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Short communication

Virus inactivation by nucleic acid extraction reagents

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Abstract

Many assume that common methods to extract viral nucleic acids are able to render a sample non-infectious. It may be that inactivation of infectious virus is incomplete during viral nucleic acid extraction methods. Accordingly, two common viral nucleic acid extraction techniques were evaluated for the ability to inactivate high viral titer specimens. In particular, the potential for TRIzol® LS Reagent (Invitrogen Corp., Carlsbad, CA) and AVL Buffer (Qiagen, Valencia, CA) were examined to render suspensions of alphaviruses, flaviviruses, filoviruses and a bunyavirus non-infectious to tissue culture assay. The dilution series for both extraction reagents consistently caused cell death through a 100-fold dilution. Except for the DEN subtype 4 positive control, all viruses had titers of at least 10^6 pfu/ml. No plaques were detected in any extraction reagent plus virus combination in this study, therefore, the extraction reagents appeared to inactivate completely each of the high-titer viruses used in this study. These results support the reliance upon either TRIzol® LS Reagent or AVL Buffer to render clinical or environmental samples non-infectious, which has implications for the handling and processing of samples under austere field conditions and low level containment.
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1. Introduction

Detection and identification of viruses have relied historically upon techniques such as isolation in cell culture or animals and serologic assays. Depending on the pathogenicity of the virus, such methods might require biological containment. Often, nucleic acid detection methods such as the polymerase chain reaction (PCR) need not be carried out under biological containment due to the low infectivity of the naked nucleic acid. Thereby, the extensive use of real-time PCR technology has enhanced the capability of laboratories to collect, process, and test samples under low containment and in remote sites.

Many assume that common methods to extract viral nucleic acids are able to render a sample non-infectious (Boom et al., 1990; Ross et al., 2001). The products used most commonly, TRIzol® LS Reagent (Invitrogen Corp., Carlsbad, CA) and AVL Buffer (Qiagen, Valencia, CA) extraction reagents have no information on the safety testing of the reagent to inactivate virus. Both reagents contain a chaotropic salt (guanidine isothiocyanate), which acts to denature macromolecules (von Hippel and Wong, 1964). Due to the caustic nature of the extraction reagent, virus inactivation is assumed. It may be that inactivation of infectious virus is incomplete during viral nucleic acid extraction methods. The reliance upon these techniques in non-containment environments may present a safety problem due to the potential for exposure during sample processing.

Accordingly, two common viral nucleic acid extraction techniques were evaluated for the ability to inactivate high-viral titer specimens. In particular, we examined the potential for TRIzol® LS Reagent and AVL Buffer to render suspensions of alphaviruses (western equine encephalomyelitis (WEE), eastern equine encephalomyelitis (EEE), Venezuelan equine encephalomyelitis (VEE)), flaviviruses (West Nile (WNV), dengue (DEN)), a bunyavirus (Rift Valley fever (RVF)) and filoviruses (Ebola, Marburg) noninfectious to cell cultures.

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2. Materials and methods

Two nucleic acid extraction buffers available commercially were used to inactivate the viral stocks used in this study. TRIzol® LS reagent (Invitrogen Corp.) is a mono-phasic solution of phenol and guanidine isothiocyanate. AVL Buffer from the Qiamp viral RNA mini kit (Qiagen) contains a chaotropic salt. High-titer virus-infected cell cultures of alphaviruses, flaviviruses, a bunyavirus; and filoviruses and filovirus tissue samples were used in these experiments (Table 1).

For each virus, three tubes were prepared. The first tube contained 560 μl of diluent (positive control). For alphaviruses, flaviviruses, and the bunyavirus, this diluent consisted of 10% heat-inactivated fetal bovine serum (FBS) in Medium 199 with Earles' salts, $NaHCO_3$, and 50 U/ml of penicillin,100 μg/ml of streptomycin, 250 μg/ml of garamycin, and 5 μg/ml of fungizone. For filoviruses, this diluent consisted of 10% heat-inactivated FBS in Earles Minimum Essential Medium (EMEM) with 400 μg/ml of penicillin and streptomycin. The second tube contained 560 μl of TRIzol® LS Reagent. The third tube contained 560 μl of AVL Buffer. One hundred and forty microliters of stock virus was added to each of the three tubes. One tube each of TRIzol® LS and AVL Buffer without virus were prepared as negative controls for each group of viruses tested. One hundred and forty microliters of the respective diluent was added to each negative control tube. All tubes were then mixed by vortex and tubes containing the extraction reagents were incubated 10 min at room temperature. The positive control tube was kept on ice until inoculation onto the cells.

Plaque assays for alphaviruses, flaviviruses, and a bunyavirus were carried out on Vero or MK2 (DEN only) cells in 6-well plates. The plaque assays for filoviruses were performed on Vero E-6 cells in 6-well plates. All virus manipulations and assays were performed at appropriate bio-safety levels (BSL-3 and BSL-4, respectively).

A 10-fold serial dilution series, using the appropriate diluent, was prepared for each virus-extraction reagent/diluent combination. One hundred microliters of each dilution was inoculated onto cell culture monolayers in duplicate. Inoculated plates were rotated to distribute the virus and were incubated at 37 °C for 1 h. After the incubation period, a medium + agar overlay was added. Alphavirus, flavivirus, and bunyavirus assays were overlaid with 2 ml of 2× EBME with Hepes, 7% FBS, 70 U/ml of penicillin and 140 μg/ml of streptomycin, 100 U/ml of mycostatin mixed 1:2 with a 1.5% agar solution. Filovirus assays were overlaid with 2 ml of 2X EBME with 10% FBS, 400 μg/ml of penicillin and streptomycin mixed 1:2 with a 1% agar suspension. Plates were incubated at 37 °C until the addition of the second medium. The second overlay was the same as the first but contained 4% neutral red for the filoviruses and 2% neutral red for all other viruses. Cells were stained on day 6 for Ebola, Marburg, RVF, and DEN viruses and plaques read on day 7. Cells were stained on day 3 for VEE, WEE, EEE, and WN viruses and read on day 4.

Table 1
Viral strains used in this study and titers in pfu/ml

Genus	Virus		
	Species	Strain	Titer
Alphavirus	Venezuelan equine encephalomyelitis	68U201	7.3
	Eastern equine encephalomyelitis	FL91-4679	7.0
	Western equine encephalomyelitis	PE1.0433	7.3
Bunyavirus	Rift Valley fever	AH-501	6.0
Filovirus	Marburg	Musoke	7.4
	Marburg	Ci67	7.5
	Marburg	Ravn	7.8
	Marburg	Monkey 18-35	9.0
	Marburg	Monkey 91-483	6.8
	Ebola	Zaire 95	6.0
Flavivirus	Dengue (subtype 1)	Hawaii	7.1
	Dengue (subtype 2)	S16803	8.0
	Dengue (subtype 3)	CH53489	6.3
	Dengue (subtype 4)	H241	4.9
	West Nile	Crow 397-99	7.0

The titer of each virus was determined by calculating the pfu/ml from the virus + diluent assay.

3. Results and discussion

In order to determine the background toxicity of the extraction reagents in cell culture, the dilution at which the extraction reagent negative control samples stopped causing massive cell death was determined. A well was considered to have experienced massive cell death if there was not a detectable live-cell sheet within the well. The TRIzol® LS Reagent dilution and AVL Buffer dilution series caused consistent cell death through a 100-fold dilution (Table 2). In a few instances, the TRIzol® LS Reagent dilution caused cell death through a 1000-fold dilution.

For each virus-positive control, the viral titer was calculated based on the average number of discrete plaques/well at the endpoint dilution. The titers were characteristic of these viruses in cell culture. Except for the Dengue virus subtype 4 positive control, which did not replicate to particularly high titer in this assay, all viruses had titers of at least 10^6 plaque-forming units per ml (pfu/ml).

The extraction reagent plus virus dilution series were evaluated by plaque assay. No plaques were detected in any extraction reagent plus virus combination in this study (Table 2). At low dilution points, massive cell death was apparent in all cases. Although this outcome could be caused by either extraction reagent toxicity or the pathogenicity of the virus, a consideration of the effect of the extraction

Table 2
Inactivation of virus by TRIzol® LS and AVL extraction reagents

Virus	Dilution (10^x)						
	U	1	2	3	4	5	6
Alphaviruses							
AVL + VEE	ND	ND	CD	0	0	0	0
TRIzol® + VEE	ND	ND	CD	0	0	0	0
Diluent + VEE	ND	ND	CD	TNTC	TNTC	20	1
AVL + EEE	CD	CD	CD	0	0	0	0
TRIzol® + EEE	CD	CD	CD	0	0	0	0
Diluent + EEE	ND	CD	CD	CD	TNTC	10	2
AVL + WEE	CD	CD	CD	0	0	0	0
TRIzol® + WEE	CD	CD	CD	0	0	0	0
Diluent + WEE	ND	CD	CD	CD	TNTC	20	2
Diluent + AVL	CD	CD	CD	0	0	0	0
Diluent + TRIzol®	CD	CD	CD	0	0	0	0
Bunyavirus							
AVL + RVF	ND	ND	CD	0	0	0	0
TRIzol® + RVF	ND	ND	CD	0	0	0	0
Diluent + RVF	ND	ND	TNTC	TNTC	10	1	0
Diluent + AVL	CD	CD	CD	0	0	0	0
Diluent + TRIzol®	CD	CD	CD	0	0	0	0
Filoviruses							
AVL + Marburg Musoke	CD	CD	CD	0	0	0	ND
TRIzol® + Marburg Musoke	CD	CD	CD	0	0	0	ND
Diluent + Marburg Musoke	CD	CD	CD	CD	TNTC	25	ND
AVL + Ebola Zaire 95	CD	CD	CD	0	0	0	ND
TRIzol® + Ebola Zaire 95	CD	CD	CD	0	0	0	ND
Diluent + Ebola Zaire 95	CD	CD	TNTC	101	11	0	ND
AVL + Marburg Ravn	CD	CD	CD	0	0	0	ND
TRIzol® + Marburg Ravn	CD	CD	CD	0	0	0	ND
Diluent + Marburg Ravn	CD	CD	CD	CD	TNTC	70	ND
AVL + Marburg Ci67	CD	CD	CD	0	0	0	ND
TRIzol® + Marburg Ci67	CD	CD	CD	0	0	0	ND
Diluent + Marburg Ci67	CD	CD	CD	CD	TNTC	29	ND
AVL + 18-35	CD	CD	CD	0	0	0	ND
TRIzol® + 18-35	CD	CD	CD	CD	0	0	ND
Diluent + 18-35	CD	CD	CD	CD	TNTC	TNTC	ND
AVL + 91-483	CD	CD	CD	0	0	0	ND
TRIzol® + 91-483	CD	CD	CD	CD	0	0	ND
Diluent + 91-483	CD	CD	CD	TNTC	70	7	ND
Diluent + AVL	CD	CD	CD	0	0	0	ND
Diluent + TRIzol®	CD	CD	CD	CD	0	0	ND
Flavivirus							
AVL + DEN 1	CD	CD	CD	0	0	0	ND
TRIzol® + DEN 1	CD	CD	CD	0	0	0	ND
Diluent + DEN 1	CD	TNTC	TNTC	TNTC	>100	14	ND
AVL + DEN 2	CD	CD	CD	0	0	0	ND
TRIzol® + DEN 2	CD	CD	CD	0	0	0	ND
Diluent + DEN 2	CD	CD	CD	TNTC	TNTC	>100	ND
AVL + DEN 3	CD	CD	CD	0	0	0	ND
TRIzol® + DEN 3	CD	CD	CD	0	0	0	ND
Diluent + DEN 3	CD	TNTC	TNTC	>150	22	3	ND
AVL + DEN 4	CD	CD	CD	0	0	0	ND
TRIzol® + DEN 4	CD	CD	CD	0	0	0	ND
Diluent + DEN 4	TNTC	TNTC	36	9	2	0	ND
AVL + WNV	ND	ND	CD	0	0	0	0
TRIzol® + WNV	ND	ND	CD	0	0	0	0
Diluent + WNV	ND	ND	TNTC	TNTC	81	10	1
Diluent + AVL	CD	CD	CD	0	0	0	ND
Diluent + TRIzol®	CD	CD	CD	0	0	0	ND

One hundred and forty microliter of a virus stock/isolate was mixed with 560 μl of AVL, TRIzol LS and diluent. A 10-fold serial dilution was made from this initial tube (U). One hundred microliter of each sample was inoculated onto 6-well cell culture plates in duplicate. The average number of plaques/well is reported for each dilution (not done (ND), cell death (CD), plaques too numerous to count (TNTC)).

reagent upon uninfected wells along with the absence of viral plaques in further dilutions appeared to implicate the former. Therefore, virus inactivation was determined based on the presence of viral plaques in the positive control dilutions to a dilution beyond the negative control dilution at which cell death stopped, combined with the absence of plaques in the plus virus dilution series. The extraction reagents, AVL Buffer and TRIzol® LS Reagent, appeared to completely inactivate each of the high-titer viruses used in this study.

This study demonstrates the ability of TRIzol® LS Reagent and AVL Buffer to inactivate members of four different viral genera. Complete inactivation is critical before specimens are manipulated outside of biological containment areas. The use of high-titer viruses and tissues shows that extraction by these reagents appeared to inactivate virus completely when the reagents were handled according to manufacturer's instructions. The ability to remove inactivated virus from BSL-3 and BSL-4 areas to work at lower level containment to conduct genetic characterization and diagnostic assay development is beneficial. Such capability should reduce the risk of accidental infection to laboratory workers by moving many operations to non-containment environments, and optimizes the utilization of equipment and space within containment laboratories. Finally, these results support the reliance upon either TRIzol® LS Reagent or AVL Buffer to render clinical or environmental samples non-infectious, which has implications for the handling and processing of samples under austere field conditions and low-level containment.

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