

# Evaluation of Animal Origin Cell Cultures for In Vitro Cultivation of Noroviruses

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## ABSTRACT

Noroviruses (NoVs) are a leading cause of acute non-bacterial gastroenteritis throughout the world. So far, in vitro growth of these viruses has not been achieved in human origin cell lines, and limited efforts have been made to evaluate the susceptibility of animal origin cell cultures. The main objective of this study was to evaluate primary and established cell cultures from a variety of animal species for in vitro growth of human NoV. A total of 19 cell cultures from 11 different animal species were evaluated. Cell monolayers were inoculated with 1 of the 2 fecal samples known to contain NoV. The infected cell monolayers were observed daily for up to 5 days for any cytopathological effects after which the infected cell suspension was inoculated in their respective fresh cells for a total of 5 blind passages. At fifth blind passage, reverse transcription polymerase chain reaction (RT-PCR) assay was used to detect the

presence of NoV RNA. None of the blind passages in any cell culture showed the evidence of any morphological change. When tested by RT-PCR, fifth cell culture passages from all cells were negative for NoV RNA. These results indicate that the 19 cell types used in this study are not susceptible to NoV and that the search should continue for a suitable cell culture system in which NoV can be isolated, propagated, and titrated.

## INTRODUCTION

Noroviruses (NoVs) are a group of single-stranded, non-enveloped, RNA viruses belonging to the family Caliciviridae.<sup>1,2</sup> Recently, these viruses have emerged as important pathogens in institutional and group settings including hospitals, nursing homes, day care centers, schools, banquet halls, cruise ships, camps, and sports grounds.<sup>3,4</sup> A relatively low-infectious dose, stability in the environment, and multiple modes of transmission make it difficult to control disease outbreaks caused by NoVs.<sup>3-9</sup> Until recently, NoVs were believed to be highly species-specific for humans,<sup>10</sup> but reports from Japan<sup>11</sup> and the Netherlands<sup>12,13</sup>

have demonstrated the occurrence of NoV-like viruses in pigs, monkeys, and calves. Of the animal caliciviruses, bovine calicivirus is considered to be more closely related to human caliciviruses.<sup>14</sup>

Until now, NoVs have been reported to be non-cultivable both in cell cultures and animal models except for limited success in some non-human primates. Most of the studies on NoVs are based on the use of human volunteers or surrogate models (e.g., feline calicivirus).<sup>15-17</sup> Recently, studies based on recombinant NoVs have highlighted their clinical and public health importance, ability to cause infection via a number of routes, and their considerable genetic diversity.<sup>18</sup>

Despite repeated attempts, experimental infection of different animal species, including non-human primates, rodents, birds, pigs, calves, and dogs, with the NoVs has not been successful.<sup>19-23</sup> Limited attempts have been made to evaluate the use of animal origin cell cultures for the growth of NoVs. The study described here is based on the premise that sequential passaging of NoVs in primary or established cell lines of animal origin may lead to its growth *in vitro*. If successful, such cell cultures would facilitate descriptive studies on the pathogenesis of NoVs and would replace the use of animal models and human volunteers.

## **MATERIALS AND METHODS**

### **Source of NoVs**

Two electron microscopy- and enzyme-linked immunoassay-confirmed Minnesota strains of NoVs obtained from an outbreak of gastroenteritis (kindly provided by the Minnesota Department of Health, St. Paul, MN) were used. Samples were in the form of human stools and were further confirmed to contain NoV genome by reverse transcription polymerase chain reaction (RT-PCR) in our laboratory.<sup>24</sup>

### **Inoculum Preparation**

A 10% suspension of the feces was made in phosphate buffered saline (pH 7.2), and the

suspension was filtered through 0.45 nm membrane filter (Millipore, Bedford, MA). The filtrate was used to inoculate cell cultures.

### **Cell Cultures**

The primary and established cell cultures used in the study are shown in Table 1. Primary cell cultures were porcine alveolar macrophages, porcine kidney, bovine and llama skin fibroblasts, and bovine, avian, and mouse embryos. The established animal cell lines BHK-21, BGM, MA-104, Vero, BT, PK-15, ST, CRFK, Frhk, QT-35, RK-13, ED, and MDCK have been used successfully to cultivate various human and animal viruses<sup>25</sup> and were evaluated in this study for the growth of NoV. Most of the cell cultures were grown in Eagle's minimal essential medium (Celox, St. Paul, MN) supplemented with 8% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (1 mg/mL), and fungizone (100 µg/mL). The same medium was used for Frhk cells except that the concentration of FBS was 16%. Porcine alveolar macrophages were grown in RPMI 1640 medium supplemented with 8% FBS, penicillin (100 U/mL), streptomycin (1 mg/mL), and fungizone (100 µg/mL). The cells were grown in 24-well microtiter plates and each well was initially seeded with approximately  $1 \times 10^6$  cells/mL in their respective growth media. The plates were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 24 hours and observed for the formation of monolayers after which they were used for sample inoculation.

### **Experimental Design**

One-day-old cultures contained in 24-well plates were rinsed twice in Hanks balanced salt solution (HBSS). Both NoV strains were inoculated in duplicate wells at 0.2 mL/well. The virus was allowed to adsorb to the cells by agitating the plates on a shaker platform at 100 rpm at 37°C for 90 minutes. Two mock-inoculated wells for each cell culture were used as negative controls. The virus inoculum was decanted and cells rinsed twice with HBSS to remove un-adsorbed virus. The cells were then flooded with 1 mL

**Table 1.** Cell Cultures Used for the Propagation of NoVs.

Name	Abbreviation Used	Origin	Source or ATCC # (if available)
<b>Primary cultures</b>			
Bovine embryo	BE	Bovine	-
Bovine skin fibroblast cells	BSF	Bovine	-
Porcine primary kidney	PPK	Porcine	-
Porcine alveolar macrophages	PAM	Porcine	-
Chicken embryo fibroblast	CEF	Avian	-
Llama skin fibroblast cells	ILF	Llama	-
Mouse embryo	ME	Mouse	-
<b>Continuous cell lines</b>			
Baby hamster kidney	BHK-21	Hamster	CCL 10
Buffalo green monkey kidney*	BGM	Monkey	-
African green monkey kidney	MA-104	Monkey	CRL 2378
African green monkey kidney	Vero	Monkey	CCL 81
Bovine turbinate	BT	Bovine	CRL 1390
Porcine kidney	PK-15	Porcine	CCL 33
Swine testis	ST	Porcine	CRL 1746
Crandell's feline kidney†	CRFK	Feline	-
Feline kidney†	Frhk	Feline	-
Quail tumor*	QT-35	Avian	-
Rabbit kidney	RK-13	Rabbit	CCL 37
Equine dermal	ED	Equine	CCL 57
Madin Darby canine kidney	MDCK	Canine	CCL 34

\*Cells were obtained from National Veterinary Services Laboratories, Ames, Iowa.

†Cells were provided by Douglas Weit, University of North Carolina, NC.

of maintenance medium containing 1% FBS followed by incubation at 37°C. Inoculated cells were observed daily for up to 5 days for any cytopathological effects. After 5 days, cells from duplicate wells were pooled individually and frozen-thawed twice, and 0.2 mL of the lysate was used for the next blind passage in fresh cell culture. An aliquot of pooled cells from each passage was stored at -70°C for future use.

### Nucleic Acid Extraction

Nucleic acid from fifth passage of all virus-inoculated cell cultures was extracted using QIAamp viral RNA mini kit (Qiagen, Valencia, CA) following manufacturer's instructions. In brief, 140 µL of cell suspension was mixed with 560 µL of AVL lysis buffer, vortexed briefly, and incubated for 15 minutes at room temperature followed by

the addition of 560 µL of absolute ethanol and vortexing. This mixture was passed through QIAamp spin columns followed by 2 washings with 500 µL of washing buffers (AWI-1 and AWI-2). RNA bound to spin column cartridge was eluted in 60 µL of QIAamp AVE elution buffer. The nucleic acid was stored at -20°C until use.

### RT-PCR

Molecular detection of NoVs was performed by RT-PCR using published primer pairs.<sup>24</sup> The primer pair sequences used in the study were JV 12 (5'-3' ATACCAC-TATGATGCAGATTA) and JV 13 (5'-3' CATCATCACCATAGAAAGAG). To avoid cross contamination, single tube RT-PCR method was adopted using Qiagen single tube RT-PCR kit. Amplification was carried out in a reaction volume of 50 µL

containing 10  $\mu$ L of 5 $\times$  RT buffer (12.5 mM MgCl<sub>2</sub>), 2  $\mu$ L of dNTP mixture (10 mM of each dNTP), 2  $\mu$ L of enzyme mix and 2  $\mu$ L of Q solution, 1  $\mu$ L of each primer (50 pmol each), 10  $\mu$ L of RNA, and 22  $\mu$ L of nuclease-free water to make the volume of reaction mix 50  $\mu$ L. Reverse transcription was carried out at 50°C for 1 hour followed by the inactivation of reverse transcriptase enzyme at 95°C for 15 minutes. Conditions for PCR used were: 40 cycles of 94°C for 1 minute (denaturation), 53°C for 1 minute (annealing), and 72°C for 2 minutes (extension) followed by a final extension at 72°C for 5 minutes after completion of 40 cycles of PCR amplification in Perkin Elmer, GeneAmp PCR system 9600. Electrophoretic separation of PCR products was performed for 1 hour at 101 V on 3% agarose gel in 1 $\times$ -TAE buffer followed by staining with ethidium bromide. The amplicons were visualized under UV trans-illuminator. For estimating product length, 1 Kb DNA ladder (Invitrogen, Carlsbad, CA) was used as a marker. To determine the specificity of virus specific primers, mock-inoculated cell culture extracts were also tested.

## RESULTS

None of the inoculated cell cultures showed any cytopathological changes in any of the 5 passages. In addition, nucleic acid extracted from the fifth cell culture passage showed no amplification of the NoV genome. The primer pairs used in this study did amplify NoVs in the original fecal samples used to infect all cell cultures with an expected amplicon of 326 bp size. No amplification was observed in any of the mock infected control cell cultures.

## DISCUSSION

Enteric caliciviruses have been reported in pigs, monkeys, calves, dogs, and chickens.<sup>26–28</sup> However, attempts to experimentally infect non-human hosts with human NoVs have largely been unsuccessful except for some encouraging results in chimpanzees, macaque, and rhesus monkeys.<sup>19–23</sup> So far, no

in vitro system for the growth of NoV has been described.<sup>18</sup> Recently, various methods have been tried to grow NoVs in vitro based on the current understanding of the binding and replication sites of NoV in vivo.<sup>29</sup> Many gastric, duodenal, and enterocyte cultures have been tried with or without the addition of supplements such as insulin, dimethylsulfoxide, and butyric acid to mimic the in vivo intestinal environment.<sup>29</sup> However, none of the approaches has been successful so far.

Attachment of virions with their specific receptors on the host cells is a major determinant of the host range and tissue tropism of a virus.<sup>30,31</sup> It has recently been discovered that recombinant Norwalk virus-like particles (rNVLPs) agglutinate red blood cells. Since histo-blood group antigens are expressed on gut mucosa as well as red blood cells, the rNVLP hemagglutination system has been used as a model for studying NoV attachment to cells to identify a potential NoV receptor.<sup>32,33</sup>

In the present study, 19 animal origin cell lines from 11 different species with an adsorption time of 90 minutes and constant shaking of the inoculum were used to achieve virus adsorption and growth. However, all efforts were unsuccessful. Other strategies such as altered adsorption time, longer incubation period, use of roller cultures, method of preparation of virus inoculum, condition for maintenance of cell monolayers, additives in maintenance medium, method of inoculation of cells, and trypsin treatment, etc., have also not been successful in the cultivation of NoV.<sup>29</sup> We conclude that efforts should continue to find a suitable NoV cultivation system.

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