RESEARCH ARTICLE

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The Development of an Alkaline Phosphatase-Based Virus Spot (ALPSpot) Detection Assay: Determination of Infectious *Rotavirus* Titer

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Bovine rotavirus (BRV) is major cause of neonatal diarrhea in calves and cause substantial economic loss in dairy and livestock production. The tissue culture infectious dose ($TCID_{50}$) is a cytopathic effect (CPE)-based microscopic examination assay designed to measure virus titers almost a century ago. It is difficult to determine the virus titers when replicating viruses do not induce CPEs in cell cultures and more accurate and reliable methods need to be developed.

Fecal samples were obtained from newborn calves and a RT-PCR assay was performed to detect BRV. A cell culture isolation method was performed to isolate BRV strains in fecal suspensions and confirmed by ELISA. All BRV strains were employed to develop and validate ALPSpot assay. Spots become visible after the addition of a chromogenic substrate that catalyzes alkaline phosphatase-conjugated antibodies.

A total of 17 fecal samples tested positive for BRV in one-step RT-PCR. Only 6 BRV isolates were successfully rescued through cell culture virus isolation studies and tested positive in ELISA (Cut-off: 0.247). Assay sensitivity was measured with a higher correlation rate (R^2=0.9833) when compared to the $TCID_{50}$ titer method and there are no significant differences between $TCID_{50}$ and ALPSpot methods in terms of virus titer. In addition, ALPSpot also determines the lowest virus titer when compared to $TCID_{50}$. In this study, an alkaline phosphatase spot (ALPSpot) detection assay has been optimized to determine BRV titers.

Key Words: *Alkaline phosphatase, ALPSpot, assay development, rotavirus, virus titration*

Alkalen Fosfataz-Bazli Virüs Odağı (ALPspot) Saptama Testi Geliştirilmesi: Enfeksiyoz Rotavirüs Titresinin Belirlenmesi

Bovine rotavirus (BRV), buzağılarda neonatal ishalin başlıca nedenidir ve süt ve hayvancılık üretiminde önemli ekonomik kayıplara neden olur. Doku kültürü bulaşıcı dozu ($TCID_{50}$), neredeyse bir asır önce virüs titrelerini ölçmek için tasarlanmış sitopatik etki (CPE) tabanlı bir mikroskobik inceleme testidir. Virüsleri çoğaltırken hücre kültürlerinde CPE'leri indüklemediğinde virüs titrelerini belirlemek zordur ve daha doğru ve güvenilir yöntemlerin geliştirilmesi gerekir.

Yeni doğan buzağılardan dışkı örnekleri toplandı ve BRV'yi saptamak için bir RT-PCR testi yapıldı. BRV suşlarını fekal süspansiyonlardan izole etmek için hücre kültür virüs izolasyon yöntemi uygulandı ve ELISA ile doğrulandı. Alkalin fosfataz temelli virüs odak (ALPSpot) testini geliştirmek ve doğrulamak için izole edilen BRV suşları kullanıldı. Alkalin fosfatazla konjuge antikorları katalize eden kromojenik bir substratın eklenmesinden sonra virüs odakları görünür hale getirildi.

Tek adımlı RT-PCR testi ile toplam 17 dışkı örneği *Rotavirüs* için pozitif test edildi. Hücre kültürü virüs izolasyon çalışmaları yoluyla 6 BRV izolatı başarıyla izole edildi ve ELISA'da pozitif test edildi (Cut-off: 0.247). Test duyarlılığı, $TCID_{50}$ titre yöntemine kıyasla daha yüksek bir korelasyon oranı (R^2=0.9833) ile ölçülmüştür ve virüs titresi açısından $TCID_{50}$ ve ALPSpot yöntemi arasında istatistiki anlamlı bir fark bulunmadı. Ayrıca ALPSpot, $TCID_{50}$ ile karşılaştırıldığında en düşük virüs titresini dahi tespit edebildi. ALPSpot testi, duyarlı hücre kültürlerinde BRV titrelerini ölçmek için indüklenmiş CPE gerektirmeyen güvenilir ve doğru bir yöntemdir ve küçük modifikasyonlarla koruma çalışmalarında aşı ile indüklenen veya pasif olarak aktarılan nötralize edici antikorları belirlemek için kullanılabilir. Bu çalışmada BRV titresi tespiti için ALPSpot saptama testi geliştirilerek optimize edilmesi tarif edildi.

Anahtar Kelimeler: *Alkalin fosfataz, ALPSpot, test geliştirme, rotavirüs, virüs titrasyonu*

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Introduction

BRV is a diarrhea-causing agent among calves, commonly seen in newly delivered neonates aged up to four weeks old. BRV often causes infection in the gastrointestinal (GI) tract after entering the body via an alimentary route (1). Accumulating evidence shows that BRV is one of the leading causative agents in newborn calf diarrhea (2, 3). Rotaviruses are also responsible for pediatric diarrhea and cause symptomatic GI infections in children. The zoonotic potential of circulating BRV also raises concerns with regard to public health (4). It is estimated that more than half a million children (≤5 years old) lose their lives due to diarrheal diseases worldwide annually. BRV belongs to the *Reoviridae* family and contains 11 identically stranded dual RNAs (dsRNA) (5). BRV genomes are encapsulated underneath the triple-protein capsid layer along with viral attachment proteins and could be accessible in the outer space after the dissociation of

surface protein capsids by enzymatically (6). The virus gains the ability to attach and enter host cells after so-called activation.

Virus identification and characterization studies can be conducted in infected fecal materials by performing reverse transcriptase and quantitative PCR analysis after the extraction of genomic dsRNAs. However, the study of antiviral agents or developing effective therapies against viruses need to be evaluated in an in vitro setting, and such observations require the measurement of the virus titers in susceptible cells in vitro. Virus isolation is the first obstacle that needs to be overcome in further characterization studies. The isolation of BRV with permissive cell culture, such as MA-104, is described for use in in vitro studies (7).

A limited number of virus measurement assays have been defined to measure the infectious *rotavirus* titers in fecal samples (8-12). The $TCID_{50}$ method was previously developed, and the calculation of virus titers was formulated by Spearman–Karber (13, 14). However, such CPE observational virus measurements can cause subjectively biased results in many cases, and results can be variable. Moreover, undefined viral GI agents other than rotaviruses can also show CPEs during initial virus isolation studies in fecal samples. Focus forming assays were applied to measure infectious virus titers for *rotavirus* in a specific antibody-mediated manner, but the quantification of the foci requires monochromatic fluorescence-emitting approaches (10, 15). Due to the highlighted concerns, the development of the most accurate and reliable virus titer assays for rotaviruses remains incompletely described.

In this study, an ALPSpot detection assay has been developed to quantify BRV on permissive cell cultures. Alkaline phosphatase-based enzymatic immunoassay has been widely used for detection of antigens in cell or tissue-based assays due to its high sensitivity and strong stability features (16, 17). The ALPSpot staining method was optimized and compared with the $TCID_{50}$-based virus titration assay with higher accuracy using cell culture-adapted BRV strains. The reproducibility of the assay was also confirmed with other cell culture-derived BRV obtained from the same study.

Materials and Methods

Screening of newborn diarrheal clinical samples for the ALPSpot assay validation: For the validation of the ALPSpot assay, fecal specimens were obtained from newborn calves presenting with diarrhea at the emergency unit of the animal hospital, Erciyes University, Turkey. To perform total viral RNA isolation from newborn diarrheal samples, fecal specimens were diluted (1:10 v:v) with PBS and centrifuge clarified at 10.000 × g for 15 min. Clarified supernatants were applied to the acid guanidinium thiocyanate–phenol–chloroform extraction method as previously described (18). *Rotavirus* genomes that consisted of 11 dsRNAs were visualized after running them in ethidium bromide-stained agarose gel to observe intact dsRNA integrity under a constant voltage of 100 V for less than 20 min. Molecular identification was directed to amplify the partial region of the VP6 gene of *rotavirus* with the following primer pairs: VP6-F sense (5′ GACGGVGCRACTACATGGT 3′) and anti-sense 157-R (5′ GTTTTCCAAGAGTDATHAHYTCAGC 3′) with modifications by Iturriza G. et al. (19). Primer pairs can amplify 405 bp of the *rotavirus* VP6 gene region. Reaction conditions were applied to the one-step amplification procedure as follows: 10 min incubation at 50°C; pre-denaturation at 95°C for 5 min; a thermal cycle loop run 35 times, including the denaturation of cDNAs at 95°C for 15 sec; an annealing step for primers at 55°C for 30 sec, and 30 sec at 72°C. Finally, the extension step was carried out at 72°C for a further 7 min. The reaction was cooled down to 4°C. RT-PCR amplified products were visualized using EtBr-treated medium-melt agarose gel (1%) electrophoresis with a constant voltage of 100 V for 15 min.

Cells and viruses for assay optimization: To optimize the ALPSpot assay, nine different BRV isolates were tested during assay development as positive controls concurrently with the $TCID_{50}$ method. For the assay positive controls, virus stocks (n=3) obtained from the Erciyes University Faculty of Veterinary Medicine, Department of Virology cell and virus collection stocks and included to optimization studies. The virus titers were measured previously which are ranged from 10^4 to 10^7 $TCID_{50}$/mL (3). Virus stocks were thawed on ice and then activated enzymatically with trypsin (10 µg/mL; SIGMA, St Louis, MO) in a 37°C water bath for 30 min. Virus suspensions were enzymatically activated and used for virus propagation on MA-104 (ATCC) cell cultures as described previously (20). Cells were maintained with 10% FBS (SIGMA, St Louis, MO) and a 1X antibiotic mix (SIGMA, St Louis, MO) containing growth media. Cells were infected when cell density reached sub-confluence in 6-well tissue culture plates (Costar, Cambridge). For the virus propagation experiments, MA-104 cells were incubated with an enzyme-conditioned media (1 µg/mL trypsin containing M199 media w/o FBS) for 3 h before the infection. Virus suspensions were diluted 10 times (1:10 v:v) in enzyme-conditioned media, and cells were infected by the virus adsorption cell-binding method at 37°C in a cell culture chamber (5% CO_2 supplied and humidified) for 90 min. The virus infection was stopped by washing the cell surface with PBS, and flasks were kept with *rotavirus* growth media (0.5 µg/ml trypsin and 1% FBS containing M199 media). Plates were left at 37°C in a cell culture chamber until cells showed robust CPEs or became nearly detachable from the bottom of the plate, between 3 and 5 days post-inoculation (dpi). Cells with virus growth media were removed entirely from the plates by

manually pipetting, and cell–virus content was stored at -80°C until use. Virus suspensions were repeatedly freeze–thawed three times. Cellular contaminants were separated from virus suspensions by centrifugation at 10.000 × g for 30 min. Virus-containing upper-clear phases were pooled and mixed, then equally dispensed in smaller volumes (100 µl/tube) to maintain an equal amount of virus titer for each assay and the repeats. Harvested BRV-containing supernatants were dispensed in several aliquots and kept at -80°C until further use. The working virus aliquots and virus-containing inoculums were discarded properly according to the waste management for biohazard safety guidelines of the Erciyes University Veterinary Medicine biosafety standard operation procedures.

Tissue Culture Infectious Dose-$TCID_{50}$ assay: BRV working stock titer was calculated after performing a Spearman–Karber $TCID_{50}$/mL assay in MA-104 cells (21). This method is based on a microscopic CPE observation and is well-established to determine infectious viruses in a given volume. MA-104 cells were transferred to a 96-well plate for 16 h before infection with $30X10^3$ cells in ml^{-1} density. Cells were washed twice with M199 media before the virus inoculation and treated with an enzyme-conditioned medium for 3 h before the infection. To determine virus $TCID_{50}$, working virus stocks were diluted ten times logarithmically in a dilution plate starting from 10^{-1} to 10^{-8} in quadruple rows. Cells were incubated in inoculums (0.05 mL) as described, and inoculums were then replaced with *rotavirus* growth media. Cells were left in the cell culture chamber and observed on the third dpi under an inverted microscope. Undiluted virus working stocks and PBS-mock diluted inoculums were used as assay controls.

ALPSpot detection assay: MA-104 cells were transferred into 24-well tissue culture plates with $30X10^4$ cells ml^{-1} density and incubated for 16 h in a cell culture chamber. Cells were washed twice with M199 media and treated with conditioned media for 3 h before the virus inoculation. Enzymatically activated (10 µg/mL of trypsin) and log10 serially diluted (10^{-1} to 10^{-6}) working virus stocks were kept in wells in a triplicate manner with 0.2 mL volume. PBS-mock diluted and undiluted inoculums were included to plate for assay controls. The plate was kept at 37°C for 90 min and followed up by hand-shaking with 30 min intervals. Inoculums were replaced with overlay media (2% carboxymethyl cellulose growth media was equally mixed with 2X M199 and supplemented with the 1X antibiotic mix; 1% of FBS; 0.5 µg/ml trypsin). Agar overlay was maintained in the wells for the next three days in the cell culture chamber. The cells were formalin-fixed (10%) at RT for 30 min without removing overlay media. Then, wells were washed with PBS. Fixed cells were permeabilized with 1% of Nonidet-P 40 in PBS (perm-wash solution) for 15 min at RT. Cells were incubated with 5% skimmed milk containing Tris-buffered (100 mM) PBS (pH 8.0) at RT for 30 min for the blocking. The blocking solution was washed away with a perm-wash solution to clear the milk residues. To detect surface-expressed viral antigens, a polyclonal mouse serum (obtained from Erciyes University Faculty of Veterinary Medicine, Department of Virology), which was raised against the whole inactivated and alum adjuvant virus (1:25600 IgG titer), was used. Sera were diluted (1/2500; v:v) in perm-wash solution and incubated with cells at RT for 45 min by slow platform agitation. Wells were washed three times and incubated with ALP-conjugated anti-mouse IgG (SIGMA, St Louis, MO) antibody (1/2000; v:v) in perm-wash for 60 min at RT by slow platform agitation. Wells were carefully washed twice with a perm-wash solution and subsequently reacted with alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$ containing alkali-pH 9.5 PBS). The chromogenic substrate NBT/BCIP (Thermo, USA) was added to wells and immediately kept in a dark place for color development. The incubation was stopped when the desired color development was observed under the inverted microscope, which varied between 15 min and 1 h. The following formula was used to enumerate virus titers:

$$\text{ALPSpot forming unit (ASFU)/mL} = \frac{\text{avarage spots}}{\text{virous inocula (mL) X dilution factor}}$$

Home-made sandwich ELISA for detection of BRV: Polyclonal rabbit anti-BRV antibody (obtained from Erciyes University Faculty of Veterinary Medicine, Department of Virology) was diluted (1 µg/ml) in a carbonate buffer (pH 9.6) and mobilized in detachable 96-well plates (Thermo, USA) overnight. Wells were washed twice with PBST-20 buffer, then subsequently blocked with skimmed milk (5%) for 1 h at 37°C. The plate was extensively washed with PBST-20 (0.01% Tween-20 in PBS) five times. Test samples were collected from infected cell supernatants at the fifth dpi and diluted in blocking buffer (1:10; v:v) before being added to wells consecutively. The plate was incubated at 37°C for 1 h. The plate washed as described previously, and HRP-conjugated monoclonal VP6 antibody (1D6 VP6 mAb; obtained from Erciyes University Faculty of Veterinary Medicine, Department of Virology) was used for the detector antibody (1:3000). The plate was incubated as before and subsequently washed with PBS-T buffer. The HRP substrate was prepared with the dissolving of a TMB tablet (1 mg; SIGMA, St Louis, MO) in DMSO followed by dilution in PBS up to 10 mL as described in the instructions. Peroxidase (30%, H_2O_2) was added (0.1%) to substrate buffer before use and the plate was incubated in a dark place for 10 min at RT for chromogenic color development. The reaction was stopped by adding 2 M H_2SO_4 to the wells. The plate was read with a spectrophotometer set to 450 nm (Allsheng, China) to obtain the optical densities. The cut-off value of the ELISA was calculated by three times the multiplication of negative control absorbance values.

Validation of ALPSpot assay*:* To validate the ALPSpot assay sensitivity, RT-PCR was confirmed, and cell culture-isolated virus stocks were tested in an ALPSpot titration assay along with $TCID_{50}$ virus titration. ALPSpot titers were obtained and statistically compared to the $TCID_{50}$ titer results to determine the ALPSpot

assay sensitivity. For this purpose, fecal samples were briefly diluted in PBS (1:10 v:v) and clarified in a centrifuge at 10,000 × g for 30 min. The upper-clear phase was filter-sterilized (0.22 µm) and equally mixed with M199 cell media supplemented with 2X antibiotics. Virus cultivation was carried out as described above in MA-104 permissive cells with at least three blind passages (P0 to P3). Cell culture supernatants were harvested from each passage and tested using an ELISA to confirm virus propagation and isolation. The collected supernatants were molecularly confirmed with RT-PCR as described in this study. Cell culture-isolated BRV stocks were kept at -80°C for ALPSpot validation experiments, and virus titers were obtained both from $TCID_{50}$ and ALPSpot assays.

Statistical analysis: Linear regression analysis was used to determine the correlation efficiency of the newly developed ALPSpot titer range of viral stocks by comparing it with the $TCID_{50}$ titer for the accuracy of the assay. The correlation matrix of the linear regression analysis and graphical outputs were obtained by GraphPad software (GraphPad PRISM 7.0; La Jolla, CA). To visualize the ELISA results, OD values were obtained using a spectrophotometer set at 450 nm and assigned in grouped table format columns using GraphPad PRISM. To compare ALPSpot assay with $TCID_{50}$ method, a non-parametric Wilcoxon two-sample paired rank test was performed for not normally distributed titer data (22) using GraphPad software (n=7). The significance level was considered as $p<0.05$ for all analyzes. To obtain $TCID_{50}$ titers, a tabulated Excel $TCID_{50}$ calculator based on the Spearman–Kärber method was used as defined and validated previously (23).

Results

Virus isolation studies: Among the obtained newborn calves' fecal samples, a total of 17 specimens tested positive for BRV in one-step RT-PCR (Figure 1). The virus isolation studies were completed in susceptible MA-104 cell culture via cultivation using three blind passages after the first inoculation of the cells (P0 to P3). Cell supernatant taken from each passage (P0 to P3) was tested using an ELISA for the success of BRV isolations (Figure 2). Six samples from the virus isolation studies were recruited successfully and tested positive in an ELISA with various OD values. BRV isolates at passage number 3 (P3) were tested both in the ALPSpot and $TCID_{50}$ for assay validation studies. A clinical sample was also included in the blind passage and assay validation studies as a mock-negative control for the assay validation (Figure 3). There is a strong correlation between two assays, and there were no significant differences except for isolate number 6; however, stock number 6 tested positive in ELISA but did not induce CPEs in cells when $TCID_{50}$ assay was performed. In addition, isolate number 6 was also tested positive in ALPSpot assay which is similar to ELISA finding. This results evidenced that ALPSpot could detect ELISA true-positive samples better higher than traditional $TCID_{50}$ technique.

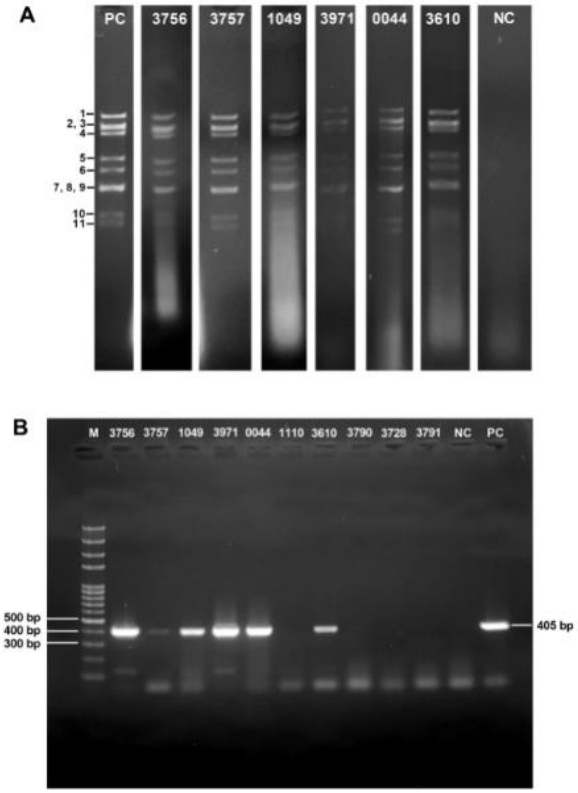

Figure 1. Viral screening of isolated RNA samples in agarose gel electrophoresis. BRV consisted of 11 dsRNAs, and genomic segmented patterns visualized in EtBr dye treated agarose gel (A) (dsRNA segment numbers lined from 1 to 11). Images were taken from various gels and then assembled into one image by using ImageJ software (NIH, Bethesda, US). (B) One-step RT-PCR performed to amplify of rotaviral VP6 gene products after obtaining total RNAs from faecal materials (M, DNA ladder; numbers between 3756 and 3610 in A and 3756 to 3791 in B indicate IDs of clinical samples; PC, positive control and NC negative control; 405 bp is showing the amplified gene product of VP6 loci of rotavirus).

Optimization of ALPSpot assay for BRV: To optimize the ALPSpot assay, cell culture-isolated BRV stocks (n=6) were used. Three more positive samples (virus stock collection of Department of Virology, Erciyes University) were also included during the validation step. Alkaline phosphatase enzyme-conjugated antibodies reacts with a substrate that catalyzes the NBT. Reaction resulted in production of NBT formazans, and so, cell spots could be visible under the microscope or by the naked eye (Figure 4). BRV stock titers were calculated after the enumeration of ALPSpot formation units (ASFU) in the given inoculum.

To optimize virus titers obtained from the ALPSpot, linear regression analysis, was performed by comparing ALPSpot titers with $TCID_{50}$. A linear regression correlation was calculated (R^2= 0.9833) between log titer for ALPSpot and log titer for $TCID_{50}$ for nine different cell culture-adapted virus stocks. ALPSpot titer results were

matched statistically in correlation to $TCID_{50}$/mL to determine the co-efficiency of the ALPSpot assay (Figure 5). The regression analysis results revealed that the ALPSpot assay had a determination efficiency relative to the $TCID_{50}$ method.

Titer results taken from ALPSpot assay were compared with those titers found using the $TCID_{50}$ method by performing Wilcoxon two-sample paired rank test since our data not log-normally distributed. In comparison to the virus titers between ALPSpot and $TCID_{50}$ infectious virus titers from ALPSpot were found to be the same as those from $TCID_{50}$. Tissue culture virus titers acquired from the ALPspot titer method and the TCID50 methods for clinical isolates are shared in Table 1.

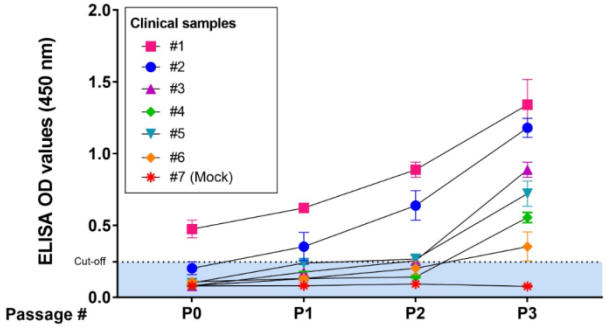

Figure 2. Blind passage isolation studies for recruitment of BRV from clinic samples. BRV VP6-positive tested fecal samples were diluted in PBS and filter sterilized (0.22µm). Suspensions were trypsin-treated and inoculated to MA-104 cells. Inoculated cells, along with supernatants, were harvested on the third dpi and freeze–thawed (P0). P0 samples were blindly inoculated to MA-104 cells and repeated as before until P3 stocks were obtained. Cell supernatants from P0 to P3 were tested using an ELISA, and OD values were obtained from 450 nm (Cut-off: 0.247).

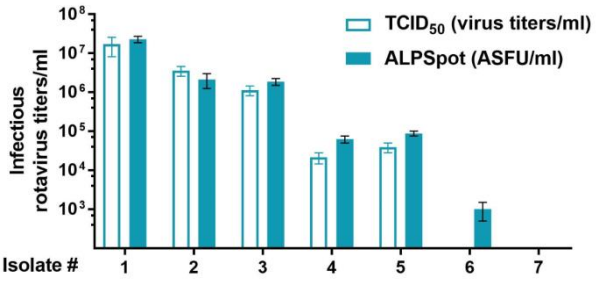

Figure 3. Validation of ALPSpot assay with clinical isolates. Blind passage no 3 (P3) from MA-104 cell supernatants was tested both in the $TCID_{50}$ and ALPSpot assay. Six isolates used were positive (isolate no 1 to 6) controls and one (isolate no 7) was included in the study as a control/mock sample. BRV titers were obtained from each respective assay and underwent statistical comparison analysis using Graphpad Prism software.

The "p" values are reported for each isolate and results showed that there are no significant differences between two methods being compared (P>0.05). However, only isolate number 6 showed positive spots in the ALPSpot assay, and the virus titers were measured as $1x10^3$ ALPSpot virus titer units (ASFUs). The study results showed that ALPSpot assay could be use in place of $TCID_{50}$ and ALPSpot assay also has a superior advantage over the traditional assay to measure as lowest virus titer.

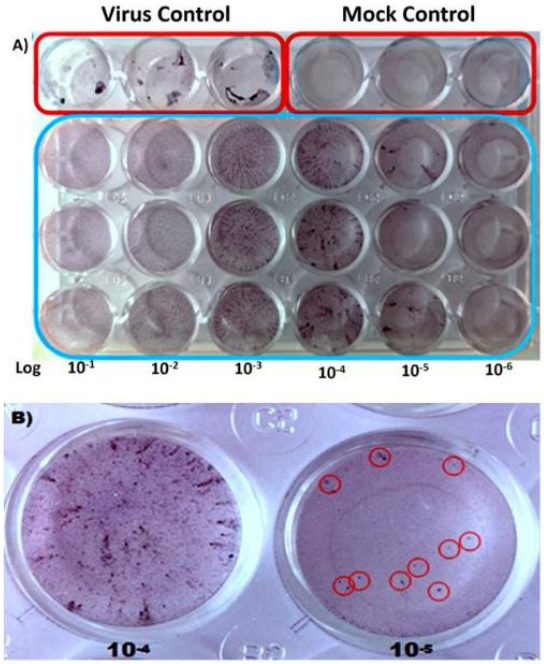

Figure 4. Establishment of ALPSpot assay in Log10 virus dilutions. MA-104 cells were infected with serially diluted BRV stock and incubated for 3 days. Cells were fixed and subsequently mobilized with BRV-specific IgGs to detect virus spots and reacted with alkaline phosphatase-conjugated IgGs. Virus-infected cell spots were stained with a chromogenic substrate (BCIP/NBT) by the enzymatic activity of alkaline phosphatase. Stained spots became visible to the naked eye in 24-well plates (A), and spots could be enumerated (B) for the determination of BRV titers (ASFU/ml). Red circles in Log 10^{-5} points ALPSpots were enumerated in this assay.

Table 1. Comparison of clinically isolated BRV stock titers (average titers taken from three replicates) in the $TCID_{50}$ and ALPSpot assays

Isolate #	$TCID_{50}$ virus titer ($TCID_{50}$/mL)	ALPSpot virus titer (ASFU/mL)	P
1	$1.7x10^7$	$2.3x10^7$	P>0.05
2	$3.6 x10^6$	$2.1x10^6$	P>0.05
3	$1.1 x10^6$	$1.9 x10^6$	P>0.05
4	$2.1 x10^4$	$6.2 x10^4$	P>0.05
5	$3.9 x10^4$	$8.7 x10^4$	P>0.05
6	0	$1x10^3$	P>0.05
7*	0	0	ND

* Negative control; ND: Not determined

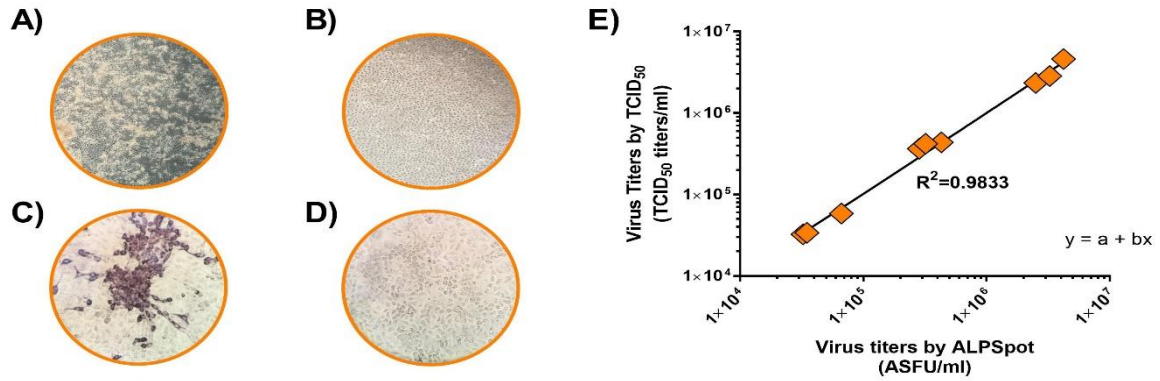

Figure 5. Correlation efficiency of the newly developed ALPSpot assay. Nine different cell culture-adapted virus stocks were employed for the determination of ALPSpot's accuracy during the establishment of the assay. $TCID_{50}$ assay results were taken at 5 dpi regarding the development of CPE (A) compared to control/mock-infected cells (B). The ALPSpot technique was evaluated after BRV-infected MA-104 cell spot (C) staining and compared to control/mock-infected and substrate-added cells (D) at 3 dpi. Linear regression analysis [(ALPSpot (x) and log titer for TCID50 (y); slope b= 1.001 ± 0.0493; a= 1333 ± 96989 y-intercept] showing the coefficient determination of ALPSpot is closely correlated (R^2=0.9833) to $TCID_{50}$ titers (E).

Table 2. An overview of the features and advantages/disadvantages of various methods for determining of BRV titers

BRV titration method	The assay	Advantages	Disadvantages	References
Cytopathic effect	$TCID_{50}$	No specialized skills and equipment needed	CPE-based evaluation could be biased and subjective.	Madin et al. (12)
Plaque formation unit	Plaque forming unit	Quantitate the number of infectious viral particles and gives unbiased results	Some rotavirus strains could not have the ability to produce plaque formation or enough large size to count.	Butchaiah and Lund (11)
Fluorescein focus unit	Fluorescent focus unit	It does not rely on plaque formation or CPE production and has high sensitivity.	Specialized skills and fluorescein microscopy are needed. The assay can cause an autofloroscein which could result in false positivity.	Butchaiah (10)
Cellular toxicity	Automated tetrazolium-based calorimetric assay	Not subjective and has higher sensitivity	The Assay depends on cellular metabolic toxicity which is not only specific for BRV. Microplate readers and spectrophotometers are needed.	Distefano et al. (24)
Amplification of genomic RNA	qRT-PCR	No cell-based experiments are needed. Results are unbiased and based on genomic material amplification.	The assay relies on genomic material amplification, and results are not relate with quantification infectious virus titer.	Schwarz et al. (25)
Immunoassay	ELISA	Results are unbiased and based on produced viral proteins.	Rely on produced viral proteins, and results are not relate with quantification infectious virus titer. The assay need a microplate readers and spectrophotometers.	Scherrer et al. (26)
	Elispot	High accuracy to quantitate infectious virus titer.	The assay need a special immunospot analyzer.	Li et al. (8)
	ALPSpot	The assay has higher accuracy and relies on BRV specific detection to quantitate infectious virus titer with high sensitivity.	Manual counting is needed and results could be varied.	This study

Discussion

Rotaviruses have been defined with multiple strains and genotypes both in human and animal hosts (27). Among the virus strains, even if they belong to the same genus, some viruses exhibit various plaque sizes from smaller to larger that can be visible to the naked eye (11). However, in some studies, some strains did not show plaque formation; however, cells eventually died because of induced apoptotic lysis. Rotaviruses show inducible cytopathology with cellular lysis and cause the rapid detachment of infected cells from the bottom of the plate, but some isolates do not induce visible CPEs (7, 20). The median $TCID_{50}$ is readily used for *rotavirus* titer assays when cells experience CPE formation (12, 28). However, results may vary between different laboratories and even between people. To eliminate biased and subjective titer results, more objective and repeatable assays need to be developed. Among the sensitive assays, fluorescein focus unit (FFU) tests were developed, and the results were observed to be more consistent than those of other assays (10, 15, 20, 29). Such a highly sensitive assay requires fluorescein-conjugated antibodies to focus on infected cells, along with the highly sensitive microscopic camera that emits fluorescein light in a dark place and must be ready during the examination of results. The mentioned specifications could be efficient for virus characterization experiments, but can be challenging for the routine analysis of *rotavirus* and virus isolation studies. Other immune-probing and enzyme catalyzing techniques, such as ELISA and enzyme-linked immunospot (ELISPOT) assay were also established to measure *rotavirus* titers (8, 24, 26, 30). Immune-probing techniques, including FFU, provide comparable advantages over CPE-based techniques such as the early detection of infectious viruses in susceptible clinical samples with higher virus-targeting sensitivities but but still need a special fluorescence equipped microscope for detection (10). Non cell-based rotavirus titration assays were also described including qRT-PCR for quantitative determination (25). In spite of the fact that, ELISA and qRT-PCR systems might have superior advantage over classical assays for virus detection but results does not relate with infectious virus titer. In terms of infectious virus titer determination we still need a cell-based methods for accurate virus quantification. A brief comparison of BRV titer quantification methods was given in Table 2.

In this study, an ALPSpot assay has been developed for determining BRV titers along with early virus detection without the consideration of CPEs or the formation of plaques. ALPSpots have divergent and distinguishable units of a dark blue/purple color that appear against the white background visible under a microscope or spots are visible in various sizes and colors, and can even be visible to the naked eye. The use of an alkaline phosphatase-based immune-probing virus quantification assay with automated focus counting was also described for the Hepatitis C virus. This colorimetric focus-forming assay was found to be the superior method among the other assays, including the fluorescence-conjugation technique (31). A similar immune-probing and enzyme catalyzing assay called the pseudo-plaque assay was developed for the determination of non-CPE viruses such as the Crimean–Congo hemorrhagic fever virus, along with the measurement of neutralization titer in susceptible cells with a higher detection limit, and this assay has been employed with higher measurement capacities than the FFU assay (32, 33). In this study, ALPSpot assay has been developed to measure the BRV titers of newborn calves, but it may also be considered in the measurement of titers for human rotaviruses. It can be suggest that the ALPSpot assay could be a suitable and repeatable method to measure vaccine-induced neutralizing antibodies and titers both in humans and animals.

During the development of virus detection or titration assays, several optimization and validation steps should be followed carefully, such as optimizing the assay with control virus stocks and validation with clinical samples (34). Overlay medium is required to restrict virus spots to limited areas in order to accurately determine infectious virus titers. In addition, spot size and the extent of spots are dependent on the growth kinetics and incubation time of the virus. An increase in incubation time after virus inoculation of up to 5 dpi would result in more extensive spots. Thus, incubation time should be optimized in laboratory settings using overlay agar.

In the current study, ALPSpot assay has been optimized and developed with the highest correlation regression unit (R^2=0.9833) when compared to the $TCID_{50}$ assay. It has also been examined and performed with various clinical samples that show that the ALPSpot assay does not require additional detection systems such as a highly sensitive microscope or even an inverted microscope to measure virus titers. The ALPSpot assay could be applied to measure and detect other non-CPE viral agents with minor modifications. However, ALPSpot assay needed a manual counting which is not required automatic counter or analyzer. In the manner of this fact, ALPSpot assay results might differ and limited according to whether spots are directly being observed. In this study, a suitable, BRV specific and reliable virus titration and detection assay that can be employed to sufficiently measure virus titers has been defined. It is suggested that this newly developed assay could be used for the early detection of viruses in a serotype-specific and characterization manner, and may also be suitable for antiviral, drug, or vaccine development studies.

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