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Research Article

Physicochemical Properties of Bovine Viral Dirrhoea Virus of an Indian Isolate

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ABSTRACT

Study of physicochemical properties of BVDV revealed that virus can be inactivated at 56°C for 30 min and by the treatment of 1% NaOH, 5% chloroform and 0.05% trypsin for an hour exposure. Ammonium chloride at 10mM concentration completely inhibited the penetration of virus in Sheep Fetal Thymus Riems (SFTR) cell line when treated for 15 min. Methyl blue dye up to 10µM had no effect on BVDV in concentration, however, crystal violet inactivated the virus completely at the same concentration, both in presence and absence of organic matter (milk powder). Acidic pH (≤3) and disulphide bond reducing agent also inactivated BVDV after 1 h exposure. Ethanol was effective at concentrations of 70, 80, and 95 percent in inactivating the virus after 15 sec of exposure at 20°C, whereas propanol inactivated the virus in same time at 30 and 45 percent concentrations. During the past few years, many healthcare workers have changed from antimicrobial liquid soaps to alcohol–based hand rubs for post–contamination treatment of hands as suggested by the CDC guideline for hand hygiene. As BVDV serves as a surrogate model for Hepatitis C virus (HCV), the present study can contribute to evaluate and devise effective methods of controlling the latter.

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INTRODUCTION

Bovine Viral Diarrhoea (BVD) is an acute, highly contagious viral disease of ruminants characterized by transient fever, diarrhoea, immunosuppression, fertility problems, thrombocytopenia, mucosal lesions, abortion, neonatal death, congenital malformations, persistent infection of the offspring, podo–dermatitis and recently reported glomerulonephritis (Baker, 1987; Thiel *et al.*, 1996; Goens, 2002; Galav *et al.*, 2007).

The causative agent for BVD, Bovine Viral Diarrhoea Virus (BVDV), belongs to the genus Pestivirus in the family *Flaviviridae* (Heinz *et al.*, 2000). There are two genotypes of BVDV, BVDV type–1 and BVDV type–2 (Pellerin *et al.*, 1994; Ridpath *at al.*, 1994; Harpin *et al.*, 1995; Van Regenmortel, 2000). In cell culture, pestiviruses appear as two biotypes, known as–cytopathic (cp) and non–cytopathic (ncp) (Kummerer *et al.*, 2000). The ncp biotypes are predominant in nature and are often responsible for majority of the disease syndromes (Nettleton and Entrican, 1995).

Very few studies have been carried out to determine the physico–chemical properties and no studies have been carried out with any Indian isolate. The physicochemical properties and replication characteristics are very important to provide suitable interventions for control of the infection. However, the knowledge regarding the effect of various alcohol–based preparations and disinfectants on BVDV is very limited. Therefore, this study was designed to determine the practical utilities of different decontaminating reagents and methods during laboratory handling of BVDV.

MATERIALS AND METHODS

Place of Work

The study was conducted at the High Security Animal Disease Laboratory (HSADL), IVRI, Bhopal, and Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Mhow, Madhya Pradesh, India.

Virus Isolate

The BVDV isolate (Ind S–17555), initially isolated from a sheep and available at HSADL, IVRI, Bhopal, was used in this study to investigate the physio–chemical properties of BVDV.

Cell line

Sheep Fetal Thymus Riems (SFTR) cell line obtained from Riems, Germany and available at HSADL, IVRI, Bhopal, was used in this study. The cells were grown in EMEM medium containing 10% horse serum and were free of any adventitious *pestivirus* contamination.

Immunoconjugates

Anti–mouse IgG–peroxidase conjugate, raised in rabbits (Sigma, cat.no.A–9044) and anti–bovine IgG (whole

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molecule) peroxidase conjugate, raised in rabbits (Sigma, cat. No.A–7414) were used in the imunoperoxidase monolayer assay (IPMA).

Effect of Temperature

In this study 1×10^6 $TCID_{50}$ dilution of BVDV was used for determining the effect of temperature on its viability. One ml of virus suspension in EMEM was treated at 56°C for 30, 45, and 60 min, respectively in circulating water bath. Then preformed monolayer of SFTR cells in 6 well tissue culture plate was infected with 300 µl of the treated inoculums. The plate was incubated at 37°C for 1h for adsorption of virus. The inoculum was removed, monolayer was rinsed slowly with 500µl EMEM, and 2 ml EMEM supplemented with 2% horse serum (infection medium) was added to the wells. The plate was incubated at 37°C in a CO_2 incubator for three to four days.

Immunoperoxidase Momnolayer Assay (IPMA)

After completion of incubation period of three to four days, the plate was tested by IPMA for detection of live/survived virus as per the method described by Wood *et al.* (2004) with certain modifications. The medium was removed and plate was rinsed with 1X PBS. The cells were then heat fixed by keeping the plate at 80°C for 1h in hot air oven and then allowed to cool to room temperature for 20 min. Cells were rehydrated by adding 300 µl of PBS (1X). Then 50 µl of 1:120 diluted WB112 Mab (specific for NS3 region) was added to each well and incubated at 4°C. After overnight incubation the wells were washed thrice (each of 5 min duration) with 1x PBST (0.05% Tween–20). Then 50µl of HRPO conjugated anti–mouse antibodies (Sigma), diluted to 1:300, was added to each well and incubated for 1 h at 37°C. Again four washings were given with 1X PBST (0.05% Tween–20). Stock chromogen mixture was prepared by dissolving 100mg of AEC (3 amino–9–ethylcarbazole) in 15 ml DMF (N'N' dimethyl formamide). From this stock, AEC working chromogen substrate was prepared by adding 300 µl of stock chromogen in 5 ml of acetate buffer (pH 4) and 5 µl of hydrogen peroxide (H_2O_2). 50 µl of this working chromogen substrate was dispensed in each well and plate was incubated for 20 min at room temperature in dark. Rinsing the plate under tap running water stopped the reaction. Development of red color in the cell cytoplasm was considered positive for infectivity / presence of BVD virus, while no color in the cell cytoplasm was considered negative for infectivity or / absence of BVD virus as shown in Figure 1.

Effect of Dyes

The effect of 1, 5 and 10µM concentrations of methyl blue and crystal violet dyes on BVDV was studied in the presence and absence of organic matter (milk powder). The BVDV was diluted (1×10^4 $TCID_{50}$ for methyl blue and 1×10^6 $TCID_{50}$ for crystal violet) in EMEM. Different concentrations (1, 5 and 10µM) of dye were prepared in 1.5 ml eppendorf tubes by adding 1, 5, 10µl of each of dye (from stock 1mM solution) in corresponding tube and final volume was made up to 1000µl by adding the diluted virus. The material was then kept under artificial light (100 lux) at room temperature for 1h. The preformed monolayer of SFTR cells (3×10^5 cells/ml) in 6 well tissue culture plate was infected with 300 µl of the treated inoculums and remaining procedure was same as described earlier.

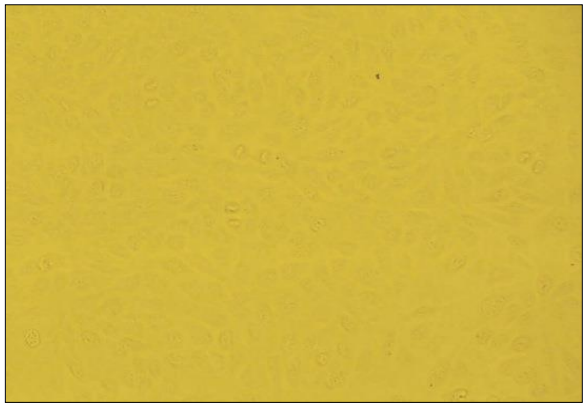
For studying the effect of dyes on BVDV in presence of milk powder, the virus was diluted in EMEM supplemented with 1% milk powder and all other procedures followed were same as described above.

Effect of Alcohols

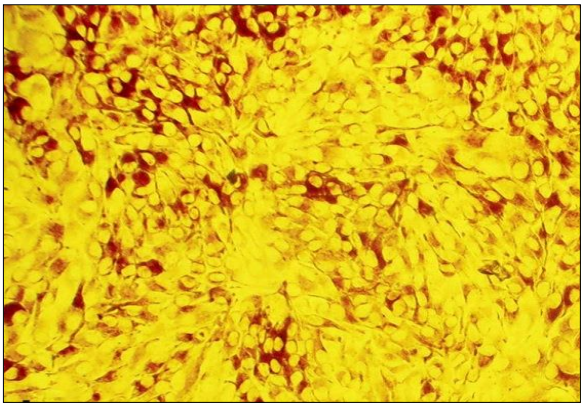
For studying the effect of alcohols treatment was given as 8 parts alcohol +1 part virus (1×10^4 $TCID_{50}$ of BVDV) +1 part medium.

Effect of Ethanol

To study the effect of 70 % ethanol, 80 µl of 87.5% ethanol, 10 µl diluted virus (1×10^4 $TCID_{50}$), and 10µl EMEM were dispensed in triplicate and mixed properly in an eppendorf tube, while for 80% ethanol 80 µl of 100% ethanol was mixed with identical volumes of diluted virus and EMEM. For 95% ethanol, 90 µl of 100% ethanol and 10 µl diluted virus (1×10^4 $TCID_{50}$) was dispensed in triplicate and mixed properly. Tubes were incubated at 20°C in a circulating water bath for 15, 30 and 60 sec, respectively and then ice–cold 900 µl EMEM was added in each tube. Subsequently preformed monolayer of SFTR cells in six well cell culture plates was infected using 300 µl of inoculum and remaining procedure was same as described earlier.



No BVDV virus present



BVDV virus detected / present

Figure 1: Detection of BVDV by immunoperoxidase monolayer assay (IPMA); Note: Presence of BVDV is indicated by precipitation of red color in cytoplasm

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Effect of Different pH

One ml of diluted virus (1×10^6 $TCID_{50}$) in EMEM was taken in each 2 ml eppendorf tubes and then pH of the medium was adjusted to 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, and 7 with glacial acetic acid. The material was then kept for 1 h at room temperature and then pH of the inocula was adjusted to 7 by using 0.1M NaOH. Preformed monolayer of SFTR cells in six well plates was infected with 300 µl of treated inoculums and then remaining procedure was followed same as that described earlier.

Effect of DTT at Different pH

DTT is a disulphide bond reducing agent. 450 µl of diluted virus (1×10^6 $TCID_{50}$) in EMEM and 50 µl DTT (stock 100mM) were dispensed in seven 2ml eppendorf tubes to make final concentration of 10mM DTT in each tube. The pH of the medium was adjusted to 4, 4.5, 5, 5.5, 6, 6.5, and 7 with HCl and kept for 1 h at room temperature. After completion of incubation period the pH of the inocula was adjusted to 7 by using 0.1M NaOH. Preformed monolayer of SFTR cells in six well plates was infected with 300 µl of treated inoculums and then remaining procedure was followed same as described earlier.

Effect of Ammonium Chloride

Different concentrations of ammonium chloride – 1, 2, 3, 4, 5, 6.5, 7.5, 8.5 and 10mM were prepared in 2 ml eppendorf tubes by adding 40, 80, 120, 160, 200µl, 260, 300, 340, and 400µl of ammonium chloride solution, respectively from 50mM stock and final volume was made up to 2000 µl by adding EMEM. The preformed monolayer of SFTR cells in six well tissue culture plates was inoculated with 500µl respective concentration of ammonium chloride and kept at 37°C for 15 min. The media was removed from wells, cells was infected with 300µl of diluted virus (1×10^4 $TCID_{50}$) and incubated at 37°C for 1 h for adsorption of virus. Then 1.5 ml EMEM containing respective amount of ammonium chloride concentration with 2% horse serum was added in the respective wells. The plate was incubated at 37°C in 5% CO_2 incubator for 3 days and rest procedure was followed same as described previously. The virus titration of the cell lysate was carried out by IPMA as per procedure of Wood *et al.* (2004).

Effect of Sodium Hydroxide

Different concentrations of sodium hydroxide – 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 percent were prepared by adding 6.25, 12.5, 25, 37.5, 50, 62.5, 75, 87.5 and 100µl of NaOH (from stock 10M NaOH), respectively and final volume was made up to 1000 µl by adding the diluted virus (1×10^6 $TCID_{50}$). The pH obtained was 8, 9, and 12 for 0.25, 0.5 and 1.0 percent NaOH, respectively, and above 14 for other concentrations. The treated inoculums were kept at room temperature for 1 h and then pH of the inocula was adjusted to 7 by using 0.1 M HCl. Monolayer of SFTR cell in 6 well tissue culture plate was infected with 300 µl of inoculums from the treated material and remaining procedure followed was same as described earlier.

Effect of Trypsin

Different concentrations of trypsin such as, 0.025, 0.05, 0.10, 0.20, 0.25 and 0.3 percent were made by adding 5, 10, 20, 40, 50, and 60µl trypsin (from 5% trypsin stock) respectively, and final volume was made up to 1000 µl by adding diluted BVDV (1×10^4 $TCID_{50}$). The inuculum was incubated at 37°C for 1 h in a circulating water bath and then horse serum was added to neutralize trypsin. Preformed monolayer of SFTR cells in six well cell culture plate was infected using 300 µl of inoculums from the treated material and remaining procedure followed was same as described earlier.

Effect of Chloroform

In 1.5 ml eppendorf tube different concentrations of chloroform such as 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 percent were made by adding 2.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60µl (from stock of 100% chloroform solution), respectively and final volume was made up to 1000 µl by adding diluted virus (1×10^6 $TCID_{50}$) in the respective tubes. Preformed monolayer of SFTR cells in six well cell culture plates was infected using 300 µl of inoculum from the treated material and remaining procedure followed was same as described earlier.

RESULTS AND DISCUSSION

Effect of Temperature

The results of the test conducted to assess the effect of temperature (56°C) on virus survivability indicated that BVDV could be inactivated at 56°C for 30 min (Table 1). This has practical implications in that bovine or ovine serum used for cell cultivation can be made free from adventitious BVDV contaminations with such treatment.

Table 1: Effect of temperature on survival of BVDV

Sr. No	Temperature	TCID	Time (min)	Virus inactivation
1	56^0C	1×10^6 $TCID_{50}$	30	yes
			45	yes
			60	yes

Effect of Dyes in Absence and Presence of Milk Powder

After treating the BVDV virus suspension with methyl blue/ crystal violet dye for 1h under artificial light (100lux) at room temperature, the results showed that the methyl blue dye had no effect on inactivating the virus at 1–10 µM concentration either in absence or presence of milk powder (organic matter) as shown in (Table 2). Whereas crystal violet dye has no effect on virus survivability up to the concentration 5 µM either in absence or presence of 1% milk powder. However, crystal violet completely inactivated the virus at and above 10µM concentration both in presence and absence of milk powder as shown in (Table 2). Kevin *et al.* (2004) studied the effect of methyl blue and methyl violet phenothiazine dyes and reported that both the dyes inactivated the BVDV completely in goat colostrum at 10 and 20µM concentration following 60 min illumination under artificial light. The results correlated well for crystal violet, but not for methyl blue, which may be due to different compositions of the dyes or different virus strains used in the study.

Effect of Alcohols

Ethanol at concentration of 70% and above and propanol at concentration of 30% and above inactivated BVDV completely when exposed for at least 15 sec at 20°C (Table 3).In a study, Gunter *et al.* (2007) observed that alcohol based hand rubs (ethanol and propanol) reduced the infection of BVD virus by ›4log_{10} steps within 15sec irrespective of the organic load.

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Table 2: Effect of dyes on survival of BVDVs

Sr. No	Dye	Concentration of dye (µM)	TCID	Time(min)	Virus inactivation
1	Methyl blue in absence of milk powder	1	1×10^4 $TCID_{50}$	60	No
		5			No
		10			No
2	Methyl blue in presence of milk powder	1	1×10^4 $TCID_{50}$	60	No
		5			No
		10			No
3	Crystal violet in absence of milk powder	1	1×10^6 $TCID_{50}$	60	No
		5			No
		10			Yes
4	Crystal violet in presence of milk powder	1	1×10^6 $TCID_{50}$	60	No
		5			No
		10			Yes

Table 3: Effect of alcohol on survival of BVDV

Sr. No	Alcohol	Concentration (%)	TCID	Time(sec)	Virus inactivation
1	Ethanol	70	1×10^4 $TCID_{50}$	15	Yes
				30	Yes
				60	Yes
		80	1×10^4 $TCID_{50}$	15	Yes
				30	Yes
				60	Yes
		95	1×10^4 $TCID_{50}$	15	Yes
				30	Yes
				60	Yes
2	Propanol	30	1×10^4 $TCID_{50}$	15	Yes
				30	Yes
				60	Yes
		45	1×10^4 $TCID_{50}$	15	Yes
				30	Yes
				60	Yes

Table 4: Effect of acidic pH on survival of BVDV

Sr. No	pH	TCID	Time (min)	Virus inactivation
1	3	1×10^6 $TCID_{50}$	60	Yes
	3.5	1×10^6 $TCID_{50}$	60	No
	4	1×10^6 $TCID_{50}$	60	No
	4.5	1×10^6 $TCID_{50}$	60	No
	5.5	1×10^6 $TCID_{50}$	60	No
	6	1×10^6 $TCID_{50}$	60	No
	6.5	1×10^6 $TCID_{50}$	60	No
	7	1×10^6 $TCID_{50}$	60	No

Table 5: Effect of DTT (10mM) at different pH on survival of BVDV

Sr. No	pH	TCID	Time(min)	Virus inactivation
1	4	1×10^6 $TCID_{50}$	60	Yes
	4.5	1×10^6 $TCID_{50}$	60	Yes
	5	1×10^6 $TCID_{50}$	60	No
	5.5	1×10^6 $TCID_{50}$	60	No
	6	1×10^6 $TCID_{50}$	60	No
	6.5	1×10^6 $TCID_{50}$	60	No
	7	1×10^6 $TCID_{50}$	60	No

Our results corroborated these results and also showed that 70% ethanol and 30% propanol can be assured to be active against clinically relevant enveloped viruses. In the earlier study (Gunter *et al*., 2007), 75% or more concentration of ethanol has been used while our study included 70% ethanol, which is common hand disinfectant used in many laboratories. We could not evaluate exposure time less than 15 sec but it has only limited clinical relevance.

Effect of Different pH and DTT at Different pH

Treatment of the virus suspension at acidic pH (pH–3 to pH–7) for 1h at room temperature showed that the BVD virus was completely inactivated at acidic pH–3 and below, while it was viable at and above pH–3.5 (Table 4). Whereas the inhibitory effect of DTT (10mM) at various pH levels showed that BVDV was completely inactivated at pH 4.5 and below only (Table 5).

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Sr. No	Concentration (mM)	TCID	Time (min)	Virus inactivation	Titre of cell lysate
1	0 (control)	1×10^4 $TCID_{50}$	15	No	10^7
	1	1×10^4 $TCID_{50}$	15	No	10^7
	2	1×10^4 $TCID_{50}$	15	No	10^7
	3	1×10^4 $TCID_{50}$	15	No	10^7
	4	1×10^4 $TCID_{50}$	15	No	10^6
	5	1×10^4 $TCID_{50}$	15	No	10^6
	6.5	1×10^4 $TCID_{50}$	15	No	10^5
	7.5	1×10^4 $TCID_{50}$	15	No	10^4
	8.5	1×10^4 $TCID_{50}$	15	No	10^2
	10	1×10^4 $TCID_{50}$	15	Yes	0
	15	1×10^4 $TCID_{50}$	15	Yes	0

Table 6: Effect of ammonium chloride on survival of BVDV

Table 7: Effect of ammonium chloride on penetration of BVDV in SFTR cells.

Sr. No	Concentration (mM)	TCID	Time (min)	Penetration of BVDV in SFTR cells
1	1	1×10^4 $TCID_{50}$	15	Yes
	2	1×10^4 $TCID_{50}$	15	Yes
	3	1×10^4 $TCID_{50}$	15	Yes
	4	1×10^4 $TCID_{50}$	15	Yes
	5	1×10^4 $TCID_{50}$	15	Yes
	6.5	1×10^4 $TCID_{50}$	15	Yes
	7.5	1×10^4 $TCID_{50}$	15	Yes
	8.5	1×10^4 $TCID_{50}$	15	Yes
	10	1×10^4 $TCID_{50}$	15	No
	15	1×10^4 $TCID_{50}$	15	No

Sr. No	Concentration (%)	TCID	Time(min)	Virus inactivation
1	0.25	1×10^6 $TCID_{50}$	60	No
	0.5	1×10^6 $TCID_{50}$	60	No
	1.0	1×10^6 $TCID_{50}$	60	Yes
	1.5	1×10^6 $TCID_{50}$	60	Yes
	2.0	1×10^6 $TCID_{50}$	60	Yes
	2.5	1×10^6 $TCID_{50}$	60	Yes
	3.0	1×10^6 $TCID_{50}$	60	Yes
	3.5	1×10^6 $TCID_{50}$	60	Yes
	4.0	1×10^6 $TCID_{50}$	60	Yes

Table 8: Effect of sodium hydroxide on survival of BVDV

Table 9: Effect of trypsin on survival of BVD virus

Sr. No	Concentration (%)	TCID	Time(min)	Virus inactivation
1	0.025	1×10^4 $TCID_{50}$	60	No
	0.05	1×10^4 $TCID_{50}$	60	Yes
	0.10	1×10^4 $TCID_{50}$	60	Yes
	0.20	1×10^4 $TCID_{50}$	60	Yes
	0.25	1×10^4 $TCID_{50}$	60	Yes
	0.30	1×10^4 $TCID_{50}$	60	Yes

Sr. No	Concentration (%)	TCID	Time(min)	Virus inactivation
1	0.25	1×10^6 $TCID_{50}$	60	No
	0.5	1×10^6 $TCID_{50}$	60	No
	1.0	1×10^6 $TCID_{50}$	60	No
	1.5	1×10^6 $TCID_{50}$	60	No
	2.0	1×10^6 $TCID_{50}$	60	No
	2.5	1×10^6 $TCID_{50}$	60	No
	3.0	1×10^6 $TCID_{50}$	60	No
	3.5	1×10^6 $TCID_{50}$	60	No
	4.0	1×10^6 $TCID_{50}$	60	No
	4.5	1×10^6 $TCID_{50}$	60	No
	5.0	1×10^6 $TCID_{50}$	60	Yes
	5.5	1×10^6 $TCID_{50}$	60	Yes
	6.0	1×10^6 $TCID_{50}$	60	Yes

Table 10: Effect of chloroform on survival of BVDV

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This showed that inactivation of BVDV can take place in presence of acid pH and disulphide bond reducing agent DTT. The survivability at pH 3.5 and above supports earlier evidence of remarkable acid resistance of pestiviruses such as BVDV and CSFV (Krey *et al*., 2005) unlike alphaviruses and flaviviruses of the family *Flaviviridae.* This has more practical consequences as BVDV can remain live in meat and meat products of P.I. animals at fairly acidic environment and can be transmitted to susceptible animals. A unique feature of BVDV is the presence of covalently linked homodimers (E^{rns}–E^{rns}, E2–E2) and heterodimers (E^{rns}–E2 and E1–E2), which may contribute to virus resistance to acids (Thiel *et al*., 1991; Krey *et al*., 2005).Inactivation of BVDV was only observed when treated with reducing agent DTT at low pH. The inactivation might be due to the reduction of disulfide bonds in the glycoproteins by DTT. The mechanism by which it occurs, however, remains to be elucidated in future studies.

Effect of Ammonium Chloride on Survival and Penetration of BVDV in SFTR cells

As shown in Table 6, it was found that BVD virus was completely inactivated at 10 and 15mM concentration of ammonium chloride, whereas the virus titre was reduced significantly at concentrations of 7.5 and 8.5mM, and there was no effect up to concentration of 3mM.

The effect of chemical inhibitors of endocytosis, such as ammonium chloride on internalization of BVDV has been studied earlier (Krey *et al*., 2005), which suggest that BVDV invades the host cell via receptor mediated clathrin dependent endocytosis. Ammonium chloride is lysosomotropic weak base, which reversibly neutralize acidification in the endosome and thereby can block completely BVDV infection of susceptible cells. Ammonium chloride at 5mM concentration decreased, upto 6 times, viral penetration into treated MDBK cells. Our study also showed almost similar results, wherein 10 times decrease in viral penetrations was noticed at 5mM ammonium chloride concentration. Whereas BVD virus penetration into treated SFTR cells was completely inhibited at ammonium chloride concentration of 10mM and above (Table7), which is an important advancement of knowledge regarding adsorption and penetration of BVDV.

Effect of Sodium Hydroxide

As shown in Table 8, after treating the virus suspension at different concentration of NaOH for 1h and after testing resulting cell lysate by IPMA, it was found that NaOH was ineffective at concentration of 0.25 and 0.50 percent (pH–8 & pH–9), while it completely inactivated the virus at a concentration of 1% and above (pH 12) exposed for 1h at room temperature. Also reports of says that NaOH at 0.1M concentrations was shown to inactivate the BVD virus to undetectable level at 60°C in 15s (Borovec et al., 1998).As NaOH solution is widely used for decontaminating biological and laboratory waste, 1% solution can be used for inactivating enveloped viruses such as BVDV, Hepatitis–C and Dengue virus.

Effect of Trypsin and Chloroform

Trypsin inactivated the virus to undetectable level at concentration of 0.05% and above when exposed for 1h at 37°C (Table 9), while, no inactivation was observed with 0.025% trypsin. However, chloroform completely inactivated the BVD virus at concentration of 5% and above after 1h exposure at room temperature (Table 10). This suggests that trypsin and chloroform could be effective virucidal agents against pestiviruses.

CONCLUSIONS

Study of physicochemical properties of BVDV revealed that pestiviruses are inactivated by various physical and chemical agents such as, temperature at 56°C for 30 min, NaOH 1% for 1h, trypsin 0.05% for 1h, chloroform 5% for 1h; Ethanol 70% within 15 sec; Propanol 30% within 15 sec, Crystal violet 10µM for 60 min, ammonium chloride for 15 min exposure, respectively and pH 3 and below after 1h exposure. Propanol (30%) can be used as an effective substitute of ethanol (70%) as a hand disinfectant in laboratory, when latter is not available due to legal restrictions.

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