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Sero- and genotyping of some marine aquatic birnavirus isolates from Norway

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ABSTRACT: Twelve isolates of aquatic birnaviruses (9 field isolates from marine fish and shellfish and the Ab/A_3, Sp/A_2 and N1 strains) were serotyped using an immunodot assay. The assay with 17 monoclonal antibodies revealed some serological variation among the Norwegian isolates, but the N1 strain and the Sp/A_2 type strain reacted identically. A genotyping assay based upon restriction fragment analysis of a polymerase chain reaction-amplified fragment from the VP2 coding region was run parallel to the serotyping. All the 9 serogroup A serotypes, except He/A_4 and Can. $3/A_8$, were assayed in addition to the Norwegian isolates mentioned above. This method differentiated among all serotypes tested, but only small differences were observed among the Norwegian isolates. The N1 and Sp/A_2 strains reacted identically. Our results indicate homogeneity between Norwegian birnavirus isolates and support earlier studies that concluded that the N1 strain belongs to the Sp/A_2 serotype.

KEY WORDS: Immunodot · PCR · IPNV

INTRODUCTION

The aquatic birnaviruses are a group within the Birnaviridae that comprises isolates from fish and shellfish from both fresh and seawater (Wolf 1988). Aquatic birnaviruses are small, icosahedral, unenveloped viruses, with a genome consisting of 2 segments of dsRNA designated A and B (reviewed by Dobos 1995). Segment B encodes the putative RNA-dependent RNA polymerase, while segment A encodes a polyprotein consisting of 3 proteins in the order 5'-pVP2-NS-VP3-3', in addition to a fourth protein (VP5) in an overlapping reading-frame. The polyprotein is processed to produce the major structural proteins VP2 and VP3 in addition to the NS protease. The VP2 coding sequence contains a hypervariable region (Håvarstein et al. 1990) that presumably codes for serotype-specific epitopes.

The aquatic birnavirus infectious pancreatic necrosis virus (IPNV) is widely distributed in Norway and is probably present in all Atlantic salmon *Salmo salar* farms along the coast (Melby et al. 1991). IPNV primarily causes infectious pancreatic necrosis (IPN) in salmonid fry, but has recently also been found to cause mortality of juvenile salmon during smoltification and among post-smolts and larger sea-reared salmon (Krogsrud et al. 1989, Taksdal 1991, Smail et al. 1992). Aquatic birnaviruses which have been tentatively considered to be IPNV have been isolated from bivalve molluscs and crustaceans and were also found to cause disease in farmed marine flatfish (Mortensen et al. 1990, 1993, Biering et al. 1994, Biering & Bergh 1996).

The most extensive classification of aquatic birnaviruses is based upon cross-neutralization using polyclonal antisera, and this classification has led to the establishment of 2 serogroups, A and B (Hill & Way 1995). Serogroup A is further divided into 9 serotypes (A_1 through A_9), while serogroup B so far contains only 1 serotype (B_1). The N1 strain, proposed by Christie et al. (1988) to be a tenth serotype within serogroup A, has since been classified as belonging to serotype Sp/A_2 (Melby & Christie 1994, Melby et al. 1994). Heppell et al. (1992) published a classification scheme based upon restriction fragment profiles of a 359 bp (base pair) polymerase chain reaction (PCR) fragment from the VP2-NS region. This classification did not

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agree with the traditional serotyping, presumably because the amplified segment is unlikely to code for any serotype-specific epitope. Recently, Blake et al. (1995) developed a primer pair that was able to amplify most of the VP2 coding region in 8 of the 9 serotypes. This primer pair was employed by Novoa et al. (1995) for restriction fragment profile analysis in a study in which several methods of typing were employed on 4 reference strains and 6 field isolates from turbot *Scophthalmus maximus*. They were not able to correlate the genomic classification with traditional serotyping.

The aims of this study were to determine the serological interrelationships of some Norwegian marine birnavirus isolates from fish and shellfish by means of an enzyme immunodot assay applying 11 monoclonal antibodies (MAbs) developed against strains within the West Buxton/A_1, Ab/A_3 and Can. 1/A_6 serotypes (Caswell-Reno et al. 1986, 1989), in addition to 6 MAbs developed against the N1 strain (Christie et al. 1990). The immunodot assay was compared with a genotyping assay based upon restriction fragment analysis of a PCR product amplified from the VP2 coding region.

MATERIALS AND METHODS

Virus. Norwegian isolates used in the various typing assays were aquatic birnavirus strains isolated from farmed halibut *Hippoglossus hippoglossus* fry in 1989 (Mortensen et al. 1990), farmed halibut fry from 2 different farms in 1991, farmed turbot *Scophthalmus maximus* fry, pollack *Pollachius pollachius* caught near a fish farm with a population of IPNV-positive Atlantic salmon *Salmo salar*, mussels *Mytilus edulis* collected near a salmon farm, scallops *Pecten maximus* (Mortensen et al. 1990), and from farmed Atlantic salmon post-smolts from 2 different farms. In addition, the Sp/A_2, Ab/A_3 and N1 strains were run in parallel in the immunodot assay. In the genotyping assay, all serogroup A serotypes except He/A_4 and Can. 3/A_8 were run in parallel (Table 1). The reference strains were kindly provided by Dr K. E. Christie at Intervet NorBio A/S (the Jasper/A_9 strain was originally obtained from Dr P. Dobos). All isolates were propagated in CHSE-214 cells as previously described (Biering et al. 1994) and cloned by end-point dilution. After a second propagation in CHSE-214 cells, all suspensions were titrated by end-point dilution using 12 parallel wells per dilution, and diluted to approximately 10^7 $TCID_{50}$ ml^{-1}.

Immunodot assay. An enzyme immunodot assay was performed using a panel of 11 MAbs produced against the West Buxton/A_1 type strain and isolates within the Ab/A_3 and Can. 1/A_6 serotypes (Caswell-Reno et al. 1986, 1989) in addition to 6 MAbs produced against the N1 strain (Christie et al. 1990). The sources of MAbs were supernatant fluids from hybridoma cell cultures propagated as described by Lipipun et al. (1989). The immunodot assay was performed as described by Caswell-Reno et al. (1989), with modifications as described by Melby et al. (1994). Briefly, the nitrocellulose membrane was placed in a dot-blot apparatus (Bio-Dot, Bio-Rad Laboratories, Richmond, USA), and 100 µl of virus containing supernatants were added to each well. After blocking, the membrane was incubated in supernatants with MAb titre 2 to 4 times the minimum titre previously determined to be required for positive result with homologous virus. As secondary antibody, a horseradish-peroxidase conjugated goat-anti-mouse antiserum (HyClone Laboratories, Logan, USA) was used. Development was performed in 0.5 mg 1-chloro-4-naphthol and 1 µl 30% H_2O_2 in 1 ml Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) until clear contrasts between samples and positive controls were observed (3 to 7 min). A known birnavirus-negative mouse MAb was used as negative antibody control, and uninfected cell culture supernatant was used as negative antigen control. Each virus isolate was assayed twice.

RNA isolation. Single-step RNA isolation was performed on 100 µl cell culture supernatant using the Trizol reagent (Gibco BRL, Life Technologies Inc., Gaithersburg, USA) according to the manufacturer's instructions. Briefly, 100 µl cell culture supernatant was mixed with 1000 µl Trizol and centrifuged at 12 000 × *g* for 15 min. The RNA in the aqueous phase was then precipitated using isopropanol and washed

Table 1. Birnavirus strains used in this study

	Strain	Origin	Host	Serotype[a]
1	West Buxton	USA	*Oncorhynchus mykiss*	A1
2	Sp	Denmark	*Oncorhynchus mykiss*	A2
3	Ab	Denmark	*Oncorhynchus mykiss*	A3
4	Te	Scotland	*Tellina tenuis*	A5
5	Can. 1	Canada	*Salmo salar*	A6
6	Can. 2	Canada	*Salmo salar*	A7
7	Jasper (Dobos)	Canada	*Oncorhynchus mykiss*	A9
8	N1	Norway	*Salmo salar*	
9	Halibut 89	Norway	*Hippoglossus hippoglossus*	
10	Halibut 1.91	Norway	*Hippoglossus hippoglossus*	
11	Halibut 2.91	Norway	*Hippoglossus hippoglossus*	
12	Turbot	Norway	*Scophthalmus maximus*	
13	Pollack	Norway	*Pollachius pollachius*	
14	Salmon 1	Norway	*Salmo salar*	
15	Salmon 2	Norway	*Salmo salar*	
16	Scallop	Norway	*Pecten maximus*	
17	Mussel	Norway	*Mytilus edulis*	

[a]According to Hill & Way (1995)

in 75% ethanol. The pellet was dried briefly under vacuum, resuspended in 8.2 µl RNAse-free H_2O, denatured for 5 min at 100°C and used directly for cDNA synthesis. RNA isolation and subsequent cDNA synthesis, PCR and restriction enzyme digestion were performed twice for each virus strain.

cDNA synthesis. cDNA synthesis was performed on 8.2 µl RNA using the First Strand cDNA Synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. The reverse transcription (RT) reaction was primed using random hexanucleotides.

PCR. The total volume of the RT reaction (20 µl) was used for PCR amplification of a 1180 bp target region in the gene coding for VP2, the major structural protein of birnaviruses. Primers 5'-TGA GAT CCA TTA TGC TTC CAG A-3' and 5'-GAC AGG ATC ATC TTG GCA TAG T-3' (Blake et al. 1995), which defined the region between bp 151 and 1330 in the published sequence of the Jasper/A_9 strain (Duncan & Dobos 1986), were used. After 2 min at 95°C, 34 cycles with the following parameters were run: 30 s at 95°C, 1 min at 58°C and 1 min at 72°C. The synthesis ended with 10 min at 72°C. The PCR was run in 100 µl volume in a MJ Research PTC-100 thermal cycler.

Restriction enzyme digestion. Digestions with the enzymes *Pvu*II, *Ban*II, *Rsa*I, *Hae*III and *Apa*I were performed in a total volume of 10 µl. Five µl PCR product (3 µl H_2O, 1 µl buffer and 1 µl enzyme) were incubated at 37°C overnight.

Agarose gel electrophoresis. The restriction fragments were analysed on 2.5% Metaphor (FMC BioProducts, Rockland, USA) agarose gels in Tris-Borate-EDTA (TBE) buffer (0.09 M Tris-borate pH 8.0, 2 mM EDTA). The gels were run for 3 h and 40 min at 55 V, stained with ethidium bromide and photographed using a Polaroid camera.

Statistics. The restriction profiles were analysed using the program GelCompar (Applied Maths BVBA, Kortrijk, Belgium). The UPGMA (unweighted pair-group method using arithmetic averages) cluster analyses were based on Jeffrey's x coefficient, taking into account only position, not concentration, of restriction fragments.

RESULTS

Immunodot

With the immunodot assay an overall similar dot pattern was observed in the Sp/A_2 and N1 strains, and in all Norwegian isolates from fish and bivalve molluscs (Fig. 1). However, the reaction patterns revealed some differences among the Norwegian isolates. The Halibut 1.91 was the only isolate in which strong reactions were observed with the W4 MAb. The Pollack isolate reacted with the E2 and E3 MAbs, and the Halibut 89 isolate differed from the general pattern by its reaction with the E3 MAb. MAb reactions with the Ab/A_3 type strain were markedly different from reactions with the Norwegian isolates.

Genotyping

The primer pair directed the synthesis of a 1180 bp fragment from all strains, except from the He/A4 and

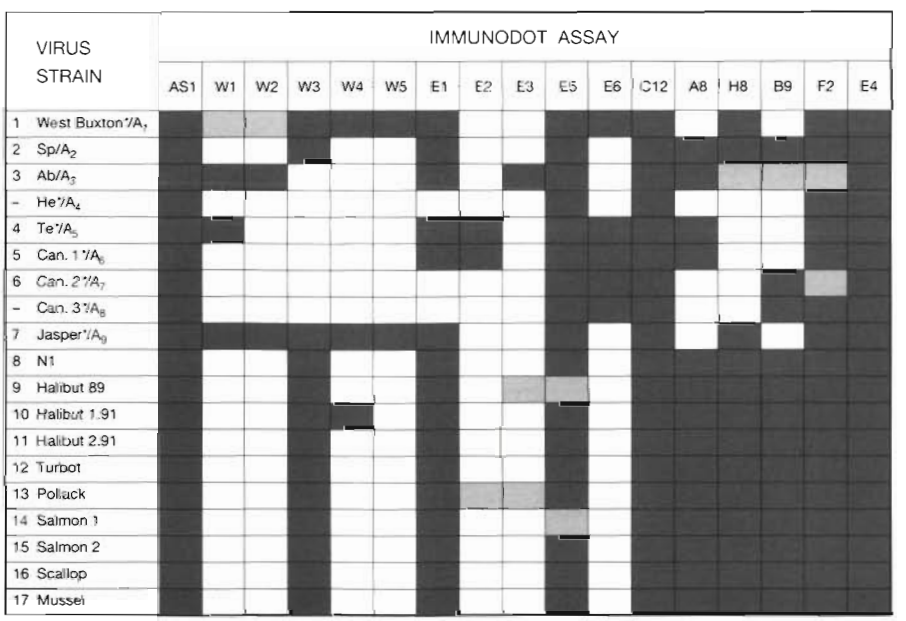

Fig. 1. Immunodot reaction pattern of aquatic birnavirus isolates. Reactions are shown as negative (white), weak or variable (light grey) or strong (dark grey). *According to Caswell-Reno et al. (1989) and Melby & Christie (1994); included for comparison. Numbering of strains corresponds to Table 1

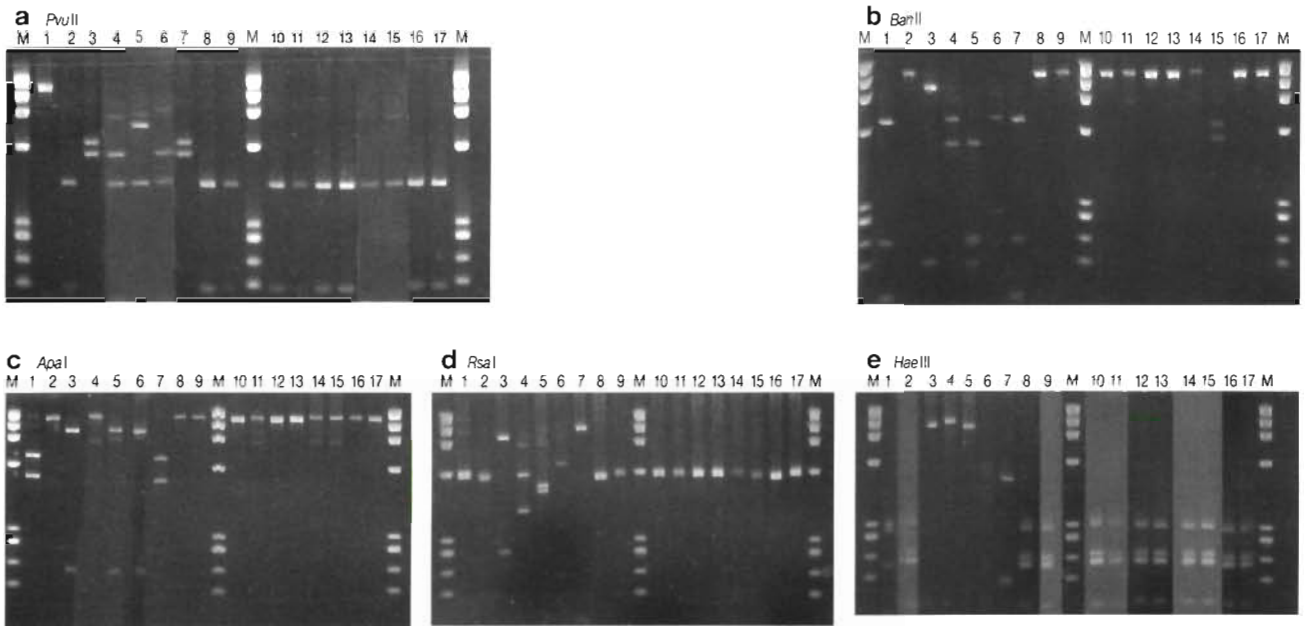

Fig. 2. Restriction fragment patterns obtained after digestion of a 1180 bp segment from the VP2 coding region of 17 isolates of serogroup A birnaviruses with: (a) *Pvu*II, (b) *Ban*II, (c) *Apa*I, (d) *Rsa*I and (e) *Hae*III. Numbering of strains corresponds to Table 1. M: DNA markers consisting of (from top to bottom) 1363, 1070, 872, 603, 310, 281/271, 234 and 194 bp respectively

Can. $3/A_8$ type strains (data not shown). Lowering the annealing temperature to 53°C did not produce any fragments from these strains. Restriction enzyme digestion of the PCR product with the enzymes *Pvu*II, *Ban*II, *Apa*II, *Rsa*I and *Hae*III generated a combination of patterns that differentiated among all 7 reference strains assayed (Fig. 2). The N1 strain (lane 8) and the other Norwegian field isolates (lanes 9–17) reacted identically to the Sp/A_2 type strain (lane 2), except for the *Rsa*I and *Ban*II digestions. In the *Rsa*I digestion, the Sp/A_2, N1, Salmon 2 (lane 15) and Scallop (lane 16) isolates produced a double band at the 603 bp marker, with slightly lower molecular weight than the double band produced by the other Norwegian isolates and the West Buxton/A_1 type strain. Furthermore, the Salmon 2 isolate differed from the other Norwegian isolates when digested with *Ban*II. Some of the Norwegian isolates and the Sp type strain appeared to differ with regard to the presence of fragments with low molecular weight (Fig 2a, e), but this difference is due to loss during reproduction. As the concentration of PCR product differed somewhat, some low molecular weight bands were faint and not accurately reproduced during photography. Also, some strains produced low amounts of unspecific products, seen as faint bands near the 1070 and 872 bp markers, during PCR. Fig. 3 shows a dendrogram that visualizes the relationship among the different isolates.

DISCUSSION

The results of the immunodot assay indicated epitope conformity among the Norwegian birnavirus isolates, as most isolates reacted identically to the Sp type strain. The genotyping also showed great similarity, as only the *Ban*II and *Rsa*I digestions revealed differences among the Norwegian isolates. Our results indicate a high degree of homogeneity in Norwegian birnavirus isolates, even among strains from different

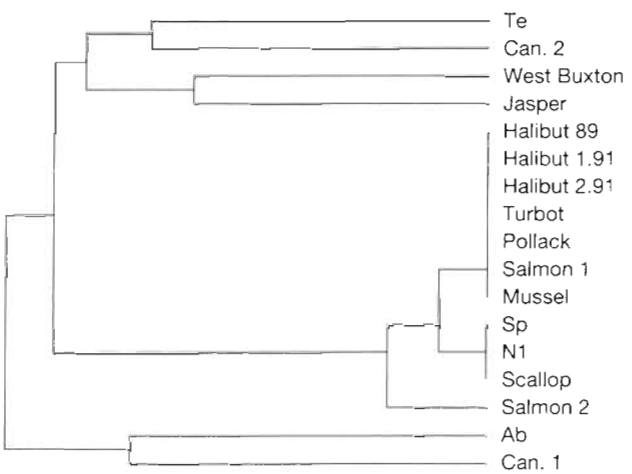

Fig. 3. Dendrogram demonstrating the genomic relationships among the birnavirus isolates tested

host species. Although this study contains too few isolates to allow us to draw firm conclusions, it is in accordance with earlier reports concerning serotyping of Norwegian birnavirus isolates with monoclonal antibodies (Christie et al. 1990, Melby & Christie 1994, Melby et al. 1994).

The isolates from marine flatfish have been associated with outbreaks of disease (Mortensen et al. 1990, 1993, Biering et al. 1994, Biering & Bergh 1996), while the scallop isolate appeared to be non-pathogenic for scallops but pathogenic for trout (Mortensen et al. 1992, Mortensen 1993). The similarity between the isolates from bivalve molluscs and salmon may therefore indicate that the bivalve isolates were environmental contaminants, rather than species-specific isolates. If epitope alteration and host specificity are related, the serological diversity observed between the isolates from marine fish could be related to host adaptation. However, the grouping and typing of aquatic birnaviruses does not reveal any strictly logical correlation between serology and host specificity, and it is therefore unlikely that serological features can be used as criteria for judging host specificity and potential pathogenicity. The variation among the Norwegian isolates observed in the present study may therefore have been due to normal variability within a group of related strains.

The N1 and Sp/A_2 strains reacted identically in both the immunodot and genotyping assays. These observations are in accordance with work done by Melby et al. (1994) and Melby & Christie (1994), which indicate that the N1 strain belongs to the Sp/A_2 serotype. Our results also agree with those of Heppell et al. (1992), who performed a genotyping study based upon restriction enzyme digestion of a 359 bp PCR fragment within the VP2-NS region. In all digestions, the N1 strain and Sp/A_2 type strain reacted identically. In a later report (Heppell et al. 1993) the same fragments were sequenced, revealing 99% homology between the deduced amino acid sequences of the N1 and Sp/A_2 strains. In contrast to earlier reports in which sero- and genotyping have been compared (Heppell et al. 1992, Novoa et al. 1995), this study demonstrates a good correlation between the assays, as all Norwegian isolates were typed as Sp/A_2 or Sp/A_2-like, irrespective of the method employed.

The negative reactions in immunodot with the E2 and E6 MAbs against serotype Ab/A_3 were not in accordance with the results of Caswell-Reno et al. (1989), while the positive reaction with E3 was in accordance with the reaction with the Ab/A_3-like isolates, but not with the Ab/A_3 type strain. The positive reaction of MAb B9 with Sp/A_2 in immunodot was not in accordance with Melby & Christie (1994). Furthermore, the digestion pattern of the Sp/A_2 type strain with *Pvu*II differed slightly between the present report and that of Novoa et al. (1995). These observations may reflect the high mutation rate of RNA viruses (Domingo et al. 1985, Steinhauer & Holland 1987) and indicate that small changes, due for instance to passages in cell culture, may influence the result of both sero- and genotyping assays. Both antigenic variation and genomic heterogeneity have been demonstrated for IPNV strains belonging to the same serotype (Berthiaume et al. 1992, Heppel et al. 1992, Lecomte et al. 1992).

Genotyping using PCR and restriction enzymes is a rapid and apparently accurate way of typing birnaviruses, and we were able to differentiate all the serotypes assayed. However, unlike Blake et al. (1995), we were unable to amplify the target region from the Can. 3/A_8 type strain. As Blake et al. (1995) used a field isolate within the Can. 3/A_8 serotype instead of the type strain, this observation indicates how sensitive a PCR-based assay may be to genomic changes, as different strains within the same serotype may produce different results during the amplification. Primers that are able to amplify all serogroup A serotypes, and preferably all strains within the types, are a prerequisite before PCR-based genotyping can be a practical substitute for serotyping. If the objective is to correlate geno- and serotyping, these primers will probably have to amplify the VP2 coding region. Due to variability within this part of the genome, it may prove difficult to find conserved sequences that are useful as primers in all birnavirus strains.

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