Isolation of a Bisegmented Double-stranded RNA Virus from Thirlmere Reservoir


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SUMMARY

A novel bisegmented double-stranded RNA virus has been isolated from water processed from Thirlmere reservoir. The virus is icosahedral, 58 nm in diam., has a buoyant density of 1.32 g/ml in CsCl, has an S value of 400 and a RNA/protein ratio of 0.087. The two linear segments of RNA have approx. mol. wt. of $2.26 \times 10^6$ and $2.09 \times 10^6$. The virus contains six polypeptides. The virus was isolated in Drosophila melanogaster cells and fails to replicate in other insect, amphibian, avian, piscine, mammalian and plant cells tested. The virus is biochemically different from infectious pancreatic necrosis virus (IPNV) and Drosophila X virus (DXV). The virus is also serologically unrelated to IPNV (strain Sp) and another invertebrate pathogenic virus, Tellina virus 1. The virus shares common antigens with DXV but is not completely identical.

INTRODUCTION

In recent years considerable interest has been shown in water as a physical medium in which viruses can both survive and be disseminated. Naturally, viruses of public health or veterinary importance have received most attention, although bacteriophages and plant viruses have also been studied. We were interested in detecting invertebrate pathogenic viruses in water, and a pilot study of a lake and a reservoir in the Cumbrian Lake District was carried out using a system of concentrating viruses from water described by Logan et al. (1981). This method involves removal of particulate material by prefiltration, adsorbing virus on to positively charged Zeta Plus 60S filters at pH 6, and subsequently eluting in a small volume at pH 9. The procedure is known to recover bacteriophages with at least 50% efficiency and should recover non-occluded invertebrate pathogenic viruses, but polyhedra or granules would be eliminated by the pre-filtration procedure.

In this pilot study we used Drosophila melanogaster (Schneider, 1972) and Spodoptera frugiperda (Vaughn et al., 1978) cells with the intention of detecting iridescent, nuclear polyhedrosis and picornaviruses. We were unsuccessful in detecting these viruses but a novel unclassified bisegmented double-stranded RNA virus was isolated and we describe the isolation and basic virological properties of this virus in this report. The virus has been designated as Thirlmere virus.

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**METHODS**

*Isolation of virus.* Virus concentrates were prepared from 210 l of lake water as described by Logan *et al.* (1980, 1981). Basically, samples were pre-filtered through 5 μm and 1 μm filters, adjusted to pH 6 to 6-5 (if necessary), and passed through a positively charged filter (Zeta Plus 60S). Bound virus was eluted in 1·5 l beef extract pH 8·5 and the pH of the eluate was lowered immediately to 7. Samples were transported on ice back to the laboratory and concentrated to about 80 ml by ultrafiltration. The percentage recovery of viruses was determined from the coliphage counts in the raw water and the final concentrate. Virus-containing samples were received at Oxford within 7 days and were filtered through a 0·45 μm Millipore filter before further use. A 1 ml amount of sample was plated on to 25 cm² Falcon flasks containing either 5 × 10⁵ *S. frugiperda* cells or 1 × 10⁶ *D. melanogaster* cells. The samples were allowed to adsorb for 1 h at room temperature, after which they were removed and replaced with 2 ml tissue culture medium. The cells were then incubated at 28 °C for a period of up to 3 weeks. The cells were monitored daily for cytopathic effects (c.p.e.) and, when a c.p.e was pronounced, part of the cell sheet was processed for electron microscopy and the remainder was stored at 4 °C for subsequent passage of virus.

*Cells.* The following cell lines maintained as described previously (Kelly & Wang, 1981) were used: *D. melanogaster*, *S. frugiperda*, *Mamestra brassicae*, *Bombyx mori*, *Helothis zea*, *Aedes albopictus*, bluegill fibroblast, rainbow trout gonad, Chinook salmon embryo, fat head minnow, chick embryo fibroblast, baby hamster kidney, and Vero cells.

*Plants.* The following species (which are collectively susceptible and sensitive to most plant pathogenic viruses) were inoculated by rubbing carborundum-dusted leaf surfaces with a gloved forefinger moistened with inoculum: *Nicotiana glutinosa*, *N. clevelandii*, *N. megalosiphon*, *N. tabacum* cv. Xanthi-nc, *Chenopodium quinoa*, *C. amaranthus*, *Phaseolus vulgaris* cv. The Prince, *Cucumis sativus* cv. Lockies Perfection and *Zea mays*. The plants were examined for symptoms for a 2-week period. The inoculum was virus-passaged twice through *D. melanogaster* cells.

*Viruses.* *Drosophila* X virus (DXV) was obtained from Dr D. Teninges (CNRS, Gif-sur-Yvette, France) and infectious pancreatic necrosis virus (IPNV strain Sp, the major European reference strain) was obtained from Dr B. J. Hill (MAFF Fish Disease Laboratory, Weymouth, Dorset, U.K.). Thirlmere virus and DXV were grown in *D. melanogaster* cells at 28 °C and IPNV in bluegill fibroblasts at 15 °C. Cells were infected at 0·1 p.f.u./cell and harvested 72 to 96 h post-infection. Cells (approx. 10⁸) were collected by centrifugation (1000 g for 15 min), resuspended in 3 ml water and sonicated for 1 min as described previously (Kelly & Leseott, 1976). The virus released from cells was centrifuged on a linear 20 to 50% (w/v) sucrose gradient at 30000 g for 2 h. The virus band was removed, the virus pelleted at 30000 g for 2 h, and resuspended in a small volume (<1 ml) of water. The virus was then centrifuged on a 34% (w/w) CsCl gradient at 35 000 g for 16 h. The virus band was removed and pelleted as above. Cell-released virus was also prepared from the medium in the same way after an initial concentration by collecting at 25000 g for 2 h.

*Plaque assay of Thirlmere virus.* Cell monolayers of *D. melanogaster* cells were seeded at 10⁶ cells per 3·5 cm plastic Petri dish and were incubated at 28 °C for 16 h in 2 ml medium. The medium was removed and 100 μl of virus was absorbed to the cells. After 1 h the inoculum was removed and replaced with a 2 ml overlay comprising equal amounts of 3% agarose (LGT agarose, Miles Laboratories, Stoke Poges, Slough, U.K.) in water and medium, and this was supplemented on solidification with 1 ml medium. The dishes were incubated at 28 °C for 72 h, and then 1 ml 0·1% (w/v) neutral red in phosphate-buffered saline was added and the dishes were incubated for another 8 h. The liquid overlay was decanted and the plaques were read 16 h after incubation at room temperature.
Table 1. Detection of virus in samples of Lake District reservoir and river water taken at time of isolation of Thirlmere virus

<table>
<thead>
<tr>
<th>Source</th>
<th>C.p.e.</th>
<th>Electron microscopic diagnosis</th>
<th>Virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S.f.*</td>
<td>D.m.*</td>
<td>S.f.</td>
</tr>
<tr>
<td>Esthwaite, inflow river</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Esthwaite, surface water</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Esthwaite, 3 m deep</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Esthwaite, bottom water</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Esthwaite, outflow river</td>
<td>—</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Thirlmere, 1 m deep</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Eluant control, pH 7:4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cell culture medium</td>
<td>—</td>
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</tr>
</tbody>
</table>

* S.f., Spodoptera frugiperda cells; D.m., Drosophila melanogaster cells.

Serology. Antiserum to Thirlmere virus was produced in rabbits by a standard procedure (Kelly et al., 1978). Antisera to DXV was provided by Dr D. Teninges, and to IPNV (strain Sp) and Tellina virus 1 by Dr B. J. Hill. A microimmunodiffusion test was performed as described by Kelly et al. (1980).

Electron microscopy. Three standard procedures were used. Cells showing c.p.e. were processed for embedding, sectioning and transmission electron microscopy as described previously (Kelly, 1976). Virus particles were negatively stained with 3% (w/v) ammonium molybdate pH 5-2, and catalase was included as a calibration standard, as described by Wrigley (1968). Double-stranded RNA was spread and visualized as described for DNA by Bud & Kelly (1977); circular PM2 DNA was used as an internal standard. Specimens were viewed on a JEOL 100 TX electron microscope. Additional calibration was achieved by photographing carbon replica grids (2160 lines/mm; EM Scope Laboratories, London, U.K.) each time the microscope was used.

RNA extraction. RNA was extracted from virus particles by resuspending virus pellets in 100 mM-Tris pH 7-8, 10 mM-EDTA, 2% SDS, 200 mM-β-mercaptoethanol, and 56% sucrose containing proteinase K (500 μg/ml). The mixture was incubated at room temperature for 30 min and deproteinized with an equal volume of buffer-saturated phenol, followed by one or two chloroform/isoamyl alcohol (24/1) extractions. B. mori cytoplasmic polyhedrosis virus RNA and reovirus type 3 RNA were gifts from Dr C. C. Payne and Dr M. A. McCrae.

Polyacrylamide gel electrophoresis of proteins and RNA. Virus structural polypeptides dissolved in SDS were resolved on 12% slab polyacrylamide gels (Laemmli, 1970) as described previously (Elliott et al., 1977). RNA was also resolved on 6% slab polyacrylamide gels (Laemmli, 1970) following dissolution in 10% SDS, 10% β-mercaptoethanol, 15% glycerol in 0-1 M-Tris pH 6-8. The gels were stacked at 20 V for 1 h and run overnight at 40 V. RNA was visualized by staining in ethidium bromide (0.5 μg/ml).

DNA, RNA and protein estimations. These were performed by diphenolamine, orcinol and folin reactions as outlined by Shatkin (1969).

RESULTS

Isolation of Thirlmere virus

Virus concentrates were prepared from five samples of Esthwaite water and one sample of Thirlmere water (Table 1). The latter sample, which contained the virus described in this report, was taken on 6th May 1980, 2 m from the edge of the reservoir at a point where the water was 1 to 2 m deep. At the time of sampling the water temperature was 9.5 °C, the pH
Fig. 1. A crystal of virus in the cytoplasm of a *Drosophila melanogaster* cell 7 days after inoculation with an extract of Thirlmere reservoir water. The virus is about 60 nm in diam. Bar marker represents 200 nm.

was 6.3 and the air temperature was 11 °C. Thirlmere is oligotrophic and this is reflected in the bacteriological counts of the raw water: 20 colony-forming units (c.f.u.)/ml on MacConkey medium, 26 c.f.u./ml on eosin–methylene blue lactose agar and 0.6 presumptive *Aeromonas hydrophila*/ml. The same sample had phage titres (determined by plating 0.5 ml samples of raw water) of 4 p.f.u./ml on *Escherichia coli* K12, 2 p.f.u./ml on *E. coli* C and 0 p.f.u./ml on *E. coli* B, *Salmonella typhimurium* G46 and *Klebsiella pneumoniae* 889. These phage titres were too low to permit an estimation of virus recovery but percentage recoveries from the Esthwaite samples were in the range 50 to 80%.
**Table 2. General properties of Thirlmere virus**

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size*</td>
<td>58.0 ± 0.2 nm</td>
</tr>
<tr>
<td>Buoyant density (CsCl)</td>
<td>1.320 ± 0.001 g/ml</td>
</tr>
<tr>
<td>RNA/protein ratio</td>
<td>8.7/91.3</td>
</tr>
<tr>
<td>Sedimentation coefficient</td>
<td>400</td>
</tr>
<tr>
<td>Mol. wt. RNA fragments†</td>
<td>$2.26 \times 10^6$, $2.09 \times 10^6$</td>
</tr>
</tbody>
</table>

* Determined from 100 individual measurements.
† Calibrated from the sizes of reovirus type 3 fragments determined by Ramig *et al.* (1977).

* *Drosophila* cells were particularly prone to exhibit c.p.e. with the sample concentrates but only one sample provided positive evidence that virus-like particles were associated with c.p.e., i.e. the Thirlmere sample. Virus was observed in most cells as small crystals of virus in the cell cytoplasm (Fig. 1). Each virus was about 60 nm in diam. The virus was readily passaged from the original cell supernatant and titres of up to $10^9$ p.f.u./ml were obtained on the second passage. The virus was amenable to purification by conventional gradient ultracentrifugation and so experiments were performed to partially characterize and to possibly identify the virus.

**Properties of the Thirlmere virus**

The basic properties of the new isolate are shown in Table 2 and they show that the virus is a bisegmented double-stranded RNA virus. An electron micrograph of negatively stained virus is shown in Fig. 2, and typically spherical particles, 58 nm in diam., were observed together with fractured particles and tubular forms of the virus. The virus contained RNA which was about 8% of the particle by weight. No DNA was detected. The structural protein comprised about six polypeptides (Fig. 3). Two segments of RNA, which were RNase-resistant and so probably double-stranded, were found in the virus (Fig. 4). The RNA was linear, about 0.92 μm in length, and showed topography typical of double-stranded RNA (Fig. 5). Bimodal distribution of the RNA was detected and this is probably because the two fragments were discrete in size. The virus had a low buoyant density (about 1.32 g/ml) and a sedimentation coefficient of approx. 400.

The experimental host range of the virus was limited to *D. melanogaster* cells. None of the other cell cultures of invertebrate, piscine, avian, amphibian or mammalian origin (listed under Methods) permitted the replication of the virus propagated from the original isolate as assessed by ability to produce c.p.e. or to produce plaques at 28 °C. Incubation of the fish cell lines at 21 °C also produced negative results. Inoculation of a number of different plant species failed to produce signs of virus infection within 2 weeks of infection. Intracerebral inoculation of suckling mice with $10^7$ p.f.u./mouse also failed to show any pathological effect within 3 weeks.

**Comparative studies on Thirlmere virus**

Viruses with similar biochemical and biophysical properties to Thirlmere virus are IPNV of trout, infectious bursal disease virus of poultry (IBDV) and DXV (Dobos *et al.*, 1979). Electrophoresis of Thirlmere virus, IPNV and DXV structural proteins and RNA showed that they were biophysically distinct viruses (Fig. 3 and Fig. 4). Serological studies using microimmunodiffusion to compare these three viruses, and which incorporated an antiserum to *Tellina* virus 1, showed that all three viruses were serologically distinct from Thirlmere virus (Table 3). Thirlmere virus shared no common antigens with the major European reference strain of IPNV (Sp) nor with *Tellina* virus. DXV shared antigens in common with Thirlmere virus but the immunoprecipitation lines possessed spurs and so identity was not complete.
DISCUSSION

A novel bisegmented double-stranded RNA (birna) virus has been isolated from a reservoir. It is probably a member of a recently recognized group of viruses which includes IPNV, IBDV, DXV and the Tellina viruses (Dobos et al., 1979). The Thirlmere virus has been shown to be distinct from IPNV, DXV and the Tellina virus and, by comparison with the work of Dobos et al. (1979), it is unlike IBDV.
One problem in this study was to convincingly demonstrate that the virus was not an endogenous virus present in *Drosophila* cells. Many *Drosophila* cell lines are known to harbour viruses adventitiously (Brun & Plus, 1980; Plus, 1980), including DXV (Teninges et al., 1979). The virus isolated is remarkably virulent producing titres of $10^9$ p.f.u./ml within 12 h of infection (X. Wang & D. C. Kelly, unpublished results) so it is unlikely that such a virus could lie dormant for the number of years we have worked with the cell line. In addition, this *Drosophila* cell line (Schneider 1) has been shown to be free of birnaviruses by Brun & Plus (1980). In extensive studies of iridoviruses, picornaviruses and retroviruses in this cell line no evidence of a double-stranded RNA virus has been obtained (N. F. Moore, R. J. Avery, J. A. Hibbin & D. C. Kelly, unpublished results), nor has there been any evidence of the virus in control cells used in these experiments. The virus isolated is markedly different from the non-segmented and 10-segmented double-stranded RNA viruses described in *Drosophila* cells by Scott *et al.* (1980) and Haars *et al.* (1980). The possibility that the virus was a 'latent' virus and is induced to replicate within the cells by a 'factor' present in serum or beef extract (Plus, 1980) was eliminated by rigorous checks of these components. Consequently, we are
Fig. 5. Double-stranded RNA extracted from Thirlmere virus examined by the Kleinschmidt technique, showing that the RNA is linear. Bar marker represents 0.5 μm.

Table 3. Summary of the serological relationships between Thirlmere virus, IPNV, DXV and Tellina virus using microimmunodiffusion

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Thirlmere</th>
<th>IPNV</th>
<th>DXV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thirlmere virus</td>
<td>+</td>
<td>–</td>
<td>+*</td>
</tr>
<tr>
<td>IPNV</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Tellina virus</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DXV</td>
<td>+*</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

* Spurring on precipitation lines indicated non-identity.
confident that the origin of Thirlmere virus genuinely was the water sample. The virus could be reisolated from this sample but not from other samples processed at the same time. We were unable to estimate the number of p.f.u. of Thirlmere virus in this sample at the time because the plaque assay for the virus had not been devised. From the c.p.e. produced it was likely to be less than 0.01 p.f.u./cell, i.e. less than 3 × 10⁴ p.f.u./ml. The sample had been concentrated approx. 2 × 10⁴-fold and, assuming 65% recovery (see Results), the virus would have been present in the reservoir at a concentration of no more than 1 p.f.u./l.

The Thirlmere virus is similar in most of its general properties to IPNV, IBDV and particularly DXV. The morphology of the viruses is remarkably similar, and all preparations contain fractured particles and tubular forms of the virus (which are smaller in diameter than spherical particles) (Teninges et al., 1979; Cohen et al., 1973). The sedimentation coefficient of 400S is similar to that of other birnaviruses (435 to 465; Dobos et al., 1979).

Estimates of the mol. wt. of the two RNA fragments are low compared to values derived for other birnaviruses (Dobos et al., 1979) and this probably reflects in part the use of different mol. wt. standards for reovirus RNA. The estimates for DXV are in good agreement with that of Teninges (1979), and the estimates for the Thirlmere virus were confirmed by electron microscopy in this study. The electron microscopy results also show that dsRNA is linear.

The polypeptides contained by the Thirlmere virus numbered about six. Intriguingly, although the overall composition was similar, yet distinct to DXV (Teninges, 1979), two polypeptides appeared to be similar in size and amount to that of IPNV (IPNV polypeptides 3 and 4, using the nomenclature of Dobos et al., 1979). We plan to peptide map these polypeptides, and also to perform hybridization studies to delineate the homology between the two viruses. A further similarity between IPNV, DXV and the Thirlmere virus is that they all possess RNA polymerase activity (Cohen, 1975; Bernard & Petitjean, 1978; Bernard, 1980; D. C. Kelly & X. Wang, unpublished results).

This work demonstrates that it is possible to recover putative invertebrate pathogenic viruses from water using the virus concentration method of Logan et al. (1980, 1981) (a method not specifically designed for use with insect viruses) and insect cell cultures. It is planned to continue this work in a general survey of lake and river water. This may reveal if any other previously unknown invertebrate pathogenic viruses are present in the natural aquatic environment and provide quantification of these viruses. One could then assess the ecological importance of these viruses. Naturally, one would like to identify the original host of Thirlmere virus but this would be impossible to undertake in a systematic fashion. It would appear from the in vitro host range of the virus that it is restricted to invertebrates, and possibly to Diptera. Fruit flies are found in the Lake District and so it is possible that it is a naturally occurring Drosophila virus.

Our interest now rests in the fact that we now possess a birnavirus which readily replicates in invertebrate cells and which will provide a model system to study the molecular biology of such viruses in invertebrate cells.

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REFERENCES


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