitability of label free detection tools, the newly developed method is convenient, less time consuming and effective for monitoring of PPR virus infection from large number of samples.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The author declares that no conflict of interest exists.

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