Cell Culture Assay for Human Noroviruses

To the Editor: We read with great interest the article on human norovirus (hNoV) by Straub et al. (*1*). By using 3-dimensional aggregates of a highly differentiated intestinal epithelial cell line, the investigators claimed to have established an in vitro cell culture model that "support[s] the natural growth of human noroviruses." While the authors provide compelling evidence of successful virus infection through microscopy, hybridization of viral RNA after 5 passages in cell culture, and preliminary evidence of viral RNA replication through limiting dilution PCR, we question the level of virus replication that is actually achieved in this system.

Straub et al. demonstrate through fluorescent in situ hybridization the presence of viral RNA through 5 passages in his system. This phenomenon could be similar to the findings of Duizer et al. (*2*), if the level of replication simply maintained the viral titer. Therefore, we argue that virus replication curve, estimated by using quantitative real-time PCR or semiquantitative endpoint dilution PCR with the end-dilution of each sample from different time points in this system, will conclusively determine the suitability of this model as a productive virus replication system. To support our hypothesis, we point to the pig model for hNoV infectivity (*3*). In that study investigators failed to observe an increase in viral shedding from symptomatic piglets upon serial passage, despite successful intracellular detection of viral RNA and newly synthesized virus-encoded protein in host cells dying of apoptosis. This suggests that the demonstration of cytopathic effect and virus internalization in cells alone may not provide direct evidence of productive virus replication. In conclusion, although we acknowledge that Straub et al. have provided evidence of successful hNoV infection in vitro, we suggest subsequent studies to characterize the level of virus replication in this system.

Martin C.W. Chan,* Y.P. Wong,* and Wai K. Leung*

*The Chinese University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China

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Address for correspondence: Wai K. Leung, Department of Medicine and Therapeutics, The Chinese University of Hong Kong, Hong Kong Special Administrative Region, People's Republic of China; email: wkleung@cuhk.edu.hk

In Response: We appreciate the comments provided by Chan et al., in response to our recently published article (*1*). The specific aim of our project was to develop an in vitro cell culture infectivity assay for human norovirus (hNoV) to enhance risk assessments when these viruses are detected in water supplies. Reverse transcription (RT) qualitative or quantitative PCR are the primary assays for waterborne hNoV monitoring. However, these assays cannot distinguish infectious from noninfectious virions. When hNoV is detected in water supplies, information provided by our infectivity assay will improve risk assessment models and protect human health, regardless of whether we are propagating hNoV. Indeed, in vitro cell culture infectivity assays for the waterborne pathogen *Cryptosporidium parvum* that supplement approved fluorescent microscopy assays do not result in amplification of the environmentally resistant hard-walled oocysts (*2*). However, identification of life cycle stages in cell culture provides evidence of infectious oocysts in a water supply.

Nonetheless, Leung et al.'s assertion regarding the suitability of our method for the in vitro propagation of high titers of hNoV is valid for the medical research community. In this case, well-characterized challenge pools of virus would be useful for developing and testing diagnostics, therapeutics, and vaccines. As further validation of our published findings, we have now optimized RT quantitative PCR to assess the level of viral production in cell culture, where we are finding increases in viral titer. The magnitude and time course of these increases is dependent on both virus strain and multiplicity of infection. We are currently preparing a manuscript that will discuss these findings in greater detail, and the implications this may have for creating viral challenge pools.

Timothy M. Straub,*
Kerstin Höner zu Bentrup,†
Patricia Orosz-Coghlan,‡
Alice Dohnalkova,*
Brooke K. Mayer,*[1]
Rachel A. Bartholomew,*
Catherine O. Valdez,*
Cynthia J. Bruckner-Lea,*
Charles P. Gerba,‡
Morteza A. Abbaszadegan,§
and Cheryl A. Nickerson†[1]

*Pacific Northwest National Laboratory, Richland, Washington, USA; †Tulane University School of Medicine, New Orleans, Louisiana, USA; and ‡University of Arizona, Tucson, Arizona, USA

[1]Current affiliation: Arizona State University, Tempe, Arizona, USA

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Address for correspondence: Timothy M. Straub, Pacific Northwest National Laboratory – Chemical and Biologic Sciences, PO Box 999, MS P7–50, Richland, WA 99352, USA; email: timothy.straub@pnl.gov

Anthrax in Red Deer (*Cervus elaphus*), Italy

To the Editor: Anthrax is hypoendemic in Italy; a few outbreaks occurred yearly involving unvaccinated herbivores on pastures in central and southern regions and the major islands. Multiple-locus variable-number tandem-repeat analysis (MLVA) with 8 variable-number tandem repeats (VNTRs) of Italian isolates of *Bacillus anthracis* has identified 9 genotypes belonging to cluster A1a (*1*). An isolate of cluster A3 has been identified recently in Sardinia, which suggests that such a strain could have been introduced into Italy from another country (*1*).

A total of 37 anthrax outbreaks occurred in a 41-day period from August 28 to October 3, 2004, in a restricted area of Pollino National Park (Basilicata region in southern Italy) and resulted in the deaths of 124 domestic or wild animals. Two suspected cases of cutaneous anthrax in humans were recorded. Pollino National Park contains several species of feral animals. Since 1990, there has been a program for reintroduction of red deer (*Cervus elaphus*) into this park from Tuscany, Italy, and Carinthia, Austria. The animals are kept in quarantine in a corral by the veterinary services of the park and given an electronic tag before their release. At the time of the anthrax outbreaks, the red deer population of the park was 45, of which 10 were living in the corral. These outbreaks killed 8 deer (4 free-ranging and 4 confined animals).

Each carcass was examined by the veterinary officer, who collected clinical samples that were examined for *B. anthracis* by using standard procedures of the Istituto Zooprofilattico Sperimentale of Puglia and Basilicata. DNA from the suspected colonies was analyzed by PCR with primers specific for *B. anthracis* (*2*) and subsequent genotyping by using MLVA with 8 VNTRs (*3*). All *B. anthracis* isolates belonged to cluster A1a, genotype 1 (A. Fasanella, unpub. data). This genotype was also identified in subsequent outbreaks that involved farm animals in the same area and resulted in the deaths of 116 domestic animals, including 81 cattle, 15 sheep, 9 goats, and 11 horses. Red deer showed the highest mortality rate during these outbreaks (Table). An ELISA (*4*) performed with 27 serum samples obtained from deer in the park detected low levels of antibodies to *B. anthracis* in 22% of the examined animals. This seroprevalence is consistent with levels found in unvaccinated livestock reared in areas endemic for anthrax (A. Fasanella, unpub. data).

A vaccination program was then instituted for farm animals, but the deer population in the park was excluded because no experimental data were available on the safety and efficacy of Carbosap vaccine (Istituto Zooprofilattico Sperimentale of Puglia and Basilicata, Foggia, Italy) in wild ruminants. Extensive vaccination limited the outbreaks in livestock and red deer, which probably prevented further spread of infection from farm animals to free-ranging deer.

These anthrax outbreaks in southern Italy suggested that red deer are highly susceptible to infection with *B. anthracis* and that the mortality rate in these deer could be even higher than that observed in domestic animals. Although epidemiologic data are limited and need to be supported experimentally by assessment of the 50% lethal dose of *B. anthracis* in red deer, the ecologic effect on deer populations in parks should not be underestimated. Moreover, concerns for public health may arise in parks in disease-endemic areas, where susceptible wild animals could represent an amplification factor for *B. anthracis* spores, which increases the probability of outbreaks in domestic animals and in humans living near, working in, or visiting the parks. This article stresses the need for evaluating the safety and efficacy of *B. anthracis* vaccines in deer and for including wild ruminants in the anthrax prophylaxis programs.

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Antonio Fasanella,*
Lucia Palazzo,*
Antonio Petrella,*
Vincenzo Quaranta,*
Bruno Romanelli,*
and Giuliano Garofolo*

Table. Mortality rates during anthrax outbreaks, Italy, 2004

Animal	Population of area	No. (%) dead animals
Cattle	≈7,000	81 (≈1.15)
Sheep	≈20,000	15 (≈0.075)
Goats	≈13,000	9 (≈0.069)
Horses	≈600	11 (≈1.83)
Red deer	45	8 (≈17.77)