

Quantitative Assay of Paravaccinia Virus Based on Enumeration of Inclusion-Containing Cells

J. C. CAMBIER AND E. S. MEEK

Department of Microbiology, University of Iowa, Iowa City, Iowa 52240

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Bovine paravaccinia virus produces cytoplasmic inclusion bodies on infection of bovine embryonic kidney cells; these were easily recognized when stained with acridine orange or May-Grünwald-Giemsa stain. The inclusions could be shown to contain newly synthesized deoxyribonucleic acid by autoradiography. Counts of inclusion-containing cells decreased when virus suspensions were treated with immune serum before being used to inoculate cell cultures. At 24 hr after infection, the number of cells containing inclusions was directly proportional to the concentration of infectious virus inoculated. These observations provide the basis for a virus assay which is simpler, faster, and more sensitive than the plaque assay.

Quantitative estimations of the agent of milker's nodules, a bovine paravaccinia virus, have previously been based on quantal assay (2) and on plaque assay (3). This virus produces easily recognizable cytoplasmic inclusion bodies in monolayers of bovine embryonic kidney cells. These inclusions have been shown to contain deoxyribonucleic acid (DNA) by autoradiography (3).

On the basis of these observations, an assay of inclusion-containing cells was developed. The inclusions could be counted after staining with acridine orange (6) or May-Grünwald-Giemsa (7). The number of cells containing inclusion bodies decreased when the virus was neutralized by specific immune serum. Under the conditions described below, the number of inclusion-containing cells was proportional to the number of infective viral particles inoculated. This assay is more sensitive than the plaque technique and can be completed in 24 hr.

MATERIALS AND METHODS

Virus. Milker's nodule (bovine paravaccinia) virus (pool 1868-10/23) was kindly provided by Wallace Rowe of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

Cells. Primary bovine embryonic kidney cells were used throughout the study. These were grown in Hanks basic minimal essential (H-BME) medium containing 10% heat-inactivated fetal calf serum. Earle's BME (E-BME) plus 10% heat-inactivated fetal calf serum was used as maintenance medium. Two-chambered tissue culture slides (Lab Tek Divi-

sion, Miles Laboratories, Westmont, Ill.) were seeded with 10^6 cells per chamber and incubated at 37 C in an atmosphere of 5% CO₂ until monolayers were formed. These were used for all inclusion assays done during the study. Monolayers for plaque assays were prepared by seeding 5×10^6 cells in 5 ml of medium in plastic petri dishes 33 mm in diameter.

Plaque assay. In parallel with inclusion assays, the virus was titrated by the plaque technique (3). Cell monolayers were infected with 0.1 ml of virus suspension. The virus was allowed to adsorb for 3 hr at 37 C. Plates were then washed with 5 ml of medium before 6 ml of warm 0.9% agar in H-BME was added. A second agar overlay was added at 5 days postinfection. Plates were stained with 3 ml of 0.01% neutral red-agar solution at 10 days, and plaques were counted after 11 days. Each dilution was assayed in triplicate.

Assay of inclusion-containing cells. Slide chambers were infected with 0.1 ml of diluted virus suspension. Virus was allowed to adsorb for 3 hr at 37 C before unadsorbed virus was removed by repeated washing with medium. After washing, 1.0 ml of fresh H-BME was added to each chamber, and the cultures were incubated for an additional 21 hr. The plastic chambers were then removed from the slides, leaving the infected monolayers attached. The cultures were fixed in 50% acetone-50% absolute methanol before staining. Cells containing inclusions were counted after staining with acridine orange (6, 7). Inclusion-containing cells were counted by light and fluorescence microscopy at a magnification of 250 times; 20 fields were counted per chamber. Average counts per field were multiplied by the number of fields per chamber, the reciprocal of the volume of inoculum, and the reciprocal of the virus dilution. Each dilution was assayed in triplicate.

Autoradiography. Autoradiography was used to

demonstrate the presence of newly synthesized DNA in inclusions. After adsorption, cultures were incubated for 21 hr in 1 ml of E-BME containing 5% heat-inactivated fetal calf serum and 1 μ Ci of tritium-labeled thymidine per ml (5 Ci/mmole). After incubation, slides were fixed and stained with acridine orange. Photomicrographs were taken of inclusion bodies with locations recorded by use of a stage micrometer. Slides were then washed to remove the stain, and were dipped in Kodak NTB-3 emulsion (Eastman Kodak Co., Rochester, N.Y.) diluted to a final concentration of 50% with distilled water. Coated slides were dried at 21 C and were stored in light-proof slide boxes at 4 C for 5 days. The slides were developed in Kodak D-19 (Eastman Kodak Co.) developer for 5 min, fixed in F-5 fixer for 8 min, and then stained with Giemsa (1). Inclusion-containing cells which had previously been photographed were relocated and rephotographed to determine whether the cytoplasmic inclusions stained by acridine orange contained newly synthesized DNA.

Neutralization. Neutralization experiments were carried out with 1:2 and 1:20 dilutions of immune serum, which were incubated at 37 C for 2 hr with equal volumes of undiluted virus before inoculation into cell cultures. Unneutralized virus was titrated by both plaque and inclusion-containing cell assays.

RESULTS

Adsorption of virus. Optimal adsorption time was determined by inclusion assay (Fig. 1). The number of cells containing inclusions was maximal after 3 hr was allowed for adsorption, and therefore 3 hr was used as the standard period of adsorption for subsequent

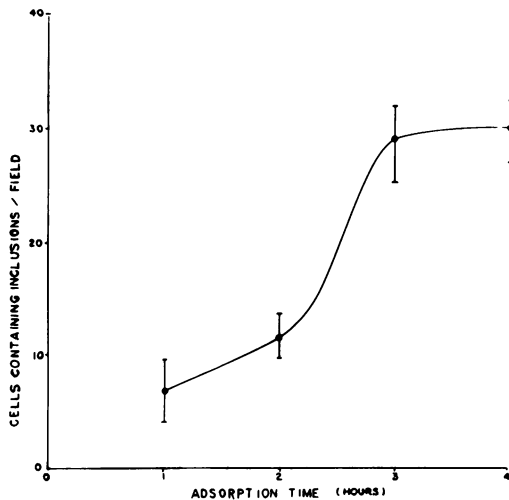


FIG. 1. Titration of virus with various adsorption times. Monolayers were inoculated with virus at a multiplicity of 0.15 cell inclusion units per cell (30 inclusion-forming units/field) and then allowed to adsorb for different periods of time before fresh medium was added and incubation was continued.

experiments.

Time of appearance of inclusions. To ascertain at which time during infection inclusion-containing cells are most numerous, assays were run in which reading times varied. Inclusions began to appear at about 15 hr after infection (Fig. 2). Counts reached a plateau at 24 hr, and began to decline at 35 hr as cells started to lyse. A period of 24 hr was chosen as the standard time after infection at which inclusions were counted.

Autoradiography. Figures 3 and 4 show that acridine orange-stained inclusions contain tritium-labeled DNA. The presence of DNA in cytoplasmic inclusions suggested that they are sites of viral DNA synthesis.

Neutralization. The neutralization experiments confirmed that inclusions are of viral origin. As shown in Table 1, a 1:2 serum dilution produced a 10-fold drop in virus titer, and a 1:20 serum dilution produced a 3-fold drop.

Quantitative evaluation of the assay. Two different batches of virus were assayed by both the inclusion-containing cell count method and the plaque method (Table 1). The results showed virus titers to be directly and linearly related to infectious virus concentration in both cases (Fig. 5). Counts made with acridine orange and May-Grünwald-Giemsa staining gave similar results, but the Giemsa-stained cultures were easier to read and this was the stain of choice.

Parallel experiments in which the plaque assay was used gave proportional results

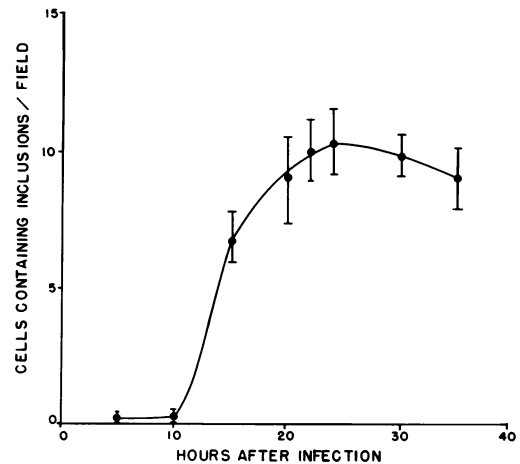


FIG. 2. Appearance of inclusion-containing cells with time. Monolayers were inoculated with virus, which was allowed to adsorb for 3 hr before fresh medium was added. Inclusion-containing cell counts were made after fixation and staining with May-Grünwald-Giemsa at various times after inoculation.



FIG. 3. Infected monolayers of bovine embryonic kidney cells stained with acridine orange. The binucleate cell in the center of the picture contains two small virus-specific cytoplasmic inclusion bodies (see arrows).

(Table 1). It should be noted that the quantitative estimation of infectious virus by the count of the inclusion-containing cells yielded a value approximately 3.5 times as high as that attained by the plaque assay method.

Bovine paravaccinia virus replicates in bovine embryonic kidney cells at a rate such that

the inclusion-containing cell counts reach a maximum at about 24 hr (Fig. 2); therefore, this time was chosen as the standard incubation period for the assay. The assay requires a total of about 26 hr to complete, as opposed to the plaque assay method which requires 11 days.

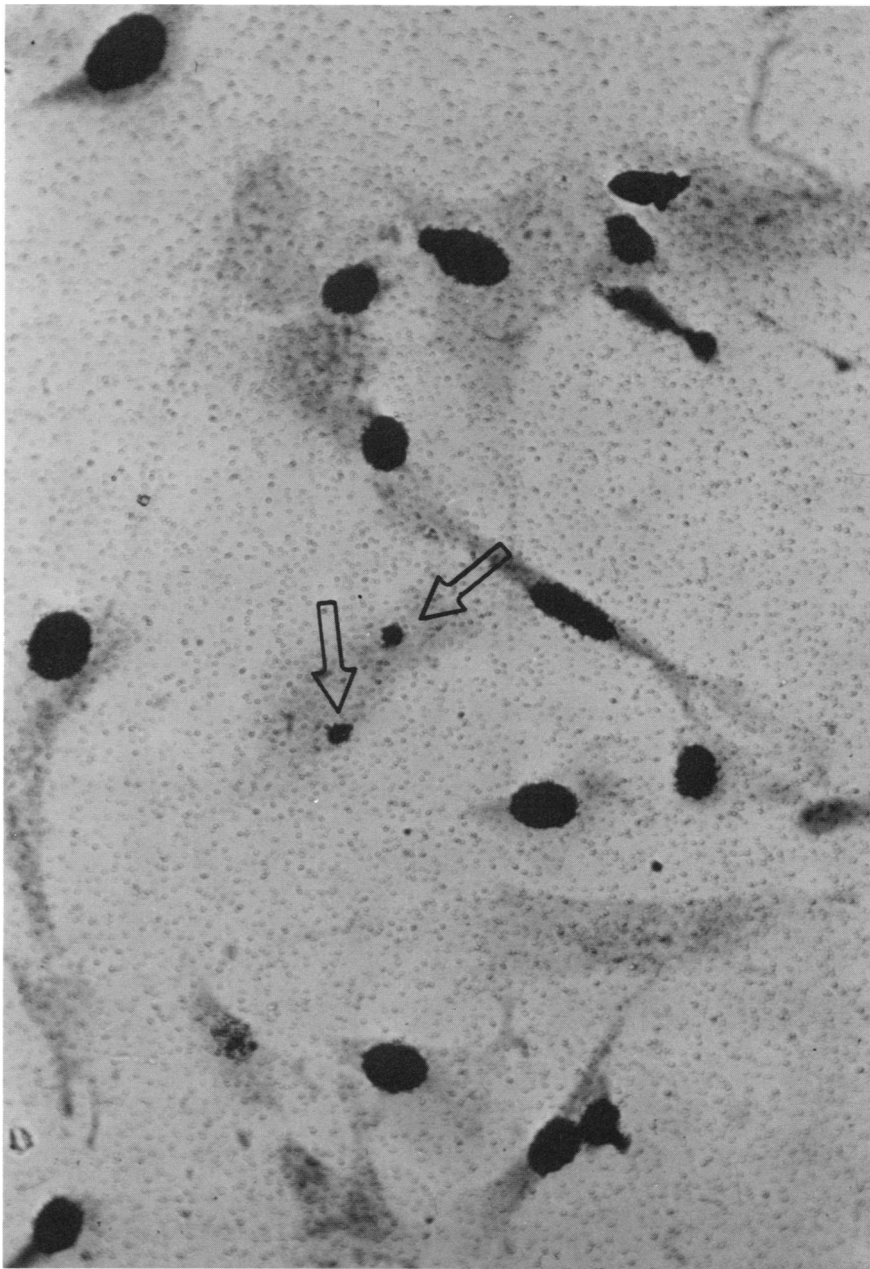


FIG. 4. Same monolayer as in Fig. 3 after completion of autoradiography. Tritiated thymidine has been incorporated in nuclei in which DNA synthesis has occurred during incubation, as well as in the virus-specific cytoplasmic inclusions shown in Fig. 3.

DISCUSSION

Methods for the quantitation of virus by enumeration of cells containing inclusions have been described for several poxviruses. Hahon (4) reported a method for assay of va-

riola virus by use of fluorescein-conjugated antiviral antibody. Hodes (5) described a similar assay for Shope fibroma virus, which involves light-field microscopy as well as immunofluorescence. Here, we have described an inclusion assay for still another poxvirus,

TABLE 1. Comparison of assays^a

Prepn	Plaque assay (PFU/ml)			Inclusion-containing cell assay (CIU/ml)		
	No antiserum	Antiserum		No antiserum	Antiserum	
		1:20	1:2		1:20	1:2
1	$8.0 \pm 1.0 \times 10^4$	$3.1 \pm 0.4 \times 10^4$	$0.69 \pm 0.06 \times 10^4$	$29.1 \pm 0.6 \times 10^4$	$9.0 \pm 0.4 \times 10^4$	$2.8 \pm 0.2 \times 10^4$
2	$26.8 \pm 2.9 \times 10^4$	—	—	$86.3 \pm 2.8 \times 10^4$	—	—
3	$7.8 \pm 0.7 \times 10^4$	—	—	$27.2 \pm 1.7 \times 10^4$	—	—

^a Results are given as plaque-forming units (PFU) or cell inclusions units (CIU) \pm the standard deviation.

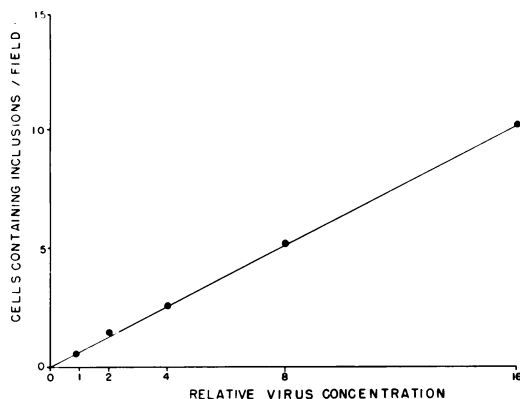


FIG. 5. Variation of number of inclusion-containing cells per field in relation to the concentration of infectious virus particles.

bovine paravaccinia.

Bovine paravaccinia virus, the agent of milk-er's nodules, has previously been assayed by plaque count (3) and quantal cytopathic effect (2). Like other poxviruses, it produces easily recognizable inclusion bodies, and therefore lends itself to inclusion-body assay.

In this study, two methods have been used to show that the cytoplasmic inclusions which appear during viral infection are of viral origin. By autoradiography, with the use of ³H-thymidine, cytoplasmic inclusion bodies were shown to be sites of DNA synthesis. Since these cytoplasmic inclusion bodies were seen only in cells infected by the paravaccinia virus, and since such sites of DNA synthesis are not seen in normal cells, it was concluded that these are areas where viral DNA is synthesized. Further evidence in support of the view

that the inclusions represent sites of viral synthesis is derived from the use of specific anti-paravaccinia antiserum which reduces the number of inclusion bodies according to its concentration.

It was also shown that the number of inclusion-containing cells is linearly related to the concentration of infectious virus applied to the culture. The relationship follows typical one-hit kinetics, suggesting that one infectious virus particle per cell is sufficient to produce the inclusion(s) found in that cell.

The inclusion-containing cell assay offers advantages over the plaque assay method because it is more sensitive and requires only 26 hr to complete.

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