# Detection of bluetongue virus and African horsesickness virus in co-infected cell cultures with NS1 gene probes

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## ABSTRACT

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The serogroup specificity of the bluetongue virus (BTV) NS1 and VP3 gene probes was confirmed by means of northern blot hybridization. Under high-stringency conditions both probes hybridized to 22 BTV serotypes (18 South African serotypes, BTV3 from Cyprus and BTV16 from Pakistan) but not to serotypes that originate from Australia and India. Furthermore, NS1 gene probes of BTV and African horsesickness virus (AHSV) were used in a dot-spot *in situ* hybridization procedure to differentiate between BTV and AHSV in co-infected cell cultures. The method detects viral RNA directly in glutaraldehyde-fixed infected cell cultures without prior nucleic-acid extraction or purification. AHSV could be detected in cells infected with AHSV at a multiplicity of infection of $10^{-4}$ PFU/cell in the presence of a hundredfold excess of co-infecting BTV. The method may have an application in epidemiological surveys to detect different orbiviruses in the same *Culicoides* population.

Keywords: African horsesickness virus, AHSV, bluetongue virus, BTV, detection, NS1 gene probes, VP3 gene probes

## INTRODUCTION

Outbreaks of bluetongue and African horsesickness in susceptible sheep and horse populations, respectively, can have severe economic implications. These viral diseases are transmitted biologically by biting midges of the *Culicoides* genus (Du Toit 1944) and are endemic in South Africa. Rapid and accurate detection of these viruses is important for efficient control and epidemiological surveillance. Several different Orbivirus serogroups and/or serotypes of the same serogroup can co-circulate in *Culicoides* populations (B.J. Erasmus & E.M. Nevil 1995, personal communication), complicating cell-culture isolation of all the viruses present in such mixed infected *Culicoides* populations.

Bluetongue and African horsesickness viruses (BTV and AHSV) represent two of the serogroups of the *Orbivirus* genus of the Reoviridae family (Knudson & Shope 1985). Virions of BTV and AHSV have ten double-stranded RNA-genome segments encapsidated in a double-layered protein capsid comprised of seven structural proteins (VP1-7), while three non-structural proteins (NS1, NS2 and NS3) are synthesized in infected cells (Huismans & Van Dijk 1990). Hybridization experiments in which cloned genome segments were used as probes, established that genome segments that encode proteins NS1 and VP3 of BTV, AHSV, equine encephalosis virus (EEV) and epizootic haemorrhagic disease virus (EHDV) are well conserved within their respective serogroups (Huismans & Cloete 1987; Bremer, Huismans & Van

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Dijk 1990; Venter, Van Dijk, Huismans & Van der Lugt 1992; Viljoen & Huismans 1989; Nel & Huismans 1990). In a dot-spot *in situ* hybridization procedure that detects virus-specified RNA directly in glutaraldehyde-fixed infected cell cultures without prior nucleic-acid extraction or purification (Paeratakul, De Stasio & Taylor 1988), the NS1 probes detected viral RNA significantly earlier than those of any of the other conserved genes (Venter, Viljoen, Nel, Huismans & Van Dijk 1991). In the case of BTV and EHDV, it has been shown that the advantage of the NS1 probe is due to the fact that the NS1 gene is transcribed at a higher frequency than are the other genome segments (Huismans & Verwoerd 1973; Huismans, Bremer & Barber 1979).

In this paper the use of the NS1 gene as group-specific probe for detecting replicating BTV and AHSV is further investigated, with particular emphasis on serogroup-specificity and the possibility of detecting viruses in cell cultures co-infected with BTV and AHSV.

## MATERIALS AND METHODS

### Viruses

All viruses were obtained from Dr B.J. Erasmus (Onderstepoort Biological Products). The BTV serotypes were virulent viruses propagated in cell cultures, with the exception of serotype 10, which was an egg-attenuated avirulent strain. The geographical origins of the different BTV serotypes are summarized in Table 1. The viruses were propagated by limited passaging in monolayer BHK-21 cells (Huismans 1979). The titres of inoculums were determined on monolayers of Vero cells and expressed as plaque-forming units per m$\ell$ (PFU/m$\ell$) (Howell, Verwoerd & Oellermann 1967).

### Northern-blot hybridization of dsRNA from different serotypes

Viral dsRNA was purified from infected BHK-cell cultures as described by Bremer (1976). Two hundred nanograms dsRNA of each of the 24 BTV serotypes was fractionated by electrophoresis on 4% polyacrylamide gels (Loening 1967). $^{32}$P end-labelled BTV4 dsRNA served as a control size marker. After electrophoresis, dsRNA was denatured by soaking the gel in 50 mM NaOH, transferred electrophoretically to a Hybond-N membrane (Amersham) and fixed by exposure to UV light, before hybridization to genomic probes (Maniatis, Fritsch & Sambrook 1982).

### Dot-spot *in situ* hybridization technique

Confluent monolayers of BHK-21 cells were infected with BTV or/and AHSV at the multiplicity of infection (m.o.i.) indicated in the text. Viruses were adsorbed to the cells for 45 min at 37 °C, after which the cells

TABLE 1 Origin of the different BTV isolates

| BTV serotypes | Country of origin |
| --- | --- |
| 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 14, 15, 18, 19, 22, 24 | South Africa |
| 3 | Cyprus |
| 16 | Pakistan |
| 17 | USA |
| 20, 21 | Australia |
| 23 | India |

were rinsed once with serum-free Eagle's medium, followed by further incubation at 37 °C. At different periods after infection the cells were harvested by trypsination, collected by low-speed centrifugation and suspended in Eagle's medium. Hundred-microlitre volumes of appropriate dilutions of cells were filtered onto Hybond-N membranes (Amersham), previously wetted with double-distilled water by means of a 96-well manifold-filtering apparatus (Bio-Rad). Cells were fixed on membranes in a solution containing 1% glutaraldehyde, 3% NaCl, 10 mM NaH$_2$PO and 40 mM Na$_2$HPO (pH 7,4) at 4 °C for 1 h, and rinsed three times with 50 mM EDTA, 100 mM Tris-HCl (pH 8,0) proteolytic buffer, containing proteinase K, at a concentration of 20 µg/m$\ell$ for 30 min at 37 °C, as described by Paeratakul *et al.* (1988). The filters were briefly air-dried and prehybridized for at least 4 h at 42 °C, in a buffer containing 50% formamide, 1 x Denhardt's solution, 4 x SSC buffer, 0,02 M Na-phosphate buffer (pH 6,8), and 0,1 mg/m$\ell$ of herring-sperm DNA.

### End-labelling of dsRNA

Unfractionated BTV4 dsRNA was radio-actively labelled to produce size markers for electrophoresis, with the use of cytidine 3´,5´- [5´-$^{32}$P] biphosphate ([$^{32}$P]pCp) triethylammonium salt and RNA ligase (England & Uhlenbeck 1978; England, Bruce & Uhlenbeck 1980). $^{32}$P-labelled RNA was separated from unincorporated labelled nucleotides by Sephadex G-75 column chromatography. The specific activity of the labelled RNA was 1 x 10$^7$ cpm/µg.

### Preparation of $^{32}$P radio-labelled probes

DNA probes were labelled with $^{32}$P-dCTP (1000 Ci/m$\ell$) by nick-translation of cloned segments of the VP3 and NS1 genes of BTV and the NS1 gene of AHSV. A commercially available BRL kit was used (Huismans & Cloete 1987; Bremer, Huismans & Van Dijk 1990). The specific activity of probes was generally in the order of 5 x 10$^7$ cpm/µg DNA.

## Hybridization

Nylon membranes containing fixed dsRNA and cells, were hybridized to the different genomic probes in a hybridization mixture containing 50% formamide, 1 x Denhardt's solution, 4 x SSC buffer, 0,02 M Na-phosphate buffer (pH 6,8), and 0,1 mg/ml of herring-sperm DNA. The probes were boiled and immediately cooled before they were added to the hybridization solution. Hybridization was performed for 16 h at 42°C, after which membranes were washed under stringency conditions requiring 90% similarity for hybridization, namely in a buffer containing 0,1% SDS and 0,1 x SSC at 65°C (four to five times for 15 min each). Autoradiography was carried out by exposure of the membranes to Cronex MRF X-ray film (Dupont, USA) at −70°C, with the use of an intensifying screen.

## RESULTS AND DISCUSSION

### Specificity of the BTV4 NS1 and VP3 gene probes

Since some reservations have been expressed about the use of dot-spot hybridization to determine the genetic relatedness of cognate RNA segments of different BTV serotypes (Unger, Chuang, Chuang, Doi & Osburn 1988), northern blot hybridizations were performed to verify the dot-spot results of Huismans & Cloete (1987) in respect of the serogroup specificity of the NS1 and VP3 gene probes. The data depicted in Fig. 1 confirms that both genes are conserved within the BTV serogroup. Under conditions requiring more than 90 % nucleic-acid-sequence similarity, both probes hybridized to the 18 South African serotypes, BTV3 from Cyprus and BTV16 from Pakistan.
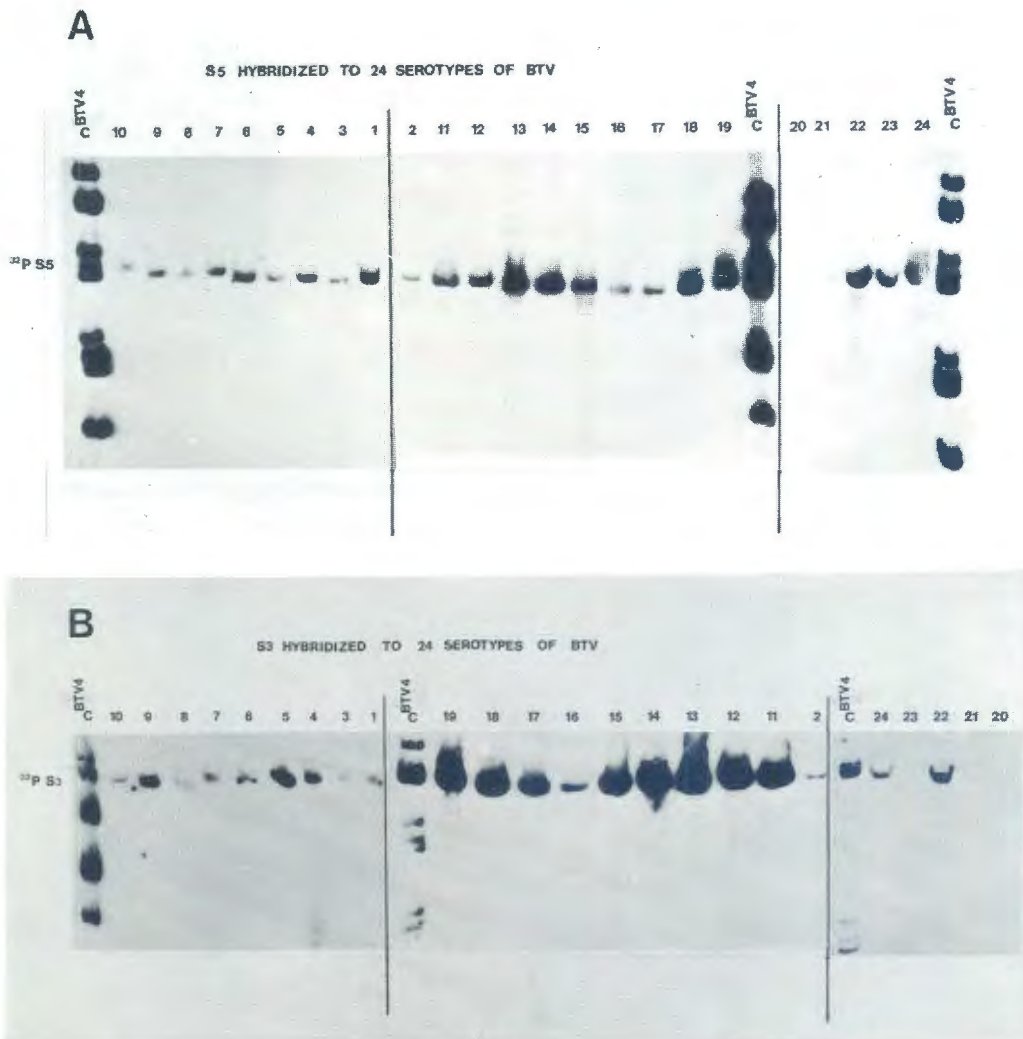


FIG. 1  A. Autoradiograph of the hybridization of a $^{32}$P-labelled BTV4 NS1-specific DNA probe to a northern blot of dsRNA of the 24 serotypes of BTV. The size marker (C BTV4) is BTV4 end-labelled dsRNA

B. Autoradiograph of the hybridization of a $^{32}$P-labelled BTV10 VP3-specific DNA probe to a northern blot of dsRNA of the 24 serotypes of BTV. The size marker (C BTV4) is BTV4-labelled dsRNA

Neither probe reacted with serotypes 20 and 21 which originate from Australia. In addition, the VP3 probe did not react with serotype 23 from India. The sensitivity of the northern blot appears to be lower than that of the dot-spot hybridization procedure, where faint signals were detected with serotypes 20 and 21 with both probes. There is a general correlation in the relative intensity of the hybridization signals between the NS1 and VP3 gene probes of the different serotypes (Fig. 1A & 1B), indicating that the variation in the intensity of hybridization signals among the serotypes largely reflects differences in the nucleotide sequence of the cognate genes. The results are also in agreement with the dot-spot hybridization results of Huismans & Cloete (1987) and the results of Gould & Pritchard (1990), who carried out a limited
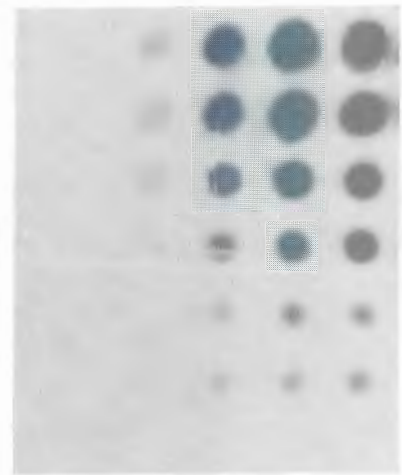
A

**BTV PROBE: CO-INFECTED CELLS**

| BTV PFU/cell | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | $10^{-2}$ | $10^{-1}$ | 1 |
| AHSV PFU/cell | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ |
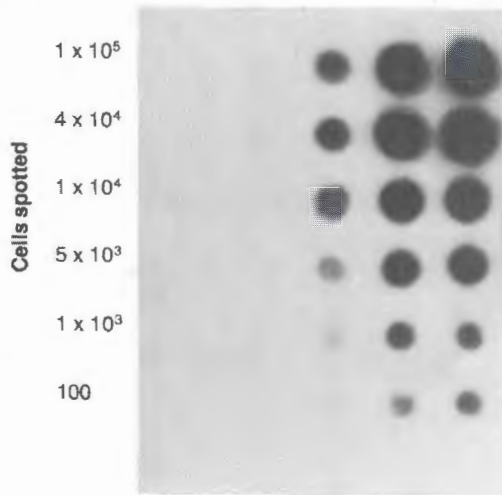
**BTV PROBE: INDIVIDUALLY INFECTED CELLS**

| BTV PFU/cell | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | $10^{-2}$ | $10^{-1}$ | 1 |

B

**AHSV PROBE: CO-INFECTED CELLS**

| BTV PFU/cell | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | $10^{-2}$ | $10^{-1}$ | 1 |
| AHSV PFU/cell | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ | $10^{-4}$ |
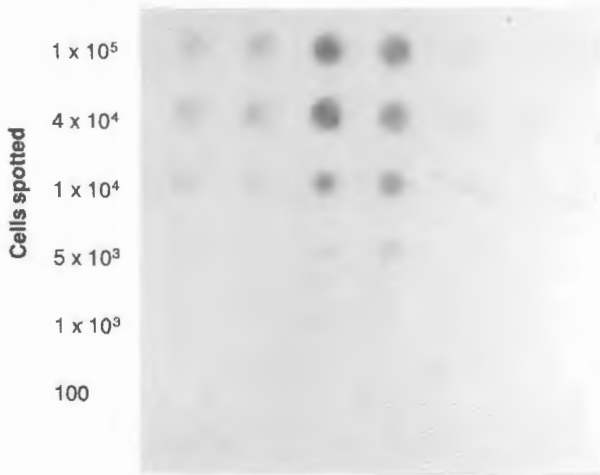
**AHSV PROBE: INDIVIDUALLY INFECTED CELLS**

| AHSV PFU/cell | $10^{-4}$ |

FIG. 2  A. Autoradiograph of the hybridization of BTV4 NS1 probe

B. Autoradiograph of the hybridization of AHSV3 NS1 probe to cells infected with BTV4 at a m.o.i. varying from 1 x $10^{-5}$–1 PFU/cell and AHSV3 virus at a m.o.i. of $10^{-1}$ PFU/cell as indicated above the lanes. The cells were harvested at 36 h p.i.

nucleotide-sequence comparison of conserved genes of isolates of the same BTV serotypes. Their results indicated that the variation in nucleotide sequence within a particular geographical region is less than that of regions which are far apart. Specifically, nucleotide sequences of conserved cognate genes of South African and Australian BTVs were reported to have a variation of about 20 %, while those of South African and North American BTVs differ by approximately 10 %.

## Detection of viral RNA in cells co-infected with BTV and AHSV

Since RNA probes have been reported to be more sensitive than DNA probes, a positive- and a negative-sense single-stranded RNA (ssRNA) and a DNA NS1 gene probe in the *in situ* dot-spot hybridization procedure were compared. It was considered that there was no advantage in using ssRNA probes. The sensitivity of the negative-sense ssRNA was the same as that of the DNA probe, and with both ssRNA probes there was considerably more non-specific background than with the DNA probe, similar to what has been reported by Squire, Stott, Dangler & Osburn (1987). The positive-sense ssRNA probe yielded only background signals (data not shown).

Experiments were carried out to investigate under which conditions the *in situ* dot-spot hybridization procedure could be used to differentiate between different orbiviruses in the case of mixed infections. In a first experiment, monolayers of BHK cells were infected with AHSV3 at a m.o.i. of $10^{-4}$ PFU/cell and co-infected with BTV4, with a m.o.i. range of $10^{-5}$–1 PFU/cell. Controls included cells individually infected, under the same conditions, with either BTV or AHSV. All infections were done in sixfold to allow harvesting at six different time intervals between 16 and 60 h p.i. Different dilutions of the harvested cells were spotted on duplicate membranes and fixed. The duplicate membranes were probed with the $^{32}$P-labelled BTV4 or AHSV3 NS1-gene DNA probe, respectively (Fig. 2).

Hybridization signals with the AHSV probe were observed from 36 h p.i. onwards, in as few as $1 \times 10^4$ cells. The results at later times yielded very little additional information. No cross-hybridization was observed between BTV and AHSV when the respective NS1 gene probes were used. As expected, the detection of AHSV in cells co-infected with BTV, depended on the ratio of infectious BTV to AHSV particles. When the cell cultures were infected with AHSV at a m.o.i. of $10^{-4}$ PFU/cell, the virus could be detected 36 h p.i., even when the cells were co-infected with BTV in a hundredfold excess (m.o.i. of $10^{-2}$ PFU/cell) (Fig. 2B). At a BTV m.o.i. of $10^{-1}$ PFU/cell (a thousandfold excess) or more, BTV completely outcompeted AHSV and no evidence of AHSV replication was observed. No significant increase in the strength of the AHSV hybridization was observed

when cells were harvested at longer periods p.i. (data not shown). To investigate the influence of a higher AHSV m.o.i. on the replication of both AHSV and BTV in co-infected cells, the above-mentioned experiment was repeated, the investigators using an AHSV m.o.i. of either $10^{-1}$ or $10^{-2}$, co-infected with BTV at the same m.o.i. range as in the previous experiment. The results indicated that at a higher m.o.i. of AHSV, AHSV could be detected at least 6 h earlier, p.i. and in the presence of increased amounts of BTV, provided—as in the previous experiment—that BTV was not present in more than a hundredfold excess (data not shown).

The dot-spot *in situ* hybridization procedure compares well with a rapid and sensitive hybridization assay in solution, for the quantitative determination of rotaviral RNA with the use of an RNA probe which detected rotavirus RNA in 5000 infected cells at 48 h p.i. (Johnson & McCrae 1988). This report is the first description of conditions under which the dot-spot hybridization procedure could be of use in orbiviral epidemiological surveillance for detection and isolation of different orbiviruses in mixed infected *Culiciodes* populations.

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