Characteristics of a new birnavirus associated with a warm-water fish cell line

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A warm-water fish cell line developed from blotched snakehead caudal peduncle (BSN) was found to have persistent birnavirus infection. Purified virus particles were of icosahedral shape and had 57±1.6 nm diameter. The BSN virus was resistant to 5-iodo-2′-deoxyuridine and induced yellowish-green cytoplasmic inclusions when stained with acridine orange. The virus was resistant to chloroform, acid and alkaline pH and heat treatment at 56 °C for 2 h. Purified virions had a buoyant density of 1.33 g/ml in CsCl and contained two genomic segments with molecular masses of 2.56 × 10^6 and 2.00 × 10^6 Da and four structural polypeptides of 112 (polyprotein, PP), 91 (VP1), 44 (VP2) and 37 (VP3) kDa. Reciprocal β cross-neutralization tests incorporating four classical strains of infectious pancreatic necrosis virus (IPNV) (WB, Sp, Ab and TV-1) and the BSN virus established the complete serological distinctness of the virus from IPNV. Considering the uniqueness of the virus, the name blotched snakehead virus is proposed for this agent.

Epizootic ulcerative syndrome (EUS) is a widespread infectious disease of wild and farmed freshwater and brackish-water fishes in Asia with a complex aetiology. Many infectious agents, including viruses, were isolated from affected fishes (Roberts et al., 1994). To aid in the study of fish viruses associated with EUS, cell lines were developed from fishes that are highly prone to the disease condition. Some of the cell lines developed from warm-water fishes were found to carry persistent virus infections (Frerichs et al., 1991) with apparently healthy carrier fish have been documented before (Frerichs et al., 1991; Chen et al., 1993). Low-level multiplication of BSNV was noticed in BSN cells, without any visible changes in cellular morphology, as evidenced by the presence of about 10^4.5 TCID_{50}/ml virus in the cell culture supernatants. Although the virus was found to belong to the family Birnaviridae, the serological, biochemical and phenotypic characteristics of BSNV differed from infectious pancreatic necrosis virus (IPNV), the representative species of the genus Aquabirnavirus (Dobos et al., 1995).

Stock BSNV used in this study was prepared by inoculating BSN cell culture supernatant onto bluegill (Lepomis macrochirus) fibroblast (BF-2) cells (Wolf et al., 1966). Once the CPE was complete, culture supernatant was clarified at 1500 g for 15 min and stored at −70 °C in aliquots. Catfish reovirus (CRV; Amend et al., 1984), provided by R. P. Hedrick (School of Veterinary Medicine, University of California, USA), sandgoby virus (SGV; Hedrick et al., 1986), provided by J. L. Fryer (Oregon State University, USA), IPNV reference strains WB and TV-1, provided by P. F. Dixon (CEFAS, Weymouth, UK), and IPNV Sp and IPNV Ab were also used in the present study. BSNV was propagated and assayed in striped snakehead (SSN-1) cells (Frerichs et al., 1991) or in BF-2 cells. The brown bullhead (BB) cell line (Wolf & Quinby, 1969) was used to grow CRV. For biochemical characterization, IPNV Sp and SGV were grown in chinook salmon (Oncorhynchus tschawytscha) embryo (CHSE-214) cells and for serological studies all the IPNV strains and BSNV were grown and assayed in BF-2 cells. The cell lines were maintained in Eagle’s minimum essential medium (EMEM) or L-15 medium with 10% foetal bovine serum and after virus inoculation the serum content was reduced to 5%. Cells inoculated with viruses other than IPNV strains were incubated at 25 °C; IPNV-inoculated cells were incubated at 20 °C. Viruses grown in the various cell lines were purified after concentrating the virus in the clarified cell culture supernatant by PEG precipitation according to the method of Mahy & Kangro (1996). The virus from pelleted cell debris was repeatedly extracted by treating with trifluoro-
trichloroethane (Sigma) after freeze–thawing the pellet. The resulting aqueous phases from PEG precipitation and tri-
fluorotrichloroethane treatment were layered on to a dis-
continuous CsCl gradient and the virus was banded iso-
pycnically by centrifuging at 130,000 \( g \) for 17 h. The virus bands were collected by puncturing the centrifuge tubes with syringe and needle, repelleted at 100,000 \( g \) for 90 min and stored at \(-20^\circ C\) after resuspending in TNE (0·01 M Tris–HCl, pH 7·5, 0·1 M NaCl, 1 mM EDTA).

BSNV grew well in SSN-1 cells at 25–30 °C and in BF-2 at 25 °C but was unable to multiply in CHSE-214, EPC (Epithelioma papillomatosum cyprini), RTG-2 (rainbow trout gonad) or FHM (fathead minnow) cells. The CPE in SSN-1 differed from that of IPNV and was characterized by the aggregation of granular refractile cells forming a mesh-like appearance in the monolayer. Within 48–72 h, the CPE spread across the entire cell sheet followed by detachment and cell lysis. Haemag-
glutination studies revealed that BSNV lacked the ability to haemagglutinate human O-type red blood cells at room temperature (25 °C).

The presence of a double-stranded RNA genome was demonstrated by the lack of inhibition of virus replication in SSN-1 cells growing in a culture medium containing 50 \( \mu \)g/ml 5-iodo-2'-deoxyuridine and the presence of yellowish-green cytoplasmic inclusions after acridine orange staining of 15 h virus-infected cell cultures (Rovozzo & Burke, 1973). The absence of a lipid-containing envelope was confirmed by isopycnic centrifugation of pelleted virus at 130,000 \( g \) for 17 h in a 20–40% discontinuous CsCl gradient and assay of recovered fractions in SSN-1 cells established a buoyant density of 1.33 g/ml for infective particles. Transmission electron micrographs of ultrathin sections of an infected SSN-1 cell culture fixed in glutaraldehyde, post-fixed in 1% osmium tetroxide and stained in uranyl acetate–lead citrate showed intracytoplasmic inclusions similar to viroplasms and scattered, fully formed virions. The virions were also sometimes found accumulated in the intercellular spaces (Fig. 1a). Preparations of the virus negatively stained with 2% phosphotungstic acid revealed the presence of icosahedral, non-enveloped, single-shelled particles with a mean diameter of 57 ± 1·8 nm (\( n = 32 \)) (Fig. 1b).

The structural protein of purified virions was analysed by SDS–PAGE according to the method of Laemmli (1970) using a Mini-V 8:10 vertical gel electrophoresis system (Gibco BRL). Broad-range molecular mass standards (Bio-Rad), IPNV Sp and SGV were also co-analysed with BSNV in 10% acrylamide gels. Separated polypeptide bands were stained with 0·1% Coomassie blue R-250 and four structural polypeptides of 112 (polyprotein, PP), 91 (VP1), 44 (VP2) and 37 (VP3) kDa were identified by extrapolation from the migration curve of molecular mass standards (Fig. 2a).

Virus nucleic acid from purified virions was recovered by proteinase K–SDS digestion and phenol–chloroform extrac-
tion as described by Burleson et al. (1992). Extracted viral RNA was electrophoresed at 1 V/cm for 14 h in 20 cm long 1·5% agarose–formaldehyde gels, essentially according to Sambrook et al. (1989), with MOPS running buffer. Viral RNAs of CRV, SGV and IPNV Sp were also co-electrophoresed with BSNV besides molecular mass markers II and III (Boehringer Mannheim). The gels were stained for 30 min in 1 \( \mu \)g/ml ethidium bromide and destained in diethyl pyrocarbonate-treated deionized water until the background fluorescence was cleared. The gels were photographed on Polaroid film after exposure to UV light (Fig. 2b, c). The molecular masses of the two genomic segments were found to be 2·56 (3·84 kbp) and 2·00 (2·99 kbp) \( \times 10^6 \) Da by extrapolation from the migration curve of the markers (conversion of 1 kbp = 6·67 \( \times 10^6 \) Da).
Serological comparison of BSNV and IPNV was performed by reciprocal cross-neutralization of BSNV and four classical strains of IPNV (WB, Sp, Ab and TV-1) by the method of Okamoto et al. (1983) with minor modifications. Antisera against two of the IPNV reference strains WB and TV-1 were provided by P. F. Dixon (CEFAS, Weymouth, UK). Antisera were serially five-fold diluted in microtitre plates (40 µl per well) and mixed with ~100 TCID₅₀ (40 µl) virus. The virus–antiserum suspension was incubated at room temperature (24 °C) for 60 min with frequent mixing on a plate shaker (Titretek, Flow Labs). BF-2 single-cell suspension (100 µl) was added to each well and the plates were incubated at the temperatures given above and observed for 10–14 days for the development of CPE. Neutralizing antibody titre (ND₅₀) of antiserum was expressed as the highest antiserum dilution protecting 50% of the inoculated cultures, as calculated by the Spearman–Kärber method (Kärber, 1931). Serological relationships (1/r) between the IPNV strains and BSNV, calculated from the formula \( r = \sqrt{r_1 \times r_2} \), where \( r_1 \) and \( r_2 \) are the titre ratios (Archetti & Horsfall, 1950), are shown in Table 1.

The biophysical and biochemical characteristics, including virus morphology, buoyant density, presence of a bi-segmented, double-stranded RNA genome, heat and pH stability and nature of capsid structural polypeptides, indicate that the virus belongs to the genus *Aquabirnavirus* in the family *Birnaviridae* (Dobos et al., 1995). Two of the proteins, VP2 (45–60 kDa) and VP3 (29–35 kDa), together make up 80–90% of the total protein content of the birnaviruses (Dobos et al., 1979; Dobos, 1996). Corresponding polypeptides (44 and 37 kDa) also formed the most abundant viral proteins of BSNV. The high molecular mass protein (112 kDa) found in BSNV is similar to the 101 kDa polyprotein of IPNV encoded by genome segment A (Duncan & Dobos, 1986). The polyprotein, not previously reported to occur in purified virus preparations, has since been found in purified preparations of IPNV (Magyar & Dobos, 1994). Although the BSNV structural proteins correspond favourably with those of IPNV, the individual molecular masses of the proteins and migration pattern are considerably different from the structural poly-peptides of IPNV. The difference in the protein pattern was unambiguously evident, as the low molecular mass protein of BSNV was 37 kDa compared with IPNV, which has two structural proteins of molecular mass falling below 32 kDa. The molecular masses of BSNV genomic RNA segments (2.56 × 10⁶ and 2.0 × 10⁶ Da) were also found to be widely different from those of the IPNV RNA segments, which have molecular masses of 2.5 × 10⁶ and 2.3 × 10⁶ Da (Dobos et al., 1977). When compared with the reported molecular masses of the genomic segments of birnaviruses (Dobos et al., 1991), BSNV has the largest size difference yet reported between the two genomic segments.

The type species of the genus *Aquabirnavirus*, IPNV, currently has 10 serotypes identified in two serogroups, A and B, which show some kind of cross-reactivity within each serogroup but not between the two serogroups (Hill & Way, 1996). The present study, involving three classical serotypes of serogroup A and the sole serotype of serogroup B, revealed that BSNV did not have any cross-reaction with strains of either serogroup. SGV, isolated from an ulcerated sandgoby in Thailand, is another tropical birnavirus reported to be significantly different from the classical IPNV strains (Hedrick et
This isolate, however, which showed some degree of cross-reaction with IPNV Ab and Sp, has later been recognized as a mixture of IPNV Ab and Sp (Hill & Way, 1996).

Apart from serological distinctness, the differences in the molecular masses and migration patterns of capsid proteins and viral RNAs also distinguish BSNV from other reported aquabirnavirus strains. Phenotypically, the virus was different from all the IPNV strains, as it was unable to multiply in CHSE-214, RTG-2, EPC or FHM cell lines (Macdonald & Gower, 1981). Although BSNV was identified as belonging to the genus Aquabirnavirus, biochemical, biological and serological characteristics demarcated it from reported strains of the type species of the genus, IPNV. Since the virus cannot be compared with any of the existing IPNV strains and also since the virus was serologically distinct from the two reported serogroups of IPNV, BSNV could be classified currently as third serogroup of the genus Aquabirnavirus. Since the virus cannot be compared with any of the existing IPNV strains and also since the virus was serologically distinct from the two reported serogroups of IPNV, BSNV could be classified currently as third serogroup of the genus Aquabirnavirus. Although BSNV was identified as belonging to the genus Aquabirnavirus, biochemical, biological and serological characteristics demarcated it from reported strains of the type species of the genus, IPNV. Since the virus cannot be compared with any of the existing IPNV strains and also since the virus was serologically distinct from the two reported serogroups of IPNV, BSNV could be classified currently as third serogroup of the genus Aquabirnavirus. Further studies would, however, be required to clearly identify whether the virus really forms a new serogroup or a new species altogether under the genus Aquabirnavirus. The name blotched snakehead virus is therefore proposed for this agent.

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**References**


**Table 1. Reciprocal cross-neutralization tests with four strains of IPNV and BSNV**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>IPNV</th>
<th>BSNV</th>
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<tbody>
<tr>
<td>WB</td>
<td>279,508* (1)</td>
<td>0</td>
</tr>
<tr>
<td>Sp</td>
<td>2991</td>
<td>0</td>
</tr>
<tr>
<td>Ab</td>
<td>200</td>
<td>0</td>
</tr>
<tr>
<td>TV-1</td>
<td>267(511-10)</td>
<td>0</td>
</tr>
<tr>
<td>BSNV</td>
<td>0</td>
<td>417,963 (1)</td>
</tr>
</tbody>
</table>

Serum titres (*) are reciprocals of 50% antiserum endpoint dilutions that neutralized the amount of virus (TCID₅₀) given in square brackets. Antigenic relationships expressed as 1/r values are given in parentheses.


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