A Genetic Probe for Identifying Bluetongue Virus Infections in vivo and in vitro

By P. ROY, G. D. RITTER, Jr., H. AKASHI, E. COLLISSON and Y. INABA

1University of Alabama at Birmingham, School of Public Health, Department of Environmental Health, University Station, Birmingham, Alabama 35209, U.S.A., 2National Institute of Animal Health, Yatabe, Tsukuba, Ibaraki, Japan 305 and 3Arthropod-Borne Animal Disease Research, P.O. Box 25327, Denver Federal Center, Denver, Colorado 80225, U.S.A.

(Accepted 8 March 1985)

SUMMARY

We have used a DNA copy of segment 3 RNA of bluetongue virus serotype 17 (BTV-17) to detect sequence homology among the equivalent segments of five U.S.A. BTV serotypes (BTV-2, BTV-10, BTV-11, BTV-13 and BTV-17) as well as 14 other BTVs isolated from different endemic areas of the world. Both by in situ and Northern hybridization all the BTV serotypes were found to have RNA that reacted with the DNA probe. No homology was detected with epizootic haemorrhagic disease virus serotype 1, a related orbivirus. The BTV-17 DNA clone has also been used to detect viral RNA in infected sheep blood. This information has led us to develop a simple and sensitive procedure for the detection of viral genome biotinylated clone DNA hybrids in vivo or in cultured cells following direct staining with either the avidin-fluorescein complex or the streptavidin-horseradish peroxidase complex.

Bluetongue is an arthropod-borne disease of sheep and cattle caused by a segmented genome, double-stranded, RNA virus, belonging to the Orbivirus genus (BTV, family Reoviridae). In sheep, the disease is characterized by high morbidity and variable but significant mortality. In cattle, mortality is usually lower, although various complications of the disease occur. The disease affects not only the reproduction of sheep and cattle but, for recovered animals, causes loss of weight, loss of wool and reduction in milk production. Cattle can become persistently infected and are capable of transmitting the virus to the foetus. Semen can also be a reservoir of the virus. In the light of these observations, BTV is of considerable concern to those involved in the export and import of animals and their germ plasm. World-wide, 22 serotypes of BTV have been identified and in the U.S.A. five serotypes of BTV have been recognized (BTV-2, BTV-10, BTV-11, BTV-13 and BTV-17). The demonstration by oligonucleotide fingerprint analyses of genetic drift and reassortment lends substance to the conclusion that in the U.S.A. BTVs form a common gene pool (Sugiyama et al., 1981, 1982).

Serological testing is used extensively to detect the presence of specific antibodies against BTV. Unfortunately, the serological procedures have limitations. Often cross-reaction occurs with other members of the orbivirus group including epizootic haemorrhagic disease virus (EHDV), Eubenangee and Palyam viruses (Moore, 1974). For EHDV, cross-reactions are of particular concern. Another drawback is that the current serological tests are not very sensitive (Bowen et al., 1980; Gorman et al., 1983). In an attempt to develop an alternative, more specific procedure, we have recently cloned into DNA and sequenced BTV-17 RNA segment 3 (Purdy et al., 1984). By Northern blot hybridization we have shown that this DNA hybridized not only to RNA segment 3 of BTV-17 but also to segment 3 of other U.S.A. BTV serotypes, namely BTV-10, BTV-11 and BTV-13. The DNA did not hybridize to RNA derived from EHDV serotype 1 (EHDV-1). Since only U.S.A. serotype viruses were analysed it was possible that the observed cross-reactions were particular to these viruses and not representative of isolates obtained.
Fig. 1. Resolution by gel electrophoresis of the genome RNA segments of BTV. Genome RNAs of BTV-1 to BTV-15 (S. Africa), BTV-16 (W. Pakistan), BTV-17 (U.S.A.), BTV-20 and BTV-21 (Australia) were purified from infected BHK-21 cells and resolved in 1% agarose gel as described previously (Purdy et al., 1984). The RNA segments of each BTV serotype were identified by staining the gel in ethidium bromide. Numbers indicate serotype number.

elsewhere. In this communication we demonstrate by in situ and Northern hybridization the cross-reactivity of the test to 19 BTV serotypes (all those that were tested). In situ hybridization has been applied not only to infected tissue culture samples but also to blood samples obtained from infected sheep.

In order to detect whether cloned DNA of segment 3 of BTV-17 shared any homologous gene sequences with other BTV serotypes (prototype strains that were originally isolated from South Africa, Pakistan and Australia), we applied the Northern blot hybridization technique. Purified double-stranded RNAs of each BTV serotype were electrophoresed and blotted on Genescreen (New England Nuclear) as described previously (Purdy et al., 1984; Sugiyama et al., 1982). Fig. 1 shows the electrophoretic patterns of the 10 RNA segments of 19 BTV serotypes. As noted previously (Sugiyama et al., 1982) for four U.S.A. serotypes (BTV-10, BTV-11, BTV-13 and BTV-17), the 19 BTV serotypes exhibited strikingly similar patterns of RNA segments in this gel system. Fig. 2 shows the results of hybridizing the blotted viral RNA segments to 32P-labelled nick-translated DNA of segment 3 of BTV-17. As shown in Fig. 2, only RNA segment 3
Fig. 2. Hybridization by Northern blotting of clone DNA of RNA segment 3 of U.S. BTV-17 to corresponding RNA segments of other BTVs. Autoradiograms of nick-translated, $^{32}$P-labelled cDNA clone hybridized to genome RNAs of 19 BTV serotypes (see Fig. 1) blotted on Genescreen paper are shown. Numbers at the top of each lane indicate serotype number; numbers on the left indicate RNA segment number. (b) shows better hybridization of RNA segment 3 of BTV-7 and BTV-8 than does (a) (see text).

of each serotype hybridized to the cDNA clone. The low level of hybridization to BTV-7 and BTV-8 in Fig. 2(a) was primarily due to the smaller quantities of RNA applied to the gel since other analyses (see Fig. 2b) gave stronger signals.

Our initial experiments to detect viral gene sequences in infected cell cultures employed a nick-translated $^{32}$P-labelled DNA probe. BHK-21 cells or hamster lung (HmLu) cells were grown on four- to eight-compartment chamber slides (Miles Laboratories) and infected at a multiplicity of 0.1 to 1 p.f.u./cell with each BTV serotype. At 16 to 20 h post-infection, slides containing infected cell cultures were removed from the growth medium and rinsed three times with phosphate-buffered saline (PBS). The cells were fixed in Carony’s B fixative (60% ethanol, 30% chloroform, 10% acetic acid) for 5 min according to the 1983 instruction manual of Enzo Bio-Chem. Inc. (N.Y., U.S.A.). The samples were then drained, air-dried and stored at 4 °C. Each chamber containing dehydrated cells was overlaid with 25 to 50 μl of the hybridization mixture containing the $^{32}$P-labelled DNA probe. The lid of the chamber slide was then sealed with tape, and the assemblies placed in a container, and heated at 80 °C in a bath for 5 min as described by Brigati et al. (1983). The slides were then transferred to a 37 °C incubator for 12 to 16 h. After hybridization the solutions were removed and kept at 4 °C for re-use. The chambers and plastic liners were removed from the chamber slides and each slide was washed in 2 × SSC and PBS as suggested by the Enzo Bio-Chem. Inc. manual. For detecting hybridized DNA containing radioactivity, the slides were simply dried and autoradiographed. Fig. 3 illustrates the results. In some wells the cells detached prior to 16 h (e.g. BTV-2, BTV-6); therefore, the analysis was repeated using a lower multiplicity of infection (Fig. 3b; m.o.i. = 0.01 to 0.1 p.f.u./cell). The presence of viral infection was detected by this procedure in each of the monolayers containing infected cells; none was detected in the control, uninfected cells.

Similar hybridization experiments were performed using a nick-translated, biotin-labelled, DNA probe instead of a $^{32}$P-labelled probe. For the fluorescence detection of hybridized DNA containing biotin, the probe was removed, the slides washed, air-dried as described above and then 25 to 50 μl fluorescein-conjugated avidin (0.1 mg/ml; Becton-Dickinson) was added to each chamber. The samples were incubated at 18 °C for 30 min, then washed three times in PBS for 5 min, blot-dried, mounted with glycerol and examined for fluorescence. Fig. 4 shows the typical results for hybrids that were identified following incubation with the conjugated avidin-
fluorescein complex. Again, strong fluorescent signals were observed with each of the BTV-infected cells. In contrast, none was observed either with the control cells or for cells infected with EHDV-1 (Fig. 4b). For BTV-10 and BTV-11 (Fig. 4c, d) intense signals of fluorescence appeared to be localized in the nucleoli. Fluorescence was dispersed throughout the nucleus of the cells infected with BTV-13 and BTV-17 (Fig. 4e, f). The reasons for the observations are not known.

For visualizing the biotinylated hybrids in blood cells we used an enzymic method developed by Singer & Ward (1982). Blood was collected in heparinized tubes from uninfected sheep or from sheep infected with either BTV-2 or BTV-11 7 days after viral inoculation. After centrifugation at 2000 r.p.m. for 20 min the sera were discarded and the cells collected and washed three times with PBS. The blood samples were then deposited on pretreated glass coverslips (Brigati et al., 1983) by cyto-centrifugation for 10 min at 2000 r.p.m. The deposited cells were then fixed, air-dried and subjected to in situ hybridization as described above, except that pieces of parafilm were used to seal the coverslips. In order to detect the hybrids by peroxidase staining, a soluble complex of biotinylated horseradish peroxidase and streptavidin (Detek-1-hrp) was used (Enzo Bio-Chem. Inc.). Samples were incubated with 25 to 50 µl of Detek-1-hrp for 30 min at 37 °C and then washed with 2 × SSC and PBS. The monolayers were stained for 2 to 5 min with 0.05 % diaminobenzidine tetrahydrochloride (DAB, Enzo Bio-Chem. Inc.) containing 0.001 % hydrogen peroxide. The reaction was stopped by washing in 2 × SSC. For counter-staining the cells, 50 µl of 0.25 % methyl green (Aldrich) in 0.03 M-sodium acetate buffer pH 4.8 was added to each sample (1 min). Each slide was then rinsed with acetate buffer, blot-dried, dehydrated by dipping in acetone:xylene (1:1), rinsed in xylene and then mounted in Permount (Fisher Scientific, Fairlawn, N.J., U.S.A.) and examined under a phase-contrast microscope. Fig. 5 illustrates the results obtained for sheep blood samples examined under a phase-microscope. The brown stains shown in the white blood cells of sheep infected by BTV-11 and BTV-2 (Fig. 5b, c) are the typical signs of the presence of biotinylated hybrids in the samples. The control, uninfected cells showed no such brown stain, indicating a lack of the biotinylated probe in the samples.

To obtain some measure of the relative sensitivity of the in situ hybridization system, HmLu cells were infected with BTV-2 at different m.o.i. (0.005 to 0.5) of virus. Infected chamber slides were fixed at different time intervals post-infection and were processed for in situ hybridization with nick-translated 32P-DNA probes. The presence of genomic RNA was detected as early as 8 h post-infection with 0.5 m.o.i. (data not shown).

Brigati et al. (1983) have recently reported (using similar procedures to those described here) the detection of various viral genomes (e.g. polyoma virus, Rous sarcoma virus, adenovirus,
Fig. 4. Fluorescence detection of the hybrids in cultured cells. Nick-translated biotinylated dUTP-labelled cDNA clone was hybridized to viral genomes present in BHK-21 cells infected with either EHDV-1 (b), BTV-10 (c), BTV-11 (d), BTV-13 (e) or BTV-17 (f) or HmLu cells infected with either BTV-11 (h), BTV-17 (q), BTV-20 (k) or BTV-21 (l) in chamber slides are shown. The hybrids were detected by fluorescence microscopy following incubation with avidin–fluorescein complex for 30 min at 18 °C. (a) Control uninfected BHK-21 cells; (g, j) control uninfected HmLu cells.
Fig. 5. Detection of BTV infection in sheep red blood cells using horseradish peroxidase following in situ hybridization. Blood samples from control sheep (a) and from sheep infected by either BTV-11 (b) or BTV-2 (c) were fixed and hybridized with a biotinylated probe as described in Methods. Each hybrid was incubated with Detek-1-hrp for 30 min at 37 °C followed by staining with DAB and counterstaining with methyl green.
Short communication

herpes simplex virus, etc.) in infected cell cultures and cytological samples. In this report we have described the detection of BTV representing 19 serotypes recovered from different areas of the world. The DNA probe evidently represents a conserved structural gene. The utility of the probe for screening both acute and inapparent BTV disease in infected animals and their germ plasm is currently under investigation.

We thank Mr L. R. Meisen for expert assistance and for helpful advice on the fluorescence microscopy and Hedeki Nago for excellent technical assistance. This work was supported by the U.S. Department of Agriculture Grant No. 82CRCR-2-1032.

REFERENCES


(Received 24 January 1985)