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Enhanced Infectivity of Bluetongue Virus in Cell Culture by Centrifugation

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The effects of centrifugation on the infection of cell culture with bluetongue virus (BTV) were investigated. Baby hamster kidney cells were infected with BTV with or without centrifugation. Viral antigen was detected by immunofluorescence at 24 h in both centrifuged and noncentrifuged cultures. However, after 24 h of infection, the production of PFU in centrifuged cell cultures was 10- to 20-fold greater than that seen in cultures not centrifuged. In addition, centrifugation enhanced the direct detection of PFU from blood samples collected from a sheep experimentally infected with BTV.

Bluetongue virus (BTV) is a member of the orbivirus genus. This pathogen of sheep, cattle, and other ruminants lacks an essential lipid envelope and has a genome composed of 10 double-stranded RNA segments (8, 9). Cell cultures, embryonating chicken eggs, and sheep have been used most frequently for the isolation of BTV (6). Results with these three systems have been varied, and no system has been shown to be superior to the others in all cases (5).

Enhancement of cytomegalovirus infection in cell cultures by use of low-speed centrifugation has been described elsewhere (7). Centrifugation of clinical samples such as urine, sputum, and bronchoalveolar lavage specimens has been found to improve the detection of virus (1, 2). In this study we evaluated the effects of centrifugation on BTV serotype 10 infection in baby hamster kidney (BHK-21) cells and on the detection of virus in the blood of a sheep experimentally infected with BTV.

BHK-21 cells (0.9 ml containing 30,000 cells) were seeded into 3-dram (ca. 12-ml) shell vials containing cover slips with basal Eagle medium to provide a monolayer in 24 h. Cells were inoculated with 0.1 ml of the virus suspension (50 to 100 PFU). The shell vials were then centrifuged for 30 min at 200 × *g* at 20°C. After centrifugation, the vials were placed in a 37°C incubator for 30 min, after which the inoculum was removed and fresh medium was added. Duplicate experiments in which the vials were incubated for 30 min at room temperature without centrifugation were performed. After 24 h, the cover slips were stained for indirect fluorescence microscopy. Cover slips were fixed in cold acetone for 10 min and then incubated with polyclonal rabbit antiserum specific for BTV serotype 10. The second antibody was goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (Hyland).

Immunofluorescence staining showed that foci of viral antigen could be detected within 24 h in BHK-21 cells infected with BTV serotype 10 with and without centrifugation. However, no clear differences between the two treatments could be distinguished by this technique (data not shown).

To determine if centrifugation enhanced the replication of BTV in BHK-21 cells, shell vials without cover slips were seeded with cells and inoculated with virus both with and without centrifugation as described above. At 24 h postinfection, these cells were sonicated in 1 ml of medium for 60 s by using an ultrasonic processor sonicator at 5 W to release virus from the cells. The cell lysate was assayed to determine the number of PFU produced in 24 h. African green monkey kidney (Vero) cell suspension (1 ml; 7×10^5 cells per ml) was added to each well of a six-well plate. The following day, 0.1 ml of dilute sonicated cell lysate was added to each well, gently agitated for 15 min at room temperature, and then incubated at 37°C for 2 h. The inoculum was removed, and an overlay medium, consisting of 0.6% agarose (SeaPlaque; FMC Bioproducts, Rockland, Maine) and medium (199E), was added to each well. The plates were incubated for 6 days at 37°C. Cells were fixed in 10% buffered Formalin and stained with 0.1% crystal violet.

The centrifugation of BHK-21 cells with an inoculum of BTV serotype 10 enhanced viral replication as determined by the production of PFU (Fig. 1). Titration values of the virus produced in the shell vials were always at least 10-fold greater in centrifuged samples than in noncentrifuged samples. Cells infected with BTV without centrifugation produced $10^{3.7}$ to $10^{4.0}$ PFU per shell vial, whereas cells infected with BTV with centrifugation produced $10^{5.1}$ to $10^{5.4}$ PFU per shell vial.

The effect of centrifugation on the direct detection of BTV in biological samples was also investigated. Heparinized blood samples collected daily following experimental infection of a sheep with BTV serotype 10 (200,000 50% chicken embryo intravascular lethal doses injected intradermally and subcutaneously) were sonicated for 5 to 10 s to release cell-associated virus. Portions of the sonicated blood samples were added to Vero cell monolayers in 24-well cell culture plates, centrifuged at 200 × *g* for 30 min at 20°C, washed with phosphate-buffered saline, and overlaid with medium 199E containing 0.6% agarose. Following incubation at 37°C for 5 to 6 days, the cells were stained with crystal violet and the plaques were counted. Duplicate 24-well plates were incubated for 30 min at room temperature without centrifugation.

Table 1 shows the effect of these treatments on the detection of PFU of virus from the blood samples. Virus was detected 1 day earlier and at least 1 day later when blood samples were centrifuged onto the Vero cell monolayer (compared with samples which were not centrifuged). In

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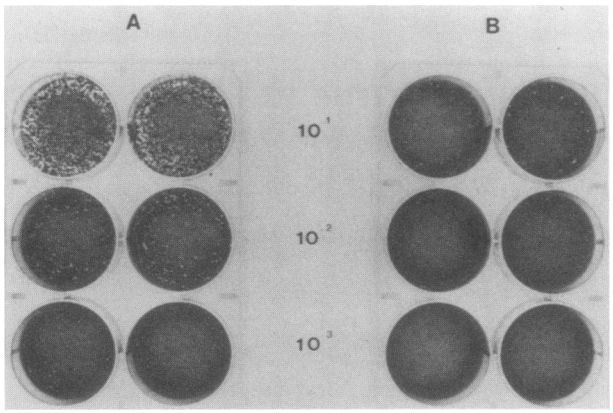

FIG. 1. Effect of centrifugation on the production of BTV. BHK-21 cells were grown in glass shell vials and infected with BTV with (A) or without (B) centrifugation. After 24 h, the infected cells were sonicated and the plaque assay was performed in Vero cell cultures.

addition, virus titers at the peak of viremia (6 to 8 days after infection) were approximately fourfold greater in the centrifuged samples than in the noncentrifuged samples.

The phenomenon of enhanced infectivity by centrifugation has been previously shown with murine and human cytomegaloviruses and with the sand rat herpesvirus. Related viruses such as herpes simplex virus types 1 and 2, bovine herpesvirus type 1, and pseudorabies virus did not show enhanced infectivity by centrifugation in the same study. In addition, infection by RNA viruses such as Sindbis and hepatic necrosis virus of fish did not show enhancement by this method (3). The mechanism of centrifugation enhancement of infection might be related to attachment and adsorption of virus. Another hypothesis is that centrifugation changes the normal interaction of virus with the cellular membrane or cytoskeleton in such a way as to enhance penetration and replication (3, 4).

Prior to this study, centrifugal enhancement of infection had not been reported for any orbivirus. We have shown that centrifugation enhances the BTV infection process. This technique could be directly applied in diagnostic procedures. For example, the use of centrifugation on clinical specimens may make the direct recovery of BTV from clinical samples in a cell culture system more reliable. The method described in this study would also save time in the inoculation of specimens and in the detection of positive BTV specimens compared with the more laborious procedure used for intravascular chicken embryo inoculation. In this study the original inoculum was in contact with cells for only 30 to 60 min. This may allow the inoculation of less dilute specimens that would otherwise be toxic to cells. Enhancement of BTV infectivity by centrifugation could also be used as an amplification step in conjunction with various diagnostic probes.

TABLE 1. Detection of BTV in the blood of an experimentally infected sheep[a]

Day after infection[b]	PFU/ml of blood	
	Without centrifugation	With centrifugation
0	0	0
1	0	0
2	0	0
3	0	0
4	0	25
5	45	180
6	80	350
7	85	330
8	65	215
9	45	135
10	40	120
11	20	90
13	0	15
14	0	0

[a] The sheep was infected by intradermal and subcutaneous injection of 200,000 50% chicken embryo intravascular lethal doses of BTV serotype 10.
[b] Heparinized blood samples were collected except on day 12, when no sample was collected.

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