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

Yashpal S. Malik, BVSc, PhD*
Sunil Maherchandani, BVSc, PhD*
Paul B. Allwood, MPH, PhD†
Sagar M. Goyal, BVSc, PhD*

*Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota

†Division of Environmental Health, Minnesota Department of Health, St. Paul, Minnesota

ABSTRACT

Noroviruses 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 noroviruses. A total of 19 cell cultures from 11 different animal species were evaluated. Cell monolayers were inoculated with one of the two fecal samples known to contain noroviruses. The infected cell monolayers were observed daily for up to 5 days for any cytopathological effects (CPE) after which the infected cell suspension was inoculated in their respective fresh cells for a total of five blind passages. At fifth blind passage RT-PCR assay was used to detect the presence of norovirus RNA. None of the blind passage 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 norovirus RNA. These results indicate that the 19 cell types used in this study are not susceptible to noroviruses and that search should continue for a suitable cell culture system in which noroviruses can be isolated, propagated, and titrated.

INTRODUCTION

Noroviruses are a group of single stranded, non-enveloped, RNA viruses belonging to the family *Caliciviridae*.[1-2] 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.[3-4] A relatively low infectious dose, stability in the environment and multiple modes of transmission, make it difficult to control disease outbreaks caused by noroviruses.[3-9] Until recently, noroviruses were believed to be highly species-specific for humans[10] but reports from Japan[11] and the Netherlands,[12-13] have demonstrated the occurrence of norovirus-like viruses in pigs, monkeys and calves. Of the animal

caliciviruses, bovine calicivirus is considered to be more close to human caliciviruses.[14]

Until now noroviruses have been reported to be non-cultivatable both in cell cultures and animal models except for limited success in some non-human primates. Most of the studies on noroviruses are based on the use of human volunteers or surrogate models eg, feline calicivirus.[15-17] Recently, studies based on recombinant noroviruses have highlighted their clinical and public health importance, ability to cause infection via a number of routes, and their considerable genetic diversity.[18]

Despite repeated attempts, experimental infection of different animal species, including non-human primates, rodents, birds, pigs, calves, and dogs, with noroviruses has not been successful.[19-23] Limited attempts have been made to evaluate the use of animal origin cell cultures for the growth of noroviruses. The study described here is based on the premise that sequential passaging of noroviruses 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 noroviruses and would replace the use of animal models and human volunteers.

MATERIALS AND METHODS
Source of Noroviruses
Two EM and ELISA confirmed Minnesota strains of noroviruses 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 norovirus genome by RT-PCR in our laboratory.[25]

Inoculum Preparation
A 10% suspension of the feces was made in phosphate buffered saline (pH 7.2) and the suspension was filtered through a 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[24] and were evaluated in this study for the growth of noroviruses. Most of the cell cultures were grown in Eagle's minimal essential medium (MEM) (Celox, St. Paul, MN) supplemented with 8% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (1mg /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 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 x 10^6 cells/mL in their respective growth media. The plates were incubated at 37°C in a 5% CO_2 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 norovirus 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 were rinsed twice with HBSS to remove un-adsorbed virus. The cells were then flooded with 1 mL 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 (CPE). After 5 days, cells from duplicate wells were pooled individually, 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 two 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 noroviruses was performed by RT-PCR using published primer pairs.[25] The primer pair sequences used in the study were JV 12 (5'-3' ATACCACTATGAT GCAGAT-TA) and JV 13 (5'-3' CATCATCAC-CATAGAAAGAG). To avoid cross contamination, single tube RT-PCR method was adopted using Qiagen sin-

gle tube RT-PCR kit. Amplification was carried out in a reaction volume of 50 μL containing 10 μL of 5X RT buffer (12.5 mM MgCl$_2$), 2 μL of dNTP mixture (10 mM of each dNTP), 2 μL of enzyme mix and 2 μL of Q solution, 1 μL of each primer (50 pmol each), 10 μL of RNA and 22 μL of nuclease-free water to make the volume of reaction mix 50 μL. Reverse transcription was carried out at 50˚C for one hour followed by the inactivation of reverse transcriptase enzyme at 95˚C for 15 minutes. PCR conditions 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 one hour at 101 volts on 3% agarose gel in 1X-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, Carlbad, 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 five passages. In addition, nucleic acid extracted from the fifth cell culture passage showed no amplification of the norovirus genome. The primer pairs used in this study did amplify noroviruses 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

Table 1. Cell Cultures Used for the Propagation of Noroviruses.

Name	Abbreviation used	Origin	Source or ATCC number if available
Primary cultures			
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 fibroblast cells	ILF	Llama	-
Mouse embryo	ME	Mouse	-
Continuous cell lines			
Baby hamster kidney	BHK-21	Hamster	CCL 10
BGM*	BGM	Monkey	-
MA 104	MA-104	Monkey	CRL 2378
African green monkey	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

*The cells were obtained from National Veterinary Services Laboratories, Ames, Iowa.
†The cells were provided by Douglas Weit, University of North Carolina, NC

in pigs, monkeys, calves, dogs, and chickens.[26-28] However, attempts to experimentally infect non-human hosts with human noroviruses have largely been unsuccessful except for some encouraging results in chimpanzees, macaque, and rhesus monkeys.[19-23] So far, no in vitro system for the growth of noroviruses has been described.[18] Recently various methods have been tried to grow noroviruses in vitro based on the current understanding of the binding and replication sites of noroviruses in vivo.[31] Many gastric, duodenal, and enterocyte cultures have been tried with or without the addition of supplements such as insulin, DMSO and butyric acid to mimic the in vivo intestinal environment.[31] However, none of these 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.[29-30] 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 RBCs, rNVLPs haemagglutination system has been used as a model for studying noroviruses attachment to cells to identify a potential norovirus receptor.[32-33]

In the present study, 19 animal origin cell lines from 11 different species with 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 noroviruses.[31] We conclude that efforts should continue to find a suitable norovirus cultivation system.

ACKNOWLEDGMENTS

REFERENCES
1. Green KY, Ando T, Balayan MS, et al. Taxonomy of caliciviruses. *J Infect Dis*. 2000;181:322-330.

2. Prasad BV, Hardy ME, Estes MK. Structural studies of recombinant Norwalk capsid. *J Infect Dis*. 2000;181:317-321.

3. CDC. Outbreaks of gastroenteritis with Noroviruses on cruise ships in the Unites States. *MMWR Morb Mortal Wkly Rep*. 2002;51:112-1115.

4. CDC. Norovirus activity—United States. *MMWR Morb Mortal Wkly Rep*. 2003;52:41-45.

5. Marks PJ, Vipond IB, Carlisle D, Deakin, D, Fey RE, Caul EO. Evidence for airborne transmission of Norwalk-like viruses in a hotel restaurant. *Epidemiol Infect*. 2000;124:481-487.

6. Deneen VC, Hunt JM, Paule CR, et al. The impact of foodborne calicivirus disease: the Minensota experience. *J Infect Dis*. 2000;181:281-283.

7. Lo SV, Conolly AM, Palmer SR, et al. The role of pre-symptomatic food handlers is a common source of outbreaks of food borne SRSV gastroenteritis in a group of hospitals. *Epidemiol Infect*. 1994;113:513-521.

8. Patterson T, Hucking P, Palmer S. Outbreaks of SRSV gastroenteritis at an international conference traced to food handled by a post symptomatic caterer. *Epidemiol Infect*. 1993;111:151-162.

9. Fankhauser RI, Noel JS, Monroe SS, et al. Molecular epidemiology of Norwalk-like viruses in outbreaks of gastroenteritis in the United States. *J Infect Dis* 1998;178:1571-1578.

10. Clarke IN, Lambden PR. The molecular biology of caliciviruses. *J Gen Virol*. 1997;78:291-301.

11. Sugieda M, Nagaoka H, Kakishima Y, Ohshita T, Nakamura S, Nakajima, S. Detection of Norwalk-like virus genes in the caecum contents of pigs. *Arch Virol*. 1998;143:1215-1221.

12. van der Poel WHM, van der Heide R, Verschoo F, Gelderblom H, Vinje J, Koopmans MPG. Epidemiology of Norwalk-like viruses infection in cattle in the Netherlands. *Vet Microbiol*. 2003;92:297-309.

13. van der Poel WHM, Vinje J, van der Heide R, Herrera MI, Vivo A, Koopmans MPG. Norwalk-like calicivirus genes in farm animals. *Emerg Infect Dis*. 2000;6:36-41.

14. Dastjerdi AM, Green J, Gallimore CI, Brown DWG, Bridge J. The bovine newburry agent-2 is genetically more closely related to humans SRSVs than to animal caliciviruses. *Virol*. 1999;254:1-5.

15. Bidawid S, Malik N, Adgburin O, Sattar SA, Farber JM. A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. *J Virol Meths*. 2003;107:163-167.

16. Slomka MJ, Appleton H. Feline calicivirus as a model system for heat inactivation studies of small round structured viruses in shellfish. *Epidemiol Infect*. 1998;121:401-407.

17. Gulati BR, Allwood PB, Hedberg CW, Goyal SM. Efficacy of commonly used disinfectants for the inactivation of calicivirus on fresh fruits, vegetables and food-contact surfaces. *J Food Prot*. 2001;64:1430-1434.

18. White LJ, Bali JM, Hardy ME, Tanaka TN, Kitamoto N, Estes MK. Attachment and entry of recombinant Norwalk virus capsid to cultured human and animal cell lines. *J Virol.* 1996;70:6589-6597.

19. Green KY. The role of human calicivirus in epidemic gastroenteritis. *Arch Virol.* 1997;13:153-165.

20. Subekti DS, Tjaniadi P, Lesmana M, et al. Experimental infection of *Macacca nemestrina* with Toronto Norwalk like virus of epidemic viral gastroenteritis. *J Med Virol.* 2002;66:400-406.

21. Cubitt WD. Human caliciviruses: characterization and epidemiology. Unpublished doctoral thesis, London University. 1985.

22. Kapikian AZ, Estes MK, Chanock RM. Norwalk group of viruses. In: *Field's Virology* (Fields BN and Knipe DM, eds), NY, Raven Press; 1999;783-810.

23. Wyatt RG, Greenberg HB, Dalgard DW, et al. Experimental infection of chimpanzees with the Norwalk agent of epidemic viral gastroenteritis. *J Med Virol.* 1978;2:89-96.

24. Parwani AV, Flynn WT, Gadfield KL, Saif LJ. Serial propagation of porcine enteric caliciviruses in continuous cell lines: effect of medium supplements with intestinal contents or enzymes. *Arch Virol.* 1991;120:115-122.

25. Vinje J, Koopmans MPG. Molecular detection and epidemiology of small round structured viruses in outbreaks of gastroenteritis in the Netherlands. *J Infect Dis*. 1996;174:610-615.

26. Granzow H, Schrmeirer H. Identification of 32 nm virus in feces of diarrheic calves by electron microscopy. *Monatshafle Veterinarmedizine.* 1985;40:228-229.

27. Mochizuki M, Kawanishi A, Sakamoto S, Tashiro S, Fujimo R, Ohwaki M. A calicivirus isolated from a dog with fatal diarrhea. *Vet Rec.* 1993;132:221-222.

28. Reckling KF. Use of electron microscopy for observation of small round structured viruses in fecal samples collected from calves and animals with diarrhea. *Monatshafle Veterinarmedizine.* 1987;42:272-275.

29. Collins AR. HLA class 1 antigen serves as a receptor for human coronavirus OC43. *Immunol Invest.* 1993;22:95-103.

30. Lamarre D, Ashkenazi A, Fleury S, Smith DH, Sekaly RP, Capon DJ. The MHC binding and gp 120 binding functions of CD4 are separable. *Science.* 1989;245:743-746.

31. Duizer E, Schwab KJ, Neill FH, et al. Laboratory efforts to cultivate Noroviruses. *J Gen Virol.* 2004;85:79-87.

32. Hutson AM, Atmar RL, Mascus DM, Estes MK. Norwalk virus like particle haemagglutination by binding to histo-blood group antigens.

32. Hutson AM, Atmar RL, Mascus DM, Estes MK. Norwalk virus like particle haemagglutination by binding to histo-blood group antigens. *J Virol.* 2003;77:405-415.

33. Hutson AM, Atmar RL, Graham DY, Estes MK. Norwalk virus infection and disease is associated with ABO histo blood group type. *J Infect Dis.* 2002;185:1335-1337.