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DOT ELISA FOR DIRECT DETECTION OF BK VIRUS IN URINE SAMPLES

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Summary. — The dot ELISA technique was applied for direct detection of BK virus in clinical urine samples. The assay was performed on nitrocellulose paper dotted with the polyethylene glycol precipitated urine samples free of cellular debris. BK virus was detected with an anti-BK virus monoclonal antibody, and the complex was visualized by immunoperoxidase staining. Positive reaction appeared as well-defined dark blue spots. Of the 110 urine samples examined, 31 were positive in the dot ELISA and 79 proved negative. Comparing with the IIF results, the dot ELISA had a 88.46% of sensibility and 90.4% of specificity, and the results agreed completely in 99 samples. The simple dot ELISA technique can be recommended for detection of BK virus excretion in routinary diagnostic.

Key words: *dot ELISA*; *BK virus*; *diagnostic*

Introduction

The BK virus (BKV), a human papovavirus, was isolated from the urine of renal transplants patients in 1971 (Gardner *et al.*, 1971). Infection with BK virus in children was described by different authors in association with respiratory symptoms (Goudmist *et al.*, 1982) and nonhaemorrhagic cystitis (Padgett *et al.*, 1983). In renal transplants patients, BKV excretion is highly frequent and was associated with urethral stenosis (Coleman *et al.*, 1978) and haemorrhagic cystitis (Arthur *et al.*, 1986).

As virus growth in culture is slow (Gardner *et al.*, 1971), following techniques to detect urinary excretion of BKV were used: electron microscopy (Lecatsas *et al.*, 1978), cytology (Coleman, 1975), immunofluorescence (Hogan *et al.*, 1980), ELISA (Arthur *et al.*, 1983) and DNA-DNA hybridization (Harley *et al.*, 1982; Gibson *et al.*, 1985). Enzyme immunoassay (ELISA) has proved to be a rapid and sensitive technique to detect the antigens of a great variety of infectious agents. However, the materials generally used in solid phase ELISA have a relatively low protein-binding capacity.

The use of nitrocellulose (NC) in Western blot has demonstrated the superior binding capacity of this substrate (Towbin *et al.*, 1979; Heberling

and Kalter, 1986). This property has been exploited by different laboratories in order to develop a technique called "dot ELISA" or "dot immunobinding" with good results (Bode *et al.*, 1984; Zerbini and Musioni, 1987; Blumberg *et al.*, 1987). The technique involves the direct application of antigens to nitrocellulose strips and detection of the antigen-antibody complex with enzyme-linked antiglobulins and substrate by forming an insoluble, coloured endproduct. We developed a method to detect BKV antigen in urines with a peroxidase conjugate and the precipitable substrate 5-bromo-4-chloro-naphtol phosphate which forms an intense blue colour on the filters.

Materials and Methods

Samples. We tested 70 urine samples from the same number of immunocompromised patients (40 renal transplants, 20 AIDS, 10 patients with malignancies submitted us four routine polyomaviruses studies) and 40 samples from the same number of people from the laboratory staff (control group).

Indirect immunofluorescence (IIF). We used the technique previously described by Hogan *et al.* (1980). The urine samples were centrifuged, the pellet was rinsed twice with 0.15 mol/l phosphate buffered saline (PBS) pH 7.4, resuspended in the same buffer, placed on the slide, air dried, and fixed with cold acetone. An anti BKV monoclonal antibody (kindly provided by J. C. Nicolas and F. Bricout) was applied in a moist chamber at 37 °C for 30 min, the slides were washed in three changes of PBS and dried. The fluorescein-conjugated goat antimouse IgG (Diagnostic Pasteur) was then applied to the test wells, the slides were incubated, washed, and dried as before and coverslips were mounted and examinated in the fluorescence microscope.

Dot ELISA. One mililiter of each clarified urine sample was mixed with 0.2 ml of 50% polyethylene glycol (PEG 6000) 0.4 mol/l NaCl in a 1.5 ml conical centrifuge tube (Eppendorf, F.R.G.) and incubated for one hour at 0 °C. Later each tube was centrifuged at 10 000 rev/min for 1 hr and the resulting pellet was resuspended in 50 ml of buffer sample (composed of 0.5% Triton X-100 in PBS). The resuspended pellet was immediately used or stored for a short time at −20 °C. The precipitates were placed in an individual well of the Hibrislot Manifold Apparatus containing a nitrocellulose membrane (0.45 ml). Thirty minutes later suction was applied to facilate the adsorption of the protein to nictrocellulose (Furuya *et al.*, 1984), then the filter was dried at 37 °C for 1 hr and then blocked for 2 hr at toom temperature with 10 ml of 2.5% bovine serum albumine (BSA) in PBS and finally rinsed twice with 0.001% Tween 20 diluted in PBS.

Immediately, the filter was incubated for 4 hr in anti-BKV monoclonal antibody (1/500 dilution), washed three times (5 minutes each) with PBS-Tween and twice with PBS alone, later on the filter was incubated for another 4 hr with a 1 : 100 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase (Inst. Pasteur Production). The monoclonal antibody and conjugate were diluted in PBS containing 0.5% BSA, and incubated in a rotatory platform at room temperature. After the last incubation, the filter was washed in the same way and the immunological reaction was developed with the substrate solution made up in 4-chloro-1-naphtol-hydrogen peroxide yielding a blue spot (for 10 − 60 min).

Controls. The supernatant of Vero cells infected with the Gardner strain of BK virus (haemagglutination titre 1 : 128) was diluted in sample buffer from 1 : 20 to 1 : 2480 and used as positive control. A urine sample with high quantity of BKV-like particles (selected by electron microscopy) was diluted 1 : 10 to 1 : 200 in PBS, treated as described above and included in all filters. As negative controls, five BKV-negative urine samples were collected from five individuals who were negative for both IF and electron microscopy; the BKV negative urine samples were processed for IF and dot ELISA as described above. To test the specificity of our assay, we applied partially purified antigen preparations of herpes simplex virus (HSV), respiratory syncytial virus (RSV) and cytomegalovirus (CMV) to the filters. Filters treated with five BKV-positive urine samples were also incubated with monoclonal antibodies against HSV, RSV or CMV, respectively. To assess the reproducibility of the assay, we tested five positive urine samples by both IF and dot ELISA in five different trials during 5 months and one positive urine sample fifteen times on the same filter.

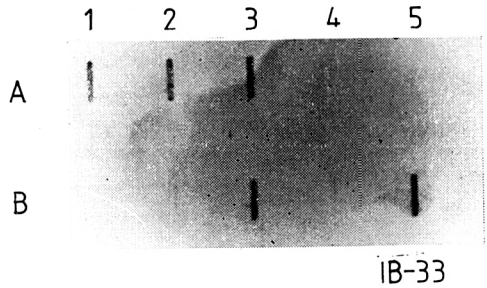

Fig. 1

Dot ELISA filter for BK virus Positions A1, A2, A3, and B3 show typical results obtained with positive samples. A4, A5, B1, and B2 were negative. B4 was the negative control (urine samples negative by electron microscopy and IIF); and B5 was the positive control (supernatant of positive culture).

Results

Fig. 1 shows the typical appearance of a dot ELISA on a NC membrane onto which urine sample has been adsorbed. One hundred and ten urine specimens were examined by this technique for the presence of virus BK and the results were compared with the IF technique. Of the urine samples tested, 31 were positive by dot ELISA (28.18%). The number of positive patients in each of the clinical categories investigated is shown in Fig. 2. BKV was detected in all groups, including the control patients (12.5% positives).

Comparing to IF results (Fig. 3), 23 samples were positive in both assays, 76 were negative in both, and 11 had discordant results. Of these, 3 urine samples were IF positive but dot ELISA negative, 8 were dot ELISA positive but IF negative. The 3 specimens which gave negative results in dot ELISA but were positive in IF revealed only 1 or 2 fluorescent cells, while the 8 negative samples by IF but positive by dot ELISA had very few cells in the urine sediment. Of the latter, 5 came from healthy subjects without evident cyturia. The infected culture supernatant was positive until dilution 160 and the urine sample showing many BKV-like particles was positive until dilution 1 : 80; the routinelly used dilution in each experiment was 1 : 20.

The urine samples used as negative controls remained as such in all assays and no cross reaction was detected between the monoclonal antibody

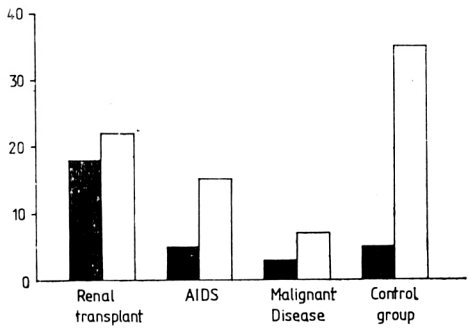

Fig. 2

Clinical state of patients and dot ELISA results.
Black columns: positive samples; white columns: negative samples.
Abscissae: per cent of positives examined.

		IF +	IF −
dot ELISA	+	23	8
	−	3	76

Fig. 3
Comparison between IF and dot ELISA for BK virus

to BKV and the other viral antigen tested. The five BKV positive urine samples assayed with monoclonal antibody to HSV, RSV, and CMV were found negative. Thus, according to these criteria, the reproducibility and repeatibility of the test was excelent.

Discussion

We have described a dot ELISA assay in which the BKV antigen from human urine was dotted by means of Hibrislot Manifold onto a NC membrane and detected with a specific monoclonal antibody, a peroxidase conjugated anti-mouse IgG, and a precipitable substrate. In preliminary experiments we applied the urine pellet directly onto the filter but the presence of cellular debris resulted in a decreased sensitivity of the assay making the interpretation of results very difficult. The precipitation of clarified urine samples with PEG-6000 provided excellent results especially when the precipitates were treated with Triton X-100 in order to expose the antigenic sites present in the inner structure of the virion. The use of filtration apparatus avoided the dots to spread on the membrane. The total time of the assay may be reduced with incubations at 37 °C but this time can vary as a function of serum and conjugate dilutions; none of them was evaluated in this study.

According to the results obtained by IF, which is routinerily used in our laboratory, dot ELISA had a 88.46% of sensibility and 90.4% of specificity, but was completely flexible with regard to the number of specimens that could be tested. With dot ELISA we could detect BKV in cell-free urine samples. This seems to us a very important point, permitting the study of healthy asymptomatic persons with very few cells in their urine.

Although the reading of this test was visual and therefore subjective, we had little difficulties in detecting positive and negative reactions. However, the spot insensity may be related to the amount of virus present in the sample (Bode *et al.*, 1984; Zerbini and Musioni, 1987; Blumberg *et al.*, 1987). The dot ELISA matches the criteria of practical and rapid assay for detecting BKV in clinical samples and may become useful tool for the rapid diagnosis of active BKV infection in man.

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